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CONTROLLING MICROBIAL GROWTH IN INNOVATIVE DIETARY SUPPLEMENT BASED ON THE BIOMASS OF YEAST *YARROWIA LIPOLYTICA*

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ABSTRACT

The growing demand for meat and dairy products necessitates the intensification of the production of feeds containing high-protein components. *Yarrowia lipolytica* biomass constitutes a rich source of easily digestible proteins and numerous vitamins, which makes it a valuable additive for both liquid and dry feeds. Unfortunately, the liquid products are susceptible to the growth of undesirable microflora. The aim of the presented research was to assess the possibility of using selected chemical substances to stabilize the high-protein liquid products acquired from *Yarrowia lipolytica* biomass. The research was designed with the use of a statistical method based on mixture designs. Microbiological stability was assessed using the results of culture tests aimed at calculating the total number of mesophilic bacteria, yeasts, molds, and bacteria from the *coli* and *Salmonella sp.* groups. Storage tests were performed at temperatures of 4 and 25 °C. Sodium benzoate, potassium sorbate and phosphoric acid were used as preservatives.
The use of preservatives resulted in a significant decrease in the numbers of bacteria during storage. After 30 days of storage, the total number of bacterial cells was 0.95±0.11 and 1.5±0.09 Log_{10} Cfu ml^{-1}, respectively, for storage tests at temperatures of 4 and 25°C. A product with optimum preservative mixture composition, stored at 4°C, should contain 1297mg l^{-1} of sodium benzoate and 1203 mg l^{-1} of phosphoric acid. In the case of products stored at 25°C, the concentration of sodium benzoate and phosphoric acid should be 1307 mg l^{-1} and 1197 mg l^{-1}, respectively.

Keywords: *Yarrowia lipolytica*, cavitation, liquid feed, microbial stability, preservatives

INTRODUCTION

Livestock production has recently seen dynamic growth, which is highly correlated with the increasing wealth of societies in developing countries. The growing demand for meat and dairy products necessitates the intensification of the production of feeds containing high-protein components. The demand for easily assimilated protein is very high, since the production of 1kg of meat requires 3 - 10 kg of grain **(Tilman *et al.*, 2002)**. Current livestock production is mainly based on the use of feeds containing soybean meal and fish meal. Soybean meal, due to its high amount of easily digestible protein and relatively low fat content, is an excellent source of protein for poultry, piglets, and calves **(Bertheau and Davison, 2011)**. By-products of the biofuel industry, such as dried distiller's grains with solubles (DDGS) created during the production of bioethanol from starch-based substrates, may also constitute a source of protein and energy. Unfortunately, the variability of this product is a significant drawback. It results from the variability of the raw materials used (e.g. corn) as well as of the conditions in which the processes of fermentation and distillation take place **(Cozannet *et al.*, 2010)**. Yeast biomass, which contains about 40% protein, may be used as an alternative to soybean meal and DDGS. According to the newest research, feed supplementation with inactive dry yeast (*Saccharomyces cerevisiae*) acquired after alcoholic fermentation does not have any negative effect on feed use by animals and provides comparable carcass gain **(Campos *et al.*, 2014)**. The yeast *Yarrowia lipolytica* is also gaining increasingly wider interest on the part of the feed industry. In 2009, a Polish company, Skotan SA, in cooperation with scientists from the Wrocław University of Environmental and Life Sciences, designed a worldwide unique technology for the industrial production of *Yarrowia lipolytica* feed yeast biomass **(Rymowicz, 2009)**. This strain is capable of intensive growth on vegetable fats, degumming, and glycerin fraction created during biodiesel production. *Yarrowia lipolytica* biomass constitutes a rich source of easily digestible proteins (41 - 45%) and numerous vitamins, which makes it a valuable feed additive **(Baszczok and Rymowicz, 2011)**. As a result of these investigations, the European Feed Manufacturers' Federation (FEFAC) decided to authorize the sale of *Y. lipolytica* feed yeast produced from crude glycerol in 2010. The powdered form of the product, acquired through spray drying, was registered under catalog number 00 575-EN **(Rywińska *et al.*, 2013)**. Unfortunately, within the last several years the price of technical glycerol, which is the main raw material in the process of producing the above-mentioned yeast, rose significantly. In 2010, it was about 90 euros/ton, while currently it is 275 euros/ton. As a result, the technology developed in 2009 was largely modified. The energy-intensive drying process was abandoned, and the powdered product was replaced with a liquid version. An innovative method is used to inactivate the yeast cells. It is based on hydrodynamic cavitation and enables effective cell disintegration. The resulting suspension, rich in easily assimilated protein, constitutes an excellent basis for the preparation of liquid animal feed. Feeding systems based on liquid feeds have numerous advantages and, therefore, are gaining popularity among breeders. The use of liquid feeds provides more flexibility and, consequently, ensures better control and modification capabilities for feeding systems. This improves the utilization of nutrients, gut health, and animal well-being, which, in turn, reduces the use of pharmaceuticals **(Plumed-Ferrer and Von Wright, 2009)**.
Unfortunately, the product obtained through hydrodynamic cavitation is characterized by high susceptibility to microbiological infections due to its high content of easily assimilated nutrients. It retains its stability in cold room conditions for several days only. From a practical standpoint, this makes transportation significantly more difficult and forces end recipients to implement costly modernizations to the storehouses in the vicinity of their barns or piggeries. Hence, the goal of the presented study was to establish the optimum dose of preservatives enabling the storage of the liquid preparation for at least 30 days at a temperature of 25°C. The preservatives which were used, i.e. sodium benzoate, potassium sorbate, and phosphoric acid, are chemical substances commonly utilized in the food industry for stabilizing different types of products **(Aderinola and Oluwamukomi, 2014; Lennerz *et al.*, 2015)**. They are characterized by a very wide spectrum of antibacterial effects, high activity in relatively low concentrations, and thermal stability in low pH environments. Moreover, these substances have no influence on the color and taste of the finished product **(Alnoman *et al.*, 2015)**. The research was designed with the use of a statistical method based on mixture designs. Microbiological stability was assessed on the basis of culture tests.

MATERIAL AND METHODS

Microorganism

The strain used in this study was *Yarrowia lipolytica* YL-A101, which belongs to the Department of Biotechnology and Food Microbiology at Wroclaw University of Environmental and Life Sciences, Poland. The strain was maintained on YPG slants stored at 5 °C. For long time storage the strain was preserved in a laboratory culture collection as a glycerol stocks stored at −80 °C.

Medium composition

Biomass production was conducted on the use of the production medium described in patent US 2011/0111090 A1 **(Baszczok and Rymowicz, 2011)**: crude glycerol (methanol-free by-product from biodiesel production, LOTOS Group, Czechowice–Dziedzice, Poland) – 50 g l^{-1}; $(NH_4)_2SO_4$ – 12.6 g l^{-1}; urea – 4.0 g l^{-1}; $MgS0_4$ x $7H_20$ – 1.0 g l^{-1}; KH_2P0_4 – 0.5 g l^{-1}; yeast extract – 0.5 g l^{-1}. All chemicals were purchased from POCh (Poland).

Production of liquid diet supplements

The inoculum development for large scale cultivation process was described on Fig. 1. The pre-inoculum was produced from the YPG slants stored at 5 °C, and microbial cells were transferred to conical flask with a 250 ml capacity containing 50 ml of growth medium. The growth medium for pre-inoculum preparation included the following composition in g l-1: glycerol: 40; yeast extract: 20; bacto peptone: 30. The flask was maintained in a shaker at 200 rpm and 30 ± 1 ºC for 48 h. After 48 h of cultivation, culture was used to inoculate the first seed fermentor containing 0,01m^3 of production medium. The 0,02m^3, 0,2m^3 and 2m^3 fermentors containing 0,01m^3, 0,1m^3 and 1m^3 of production medium were used for the first, second and third-stage liquid seed culture, respectively. All cultures were performed batch-wise in aerobic conditions. The last stage of liquid supplement production was the cultivation of the *Yarrowia lipolytica* YL-A101 strain in an industrial scale bioreactor with total capacity of 15m^3 and working volume of 12m^3. The culture was maintained at 30±1 °C and the pH was adjusted automatically to 3.5 with 10M sodium hydroxide solution. The dissolved oxygen level was automatically controlled by adjusting the agitation within the range of 120–400 rpm to maintain over 20% of saturated dissolved oxygen (at a constant aeration rate of 1.5 vvm). Foam formation was controlled by automatic addition of Acepol 83E (Emerald Foam Control, Germany). Biomass productivity in the above-mentioned conditions was about 2.5g $l^{-1}h^{-1}$, while final cell concentration in post-culture liquid was 33g dry weight l^{-1}. After the cultivation process, the cell suspension was pumped into a storage tank.
The goal of the next stage was the disintegration of cell biomass leading to cell death and the release of cell contents into the post-culture liquid. This process was conducted with the use of a hydrodynamic continuous cavitation device (Unister Plus, Poland). The cavitation device was equipped with a high-pressure piston pump and a cavitation head. The maximum operating pressure achieved during cavitation was 10 MPa. The yield of the device under the said pressure was 3.5 m^3 h^{-1}. Hovewer, the cell biomass disintegration was conducted at a pressure of 8.2 MPa; the number of suspension passages through the cavitation head was 18; the temperature during cavitation was 72±2 °C. A microprocessor controller equipped with a resistance temperature sensor (Pt-100, Apar, Poland) coupled with an actuator in the form of an electromagnetic valve assembly (Danfoss, Denmark) and a shell and tube heat exchanger with straight corrugated tubes (Secespol, Poland) was used to maintain constant temperature. After the cavitation process, the suspension was cooled down on a heat exchanger to a temperature of 10 °C and pumped into a storage tank feeding a distribution line for 2 L polyethylene bags, which were equipped with secure stoppers and silicone rubber membranes. The storage tanks and the distribution line were not aseptic. The product was then used for further research aimed at establishing the optimum composition of the preservative mixture.

Experimental design

A characteristic feature of experimental design for mixtures is the condition of factor summability and linear restrictions on the values of these factors. In practice, this means that in a given experimental variant individual factor levels are fractions whose sum always equals 1 **(Cox and Reid, 2000)**. Three-factor D-optimal design, involving the performance of 20 experiments, was used to establish the influence of individual preservatives on the microbiological stability of the product (Table 1). The minimum and maximum factor levels used in the research were the following: sodium benzoate, SB (0 – 2500mg l^{-1}), sodium sorbate, SS (0 – 2500mg l^{-1}) and 75% (w/w) phosphoric acid, PA (0 – 2500mg l^{-1}). The order in which the experiments were performed was randomized, which fulfils the requirement for independent and random distribution of observations. Such practice helps avoid the influence of unknown nuisance variables. All preservatives were purchased from Sigma-Aldrich (Germany).

Product preparation with the addition of preservatives

The preservatives in a given mixture (Tab. 1) were dissolved in distilled water and filtered using a filter with pore diameter of 0.22µm (Millipore, USA). In order to reduce the risk of recurrence of microbiological infection, the preservatives were added to the product in aseptic conditions. The outside of the bags was cleansed with a 75% (v/v) ethanol solution. Then the bags were placed in a laminar flow cabinet (Holten, Denmark). The preservative solutions were introduced through a membrane (previously washed with a 75% (v/v) ethanol solution) with the use of a sterile 50ml syringe with a sterile needle. After mixing, the bags were moved to climate chambers with temperatures of 4 or 25°C, depending on the variant of the experiment (Philips, Netherlands). The bags were stored (protected from light) for 30 days. After that time, the bags were once again placed in a laminar flow cabinet; 200ml samples were collected for microbiological analyses. Each experiment was repeated three times. Bags containing the product without any preservatives added were used as control samples.

Microbiological analysis

The samples for microbiological analyses were prepared in accordance with the Polish standards concerning the microbiological examination of food and animal feeds **(PN-EN ISO 6887-1:2000; PN-EN ISO 7218:2008)**. Total aerobic mesophilic bacteria count was calculated in Plate Count Agar (PCA) (Biocorp, Poland) and incubated at 30 °C for 72 h **(PN-EN ISO 4833-2:2013-12)**. Total yeast and mold count was calculated in modified Dichloran Rose Bengal Chloramphenicol agar (DRBC) (Biocorp, Poland) and incubated at 25 °C for 5 days **(PN-ISO 21527-1:2009)**. The presence of *Escherichia coli* was determined using Lauryl Sulfate Broth (LSB) (Biocorp, Poland) after incubation at 37 °C for 24h **(PN-ISO 4831:1998)**. The presence of *Salmonella spp*. was established with the use of Xylose Lysine Deoxycholate Agar (XLDA) (Biocorp, Poland) after incubation at 37 °C for 24h **(PN-EN ISO 6579:2003)**. Microbiological data was expressed as a logarithm of colony forming units per ml (Log_{10} Cfu ml^{-1}).

Data analysis and process optimization

The data from the microbiological assays was used to calculate the coefficients of the Scheffé quadratic equation:

$$Y = \sum_{i=1}^{q} \beta_i x_i + \sum_{i<j}^{q-1} \sum_{j}^{q} \beta_{ij} x_i x_j \qquad (1)$$

Where Y is the dependent variable and β is the regression coefficient for each component. This model contains q linear terms $(\beta_i x_i,\ i= 1,2....,q)$ and $Q = q\ (q - 1)/2$ quadratic cross product terms $(\beta_{ij} x_i x_j,\ 1 \leq i < j \leq q)$. Statistical tests were performed at a significance level of α=0.05. The accuracy and general ability of the above mathematical model were evaluated using the adjusted coefficient of determination Adj-R^2 and model p value. A regression model was used to establish the optimum composition of the preservative mixture, enabling efficient reduction of the growth of foreign microflora in the product during storage. In order to verify the pertinence of the mathematical model and the developed optimum conditions, two confirmation experiments were run on the product with the addition of preservatives. Using the point prediction capability of the software, the number of viable bacterial cells after the storage period was predicted, together with the 95% prediction intervals, and compared with the observed values. The Design-Expert 9 (Stat-Ease, USA) and Origin 9.1 Pro (OriginLab, USA) commercial software was used for the regression and graphical analyses of the obtained data.

RESULTS

Microbiological quality assessment of the control sample

The production technology of the diet supplement based on the yeast *Yarrowia lipolytica* consists of several stages (Fig.1). Analysis of the production system revealed two critical control points which may constitute a potential source of secondary microbiological contamination on the production line. The first point was the hydrodynamic cavitation system, the purpose of which is the disintegration of yeast cells. The potential operation of this device with non-optimal parameters (lower temperature, cavitation pressure or lower number of passages) may reduce the efficiency of the process. As a result, the living microbial cells present in the product were capable of further growth during storage. The storage tanks and the distribution line, which was not aseptic, constituted another critical point. Taking into consideration the two control points, the assessment of the microbiological quality of the product was performed directly after hydrodynamic cavitation and distribution to polyethylene bags.

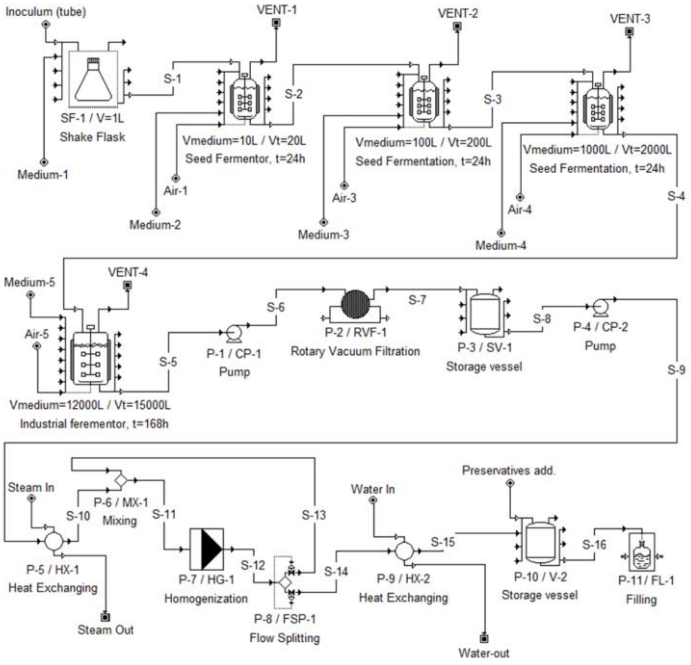


Figure 1 Processing line for the production of the diet supplement based on the yeast *Yarrowia lipolytica*. S-1...S-16 – stream numbers; P-1...P-11 – procedure numbers

In the first sample, collected directly after the head of the cavitation device, none of the studied groups of microorganisms were found. Therefore, both yeast cells and any potential foreign microflora was inactivated during disintegration. The second sample, constituting the finished product after distribution to polyethylene bags, contained no yeasts, molds, or bacteria from the *coli* and *Salmonella sp.* groups. However, the large number of aerobic mesophilic microorganisms was of concern, as it amounted to 2.01±0.04 Log_{10} Cfu ml^{-1}. This number was growing during storage and after 30 days it amounted to 3.52±0.28 and 6.21±0.15 Log_{10} Cfu ml^{-1}, respectively, for samples stored at temperatures of 4 and 25°C. In accordance with the national standards concerning the microbiological quality of food and animal feeds, the maximum total number of aerobic mesophilic bacteria should not exceed 3×10^6 Cfu g^{-1} (6.47 Log_{10} Cfu g^{-1}) **(PN-EN ISO 7218:2008)**. Therefore, the product stored for a period of 30 days in cold room conditions met these quality criteria. The results for samples stored at room temperature provided significantly worse results. After 30 days, the number of cells was slightly lower than the upper limit set by the above-mentioned national standard. It needs to be pointed out that the European Union currently has no explicit criteria concerning the maximum acceptable limits for microbiological contamination in animal feeds **(Kwiatek *et al.*, 2008)**. According to the currently applied criteria, only the presence of *Salmonella* in 25g of feed and *C. perfringens* in 1g of animal meal is considered unacceptable. The acceptable number of bacteria from the *Enterobacteriaceae* family in animal meals has also been reduced (it is assumed that in 2 out of 5 analyzed samples, the level of contamination with these bacteria cannot exceed 300 Cfu g^{-1}). Taking this into consideration, the product could theoretically be put on the market in its original form (without any addition of preservatives), provided that the rules concerning the so-called continuity of the cold chain are observed. Otherwise, the intensive growth of foreign microflora will cause a gradual decrease of the nutrient content released from yeast cells during disintegration. The fulfillment of this condition is usually quite difficult and is associated with additional investment costs, both on the part of the distributor and end recipient. In order to eliminate the necessity of using lowered temperatures during transportation and storage of the finished product, a decision was made to use preservatives.

The influence of preservatives on product stability

As indicated by the data presented in Tab. 1, the number of bacteria, expressed as Log_{10} Cfu ml^{-1}, differed significantly depending on the composition of the preservative mixture. In the case of samples stored in cold room conditions, depending on the variant of the experiment, the number of cells varied from 1.0 to 3.62 Log_{10} Cfu ml^{-1}. Storage at 25°C resulted in slightly higher infection levels, with the number of cells ranging from 1.0 to 3.72 Log_{10} Cfu ml^{-1}. Therefore, in the case of samples containing preservatives, storage temperatures were not a very significant factor. However, the applied additives enabled a reduction of the number of cells by several orders of magnitude in comparison to the control sample. In order to establish how the composition of the preservative mixture influenced the change of the number of bacterial cells during storage, the collected data was analyzed with the use of backward stepwise regression.

Table 1 Component concentrations in the preservative mixtures prepared in accordance with mixture designs and experiment results expressed as Log_{10} $Cfuml^{-1}$ from the total number of mesophilic bacteria (TMB).

Run	Components in actual scale			Components in real scale*			Dependent variables	
	Sodium benzoate [mg l^{-1}]	Sodium sorbate [mg l^{-1}]	Phosphoric acid [mg l^{-1}]	Sodium benzoate X_1	Sodium sorbate X_2	Phosphoric acid X_3	Log_{10} Cfu ml^{-1} TMB T=4°C	Log_{10} Cfu ml^{-1} TMB T=25°C
1	1248	0	1252	0.499	0.000	0.501	1.000	1.000
2	1240	1260	0	0.496	0.504	0.000	3.301	3.602
3	0	610	1890	0.000	0.244	0.756	3.623	3.716
4	1240	1260	0	0.496	0.504	0.000	3.204	3.415
5	0	2500	0	0.000	1.000	0.000	2.973	2.903
6	618	1457	425	0.247	0.583	0.170	2.806	2.934
7	0	0	2500	0.000	0.000	1.000	2.342	3.301
8	0	1256	1244	0.000	0.503	0.497	2.079	2.380
9	2500	0	0	1.000	0.000	0.000	2.716	2.982
10	632	0	1868	0.253	0.000	0.747	1.623	1.845
11	0	1934	566	0.000	0.774	0.226	2.914	2.914
12	1248	0	1252	0.000	1.000	0.000	3.623	2.954
13	1240	1260	0	1.000	0.000	0.000	2.833	2.732
14	0	610	1890	0.000	0.000	1.000	2.176	2.602
15	1240	1260	0	0.730	0.038	0.232	1.000	1.477
16	0	2500	0	0.340	0.331	0.329	1.477	2.255
17	618	1457	425	0.499	0.000	0.501	1.000	1.000
18	0	0	2500	0.340	0.331	0.329	2.000	2.663
19	0	1256	1244	0.000	0.503	0.497	2.892	2.903
20	2500	0	0	0.496	0.504	0.000	2.663	2.663

*Real scale - each component was re-scaled so that the total was 1.0

The statistical analysis results of the data concerning the products stored at 4°C indicated that all ingredients of the preservative mixture had significant influence on the final number of cells. The statistical analysis results of the samples stored at 25°C were similar. Both regression models accounted for about 78% of variations (Tab. 2).

Table 2 Summary of ANOVA results for mixture design

Storage temperature	Source	Sum of Squares	df	Mean Square	F value	p-value	Adj-R^2
4 °C	**Model**	10.54	3	3.51	19.26	< 0.0001	0.773
	Linear Mixture	4.87	2	2.43	13.34	0.0004	
	AC	5.67	1	5.67	31.11	< 0.0001	
	Residual	2.92	16	0.18			
	Lack of Fit	1.98	8	0.25	2.12	0.1545	
	Pure Error	0.94	8	0.12			
	Cor Total	13.46	19				
25°C	**Model**	9.30	4	2.33	18.25	< 0.0001	0.784
	Linear Mixture	2.15	2	1.07	8.43	0.0035	
	AB	0.44	1	0.44	3.42	0.0841	
	AC	6.34	1	6.34	49.78	< 0.0001	
	Residual	1.91	15	0.13			
	Lack of Fit	0.92	7	0.13	1.06	0.4621	
	Pure Error	0.99	8	0.12			
	Cor Total	11.21	19				

Their usefulness for predicting the number of cells in the space established by the studied independent variables was finally confirmed by a lack-of-fit test. In both cases, the probability value *p* was higher than 0.05, which clearly suggests that the experimental models described the relationship between independent and dependent variables, expressed with the use of the equations presented in Tab. 3, with a satisfactory degree of quality.

Table 3 Final equations for the regression models describing the number of microbial cells in the product during storage at 4 and 25°C.

Storage temperature	Componets scale	Regression equation	Eq.
4 °C	**Real**	$\mathrm{Log}_{10}Cfuml^{-1} = 2.706\mathrm{X}_1 + 3.197\mathrm{X}_2 + 2.498\mathrm{X}_3 - 6.961\mathrm{X}_1\mathrm{X}_3$	(1)
	Actual	$\mathrm{Log}_{10}Cfuml^{-1} = 1.08 \times 10^{-3}\mathrm{X}_1 + 1.27 \times 10^{-3}\mathrm{X}_2 + 9.99 \times 10^{-4}\mathrm{X}_3 - 1.11 \times 10^{-6}\mathrm{X}_1\mathrm{X}_3$	(2)
25°C	**Real**	$\mathrm{Log}_{10}Cfuml^{-1} = 2.995\mathrm{X}_1 + 3.047\mathrm{X}_2 + 3.068\mathrm{X}_3 - 7.574\mathrm{X}_1\mathrm{X}_3$	(3)
	Actual	$\mathrm{Log}_{10}Cfuml^{-1} = 1.19 \times 10^{-3}\mathrm{X}_1 + 1.21 \times 10^{-3}\mathrm{X}_2 + 1.22 \times 10^{-3}\mathrm{X}_3 - 1.21 \times 10^{-6}\mathrm{X}_1\mathrm{X}_3$	(4)

Apart from the main factors, the interaction between the concentration of sodium benzoate and phosphoric acid was also statistically significant (Tab. 2). This dependency was parabolic with a clearly marked minimum. The preservative mixture with a composition corresponding to this point was characterized by the highest effectiveness in inhibiting undesirable microflora growth during product storage (Figure 2a, b).

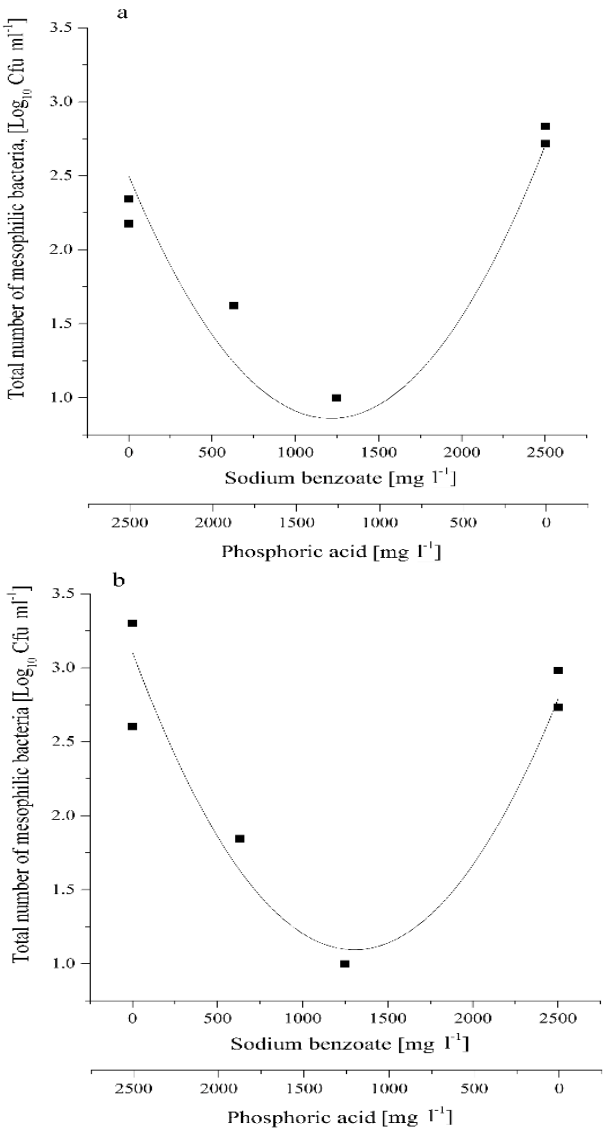


Figure 2 Interaction plot for sodium benzoate and phosphoric acid for products stored at 4 (a) and 25°C (b)

Increased concentration of sodium benzoate in the preservative mixture with a simultaneous decrease in the concentration of phosphoric acid had a negative influence on its effectiveness. This was caused by increased mixture pH, resulting from a lower content of phosphoric acid (Figure 3).

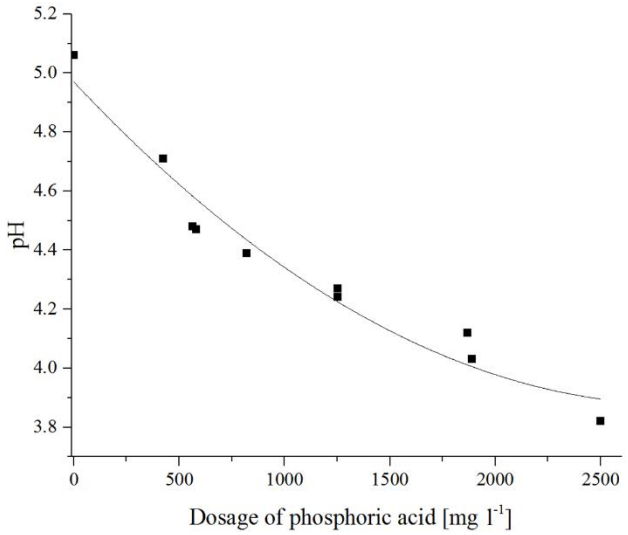
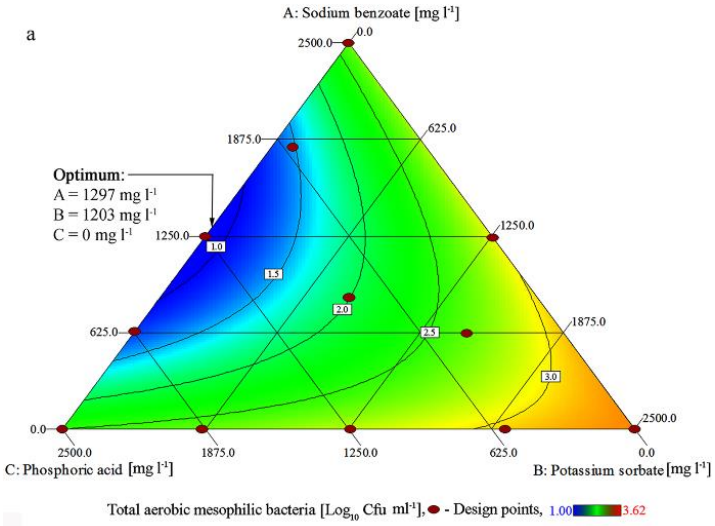


Figure 3 Influence of phosphoric acid concentration in the mixture on product pH.

The antimicrobial activity of weak organic acids (benzoic and sorbic) and their salts (sodium benzoate and potassium sorbate) is highly dependent on the pH of the environment. In general, the lower the pH, the higher the effectiveness of these substances **(Alnoman *et al.*, 2015)**. This mainly results from the fact that in solutions with highly acidic pH, the majority of conjugated benzoic acid occur in their undissociated form. In accordance with the pKa value of benzoic acid, in order for the concentration of the undissociated form to equal 50%, the pH of the solution should be 4.20. The low pH and clearly lipophilic nature of this molecule facilitates its penetration of the cell membrane, which contains a hydrophobic lipid layer. When inside a cell, benzoic acid are ionized, which results in the disturbance of substrate transportation process (including amino acids) and oxidative phosphorylation in the electron transport system. As a results of the ionization of acid molecules, protons are released inside the cell, which then becomes acidified. Consequently, not only the permeability of the cell membrane is altered, but also the intensity of some metabolic transformations (e.g. the Krebs cycle), which leads to the inhibition of cell growth **(Chipley, 2005; Davidson *et al.*, 2013)**. In the experiments performed with the use of preservative mixtures without phosphoric acid, the pH was 5.06. Therefore, the concentration of the undissociated form of benzoic acid, calculated using the Henderson–Hasselbalch equation, was about 12%, which is in accordance with the literature **(Baird-Parker, 1980)**. With such a low content of the undissociated form in the solution, the amount of preservative molecules penetrating into cells was most likely low, which explains the reduction of effectiveness in the case of preservative mixtures with no or low phosphoric acid content. The preservative mixture with a composition corresponding to the minimum on the parabola presented in Fig. 2a, b contained 1247.85 mg l^{-1} of sodium benzoate and 1252.15mg l^{-1} of phosphoric acid. With this concentration of phosphoric acid, the pH of the product was 4.26, which means, in accordance with the previously mentioned Henderson–Hasselbalch equation, that about 46% of benzoic acid molecules were in their undissociated form, which could easily penetrate bacterial cells envelope and cause growth inhibition.

Process optimization and validation

In order to establish the optimum composition of the preservative mixture, optimization calculations were conducted using the simplex method, which provides excellent results in the search for the local minimums of functions of several variables **(Nelder and Mead, 1965)**. According to these calculations, the product should contain only benzoic and phosphoric acids in its composition (Figure 4a, b). There is also no need to use the third of the studied preservatives, potassium sorbate, which is very beneficial from the point of view of the costs associated with the production of the *Yarrowia lipolytica* yeast biomass-based supplement. The predicted number of cells, expressed as Log_{10} Cfu ml^{-1}, after 30 days should be equal to 0.914 ± 0.23 and 1.095 ± 0.19, respectively, for samples stored at temperatures of 4 and 25°C.



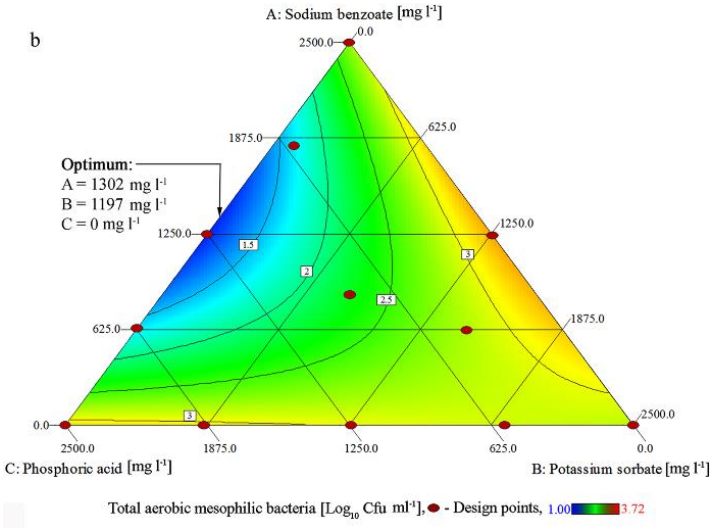


Figure 4 Contour plot depicting the relation between sodium benzoate, potassium sorbate, and phosphoric acid and the total number of mesophilic bacteria in the product during storage at 4 (a) and 25°C (b).

In order to verify the acquired data, a validation study was performed in accordance with the new technological premises resulting from optimization and the analysis of the control points on the production line. In accordance with the new guidelines, a concentrate of preservative substances was added to cell homogenate in the process of hydrodynamic cavitation during the 16 passage of the cell suspension through a cavitation head. The product was stored at 4 and 25°C for a period of 60 days.

The applied production process modifications enabled the uniformity of the cell homogenate and the reduction of the levels of infection with foreign microflora during distribution to packages. The product maintained its stability throughout a period twice as long as the one originally assumed (Figure 5). The number of bacterial cells was within the prediction interval established during optimization calculations, which explicitly confirms their correctness.

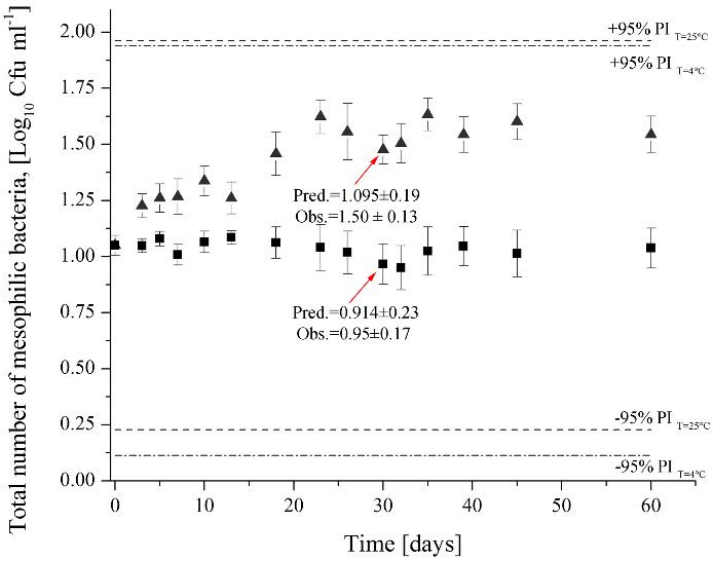


Figure 5 Changes in the total number of mesophilic bacteria during 60 days of storage at temperatures of 4 (■) and 25°C (▲). ·-·-·-·-·- prediction interval for samples stored at 4°C, ------ - prediction interval for samples stored at 25°C

DISCUSSION

Sodium benzoate, potassium sorbate, and phosphoric acid were selected for research focusing on the improvement of microbiological stability of innovative dietary supplement for farm animals. These substances were chosen mainly because of the fact that their use in food and animal feeds is authorized and that they possess the GRAS (Generally Recognized As Safe) status **(Busta *et al.*, 2005; Chipley, 2005)**. Other factors that were taken into consideration included the ease of their introduction into the product, lack of taste, smell, or color, as well as the relatively low cost of purchase. It was also taken into account that these compounds, especially benzoic acid, naturally occur in many fruit (grapes, blackcurrants, apples, cherries, strawberries), fermented products (black tea, smear-ripened cheese, wine, beer), and other products, such as honey or mushrooms. Benzoic acid is also produced by the *Lactobacillus plantarum* probiotic bacteria **(Niku-Paavola *et al.*, 1999)**. When it comes to sorbic acid, it naturally occurs in rowanberries **(Busta *et al.*, 2005)**. The concentration of these substances in products of natural origin is very diverse, e.g. the content of benzoic acid ranges from 10 to 1000 mg kg^{-1} **(Chipley, 2005)**. In the presented work, the optimum concentration of sodium benzoate in cell homogenate of the yeast *Yarrowia lipolytica* (constituting a diet supplement for farm animals) stored at 4°C was 1297mg l^{-1}, while for products stored at 25°C it equaled 1307mg l^{-1}. A very similar combination, in terms of the concentration of benzoic acid and pH (1250mg l^{-1} and pH 3.9), was used for the stabilization of pasteurized papaya juice **(Okoli and Ezenweke, 1990)**. The bottled drink was stored for 80 weeks at temperatures of 10 and 30 °C, while the control sample, which underwent the process of pasteurization only, remained fresh for just 20 weeks. The use of sodium benzoate at a concentration of 1000mg l^{-1} also significantly extended the shelf life of a tamarind drink. Significant deterioration of the control sample was visible as early as after two days of storage at room temperature; the addition of sodium benzoate extended shelf life by forty days **(Adeola and Aworh, 2014)**. According to the data found in the literature, the minimum inhibitory concentration (MIC) of benzoic acid for bacteria ranges from 50 to 3000 mg l^{-1} **(Chipley, 2005)**. The resistance of yeasts and fungi to benzoic acid is also very diverse. In the case of *Sporogenic yeast*, MIC ranges from 20 to 200 (pH 2.6-4.5), while successful inhibition of the growth of *Zygosaccharomysec baili* requires the use of concentrations between 1200 and 4500mg l^{-1} (pH 4.0-4.8) **(Praphailong and Fleet, 1997)**. Among fungi, the highest resistance to benzoic acid is exhibited by the *Aspergillus parasiticus* (>4000mg l^{-1}, pH 5.5) and *Aspergillus niger* (2000mg l^{-1}, pH 5.0) species **(Steels *et al.*, 1999; Davidson *et al.*, 2001; Chipley, 2005)**. Our results concerning lower antimicrobial activity of potassium sorbate, when compared to the activity of sodium benzoate, could be probably attributed to the fact, that certain bacterial strains are not inhibited by sorbate, and some may even metabolize the compound **(Sofos, 1989)**. Overall, potassium sorbate is considered as a more effective inhibitor of yeasts and molds than bacteria **(Sofos, 1989; Marie Skirdal and Eklund, 1993; Busta *et al.*, 2005)**. In general, the effectiveness of such preservatives as weak organic acids is dependent on the type of strain and environmental conditions (pH, ionic strength, surfactant presence). A single strain may exhibit different resistance to a given preservative depending on the environmental conditions in which it occurs **(Busta *et al.*, 2005)**. Therefore, a series of optimization studies needs to be conducted each time to establish the actual doses of preservative substances required to effectively inhibit the growth of undesirable microflora.

In accordance with the applicable legislation, the studied preservatives are authorized for use as feed additives (70/524/EEC; EC 1831/2003; Comission, 2004). Moreover, in May 2011, the European Commission passed a regulation concerning the authorization of sodium benzoate as a feed additive for weaned piglets. This product is intended for use in weaned piglets from weaning to 120 days of age (or 35 kg bw) at a dose of 4000 mg kg-1 of complete feeding stuffs. In accordance with the opinion prepared by experts from the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) of the European Food Safety Authority (EFSA), sodium benzoate is safe for target species at the proposed dose with a margin of safety of approximately three (EFSA Panel, 2011). The research on which the above-mentioned directive was based indicates that benzoate is rapidly excreted and no residues or accumulation occur. Moreover, the initiator (Kemira, Sweden) provided five efficacy trials, two of which showed a significant benefit in terms of final body weight. In addition, a meta-analysis made by pooling data from the five trials showed a significant improvement of the feed to gain ratio. Consequently, the FEEDAP concludes that sodium benzoate at a dose of 4000 mg kg-1 of complete feed has the potential to improve performance in weaned piglets. In 2012, the FEEDAP prepared an expertise on the safety and efficacy of sodium benzoate as a silage additive for pigs, poultry, bovines, ovines, goats, rabbits and horses. Three studies with laboratory-scale silos are described, each lasting at least 90 days **(EFSA Panel, 2012)**. The results showed that the application of sodium benzoate in the preparation of silage up to the maximum proposed dose of 2400 mg kg^{-1} of forage was safe for the target animals. It was demonstrated that sodium benzoate at concentrations between 250 and 2500 mg kg^{-1} forage also has the potential to increase the aerobic stability of ensiled materials at a wide range of dry matter contents.

CONCLUSIONS

Our experiments showed significant improvement in the microbial quality of diet supplement, when compared to the control sample without preservatives addition. In this study, the established optimum doses of chemical preservatives were lower than the doses included in the above-mentioned documents of the European Commission. It can be concluded that the novel diet supplement based on the homogenate of the cells of yeast *Yarrowia lipolytica* can be considered safe for animals.

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STATISTICAL APPROACH FOR PECTINASE PRODUCTION BY *Bacillus firmus* SDB9 AND EVALUATION OF PECTINO-XYLANOLYTIC ENZYMES FOR PRETREATMENT OF KRAFT PULP

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ABSTRACT

The present study originated with the concomitant production of 1.94 IU.mL^{-1} of pectinase, 0.34 IU.mL^{-1} xylanase and 0.086 IU.mL^{-1} cellulase from a newly isolated *Bacillus firmus* SDB9 in pectin salt media. Plackett-Burman Design (PBD) and Box-Behnken design (BBD) were used for optimization of mass production of pectinase using agro-residues. Statistical optimization of medium enhances the pectinase production to 17.55 IU.mL^{-1}, 17.7% higher activity than that of unoptimized medium. The optimal pectinase activity was found at pH 8.5 and 45°C temperature. The enzyme was alkali-stable over a range of pH 7.0 to 10.0 for 1 h and thermostable at 35 to 70°C for 1h. Out of 12 tested metal cations at 1mM concentration, the enzyme was found to be hindered by the presence of three cations, whereas four cations were reported to augment, and the rest marginally subdued the activity. Pretreatment with 15 IU pectinase and 2.7 IU xylanase per gram of OD pulp reduce kappa number by 7.9%. After bleaching sequence D_0-E_p-D_1, the increment in pulp brightness was 2.7% with acceptable whiteness level. Pretreatment also led to a reduction in ClO_2 consumption by 15% with superior brightness level. The 0.83% increase in pulp viscosity along with 3.2% gain in double fold number indicated the maintenance of relative cellulose content and strength of paper. Minor variations in burst index, tensile index and tear index reflected the conserved properties of the treated pulp. Synergistic application of mixed enzymes preparation produced from raw agro-residue headed by pectinase in preference to xylanase is first time set forth in the present study to help in cost economization and propel the pulp and paper industries towards environmental friendly future.

Keywords: Pectinase; xylanase; optimization; *Bacillus firmus* SDB9; pretreatment; kraft pulp

INTRODUCTION

The pulp and paper industry uses an immense amount of hazardous chemicals to process inordinately high quantities of raw materials for the production of virgin pulp. Pulp and paper industries account for creating extremely high pollution in the environments globally (**Sumathi and Hung, 2006; Thompson *et al*., 2001**). Therefore, key attention has been to develop cost effective and environmentally benign bleaching technologies for a reduced adsorbable organic halogens (AOX) generation as many countries have now set discharge limits for these compounds in the generated effluents.With the progress in the field of biotechnology; enzymes have found their way into many new industrial processes. Enzymes are already well established in the processing of pulp and paper **(Gavrilescu and Chisti, 2005)**. The earlier reports have shown interesting implications of the enzymes namely xylanase, pectinase, mannanase and laccase (**Lahtinen *et al*., 2009; Várnai *et al*., 2011**) in pretreatment and processing of kraft pulp that collectively reduce the toxic discharge of pulp and paper industries.

Xylanase pretreatment of kraft pulp is already being employed across the globe with initial efforts by **Viikari *et al*., (1986)**. However, the combined use of xylanase with pectinase prove a better option for treatment of Kraft pulp (**Dhiman *et al*., 2009**). Parenthetically the use of pectinase in the Kraft pulp pretreatment was proposed by **Ahlawat *et al*., (2007)**. Since then the combination of xylanase with pectinase is being tried (**Kaur *et al*., 2010**). Nevertheless, Xylanase is the key enzyme in the kraft pulp pretreatment due to its significant role in the kappa number reduction, which reflects the lignin proportion due to its better penetration as a bleaching agent after removing hexenuronic acid from the pulp (Gangwar, Prakash, & Prakash, 2014).In contrast, pectinase depolymerize polymers of galacturonic acids, and subsequently lower the cationic demand of pectin solutions and the filtrate from peroxide bleaching during paper making (**Reid and Ricard, 2000; Viikari and Tenakanen, 2001**). Hence, the combinatorial approach with the reverse proportion of xylanase and pectinase (where pectinase is more than xylanase) may serve as an innovative solution in kraft pulp pretreatment. Nonetheless, this area remains unexplored.

Production of xylanase from *Bacillus* species has been reported by several researchers (**Ersayin *et al*., 2010; Nagar *et al*., 2010**). Similarly, pectinase production from *Bacillus* species has also been reported (**Basu *et al*., 2008; Sharma & Satyanarayana, 2006**). Moreover there is a single report on simultaneous production of xylano-pectinolytic mixture from the same strains of *B. pumilus* (**Kaur *et al*., 2010**) but there is no report available till date regarding simultaneous production of pectino-xylanolytic mixture from single strain of *Bacillus*.The possibility to use crude enzymes for pretreatment is the first and foremost step in reducing the cost of the process (**Viikari *et al*.,1987; Viikari *et al*., 1986).** The second step is the cost-effective production of pectino-xylanolytic enzymes to improve the process economics of biobleaching at industrial scale. Pure substrates being highly expensive, it cannot be afforded at the industrial level bulk production of enzymes (**Adhyaru *et al*., 2014**).This goal can be achieved by employing a huge amount of residual plant biomass considered as waste. In Indian subcontinents, orange peel is massively generated waste agri-residues suitable for pectinase production. However, to use crude and raw substrates for the enzymes production, successful optimization of media is a pre-requisite (Kumar et al., 2014). The traditional practice using OVAT approach is time consuming and cumbersome as against the statistical approach that additionally offers the analysis of interactive effects of various process parameters (**Embaby *et al*., 2014**).Response surface methodology is a well-accepted statistical approach to enhance the enzyme yield though the magnitude of increase may vary **(Ali *et al*., 2013)**. However, finally RSM renders the process more feasible at industrial scale.

Assessment of the bleaching potential of crude enzymes in terms of kappa number reduction and improvement in brightness is a well-established strategy (**Choudhury *et al*., 2006**). Moreover, the positive inputs in optical and strength properties of pulp and paper support the same. Several studies of the application of crude xylanase with pectinase in pulp bleaching have been reported, but the use of the high content of pectinase than xylanase was not being tried earlier that is presented herewith.

METHODOLOGY

Isolation, Screening and Identification of pectinase and xylanase producer strain

A bacterial strain was isolated from soil samples contaminated with the effluent of an oil refinery, Digboi, Assam, India. The pooled soil samples were homogenized and serially diluted in sterile distilled water followed by plating on pectin agar medium containing pectin 5.0 g.L $^{-1}$ and yeast extract 1.0 g.L^{-1} (pH 8.0). All plates were incubated at 35±2.0ºC for 24 to 72h. Morphologically distinct colony was purified by repeated streaking. Primary evaluation of potent bacterial strains for pectinase production was carried out by plate assay method (**Wood *et al*., 1988**) using nutrient agar supplemented with 0.5% w/v of pectin. The plates were incubated for the growth and then flooded with 1% CTAB. Positive pectinase activity was detected by the clear zone surrounding the colonies. Similarly, the strains were screened for xylanase and cellulase activities using Bushnell-Haas medium containing 0.5% w/v xylan and 1 % w/v carboxymethyl cellulose (CMC) as a substrate. The plates were incubated for growth and stained with 1% w/v Congo red dye and destained with 1.0 M NaCl. Positive xylanase and cellulase activities were detected by the presence of yellow halo against a red background. Potential strainSDB9 was identified based on 16S rRNA gene sequencing. The phylogenetic relationships with some reference strains were determined using the neighbor-joining method using Mega 5.0.

Concurrent production of enzymes and growth profile

Pectino-xylanolytic enzymes were produced under submerged fermentation in 250mL Erlenmeyer flasks containing 100mL of production medium consisting of Yeast extract (0.1 %), $(NH_4)_2SO_4$ (0.2 %), NA_2HPO_4 (0.6 %), KH_2PO_4 (0.3 %), $MgSO_4.7H_2O$ (0.1%),$CaCl_2$ (0.001%) and Pectin (0.5 %) as a sole carbon source (pH 8.0) incubated under shaking (120 rpm) conditions at 40 °C. After 48 h, the fermented broth was centrifuged, and the cell-free supernatant was subjected to purification. The enzyme was partially purified by precipitation with ammonium sulfate and subsequent dialysis from the supernatant obtained by separating the biomass through refrigerated centrifugation at 10,000 g for 20 min and used for further studies.

Enzyme assay

The pectinase activity was assayed by measuring the amount of reducing sugars (xylose equivalent) liberated from polygalacturonic acid using 3, 5-dinitrosalicylicacid (**Miller, 1959**). The xylanase activity was assayed according to the method of **Bailey *et al*., (1992)**. Cellulase activity (carboxymethyl cellulase) was determined as per IUPAC method (**Ghose, 1987**). One unit (IU) of pectinase, xylanase and cellulase activities were defined as the amount of enzyme that catalyzed the release of 1.0 micro mol of reducing sugar as galacturonic acid, xylose and glucose equivalent per minute under the specified assay conditions respectively.

Effect of nitrogen and crude pectin sources on pectinase production

Three inorganic nitrogen sources KNO_3, $NaNO_3$, NH_4Cl and three organic nitrogen sources peptone, yeast extract and casein at 0.5% w/v were studied for optimum enzyme production. A control lacking nitrogen source was run. The pectinase production was also tested by replacing 0.5% w/v pectin with agricultural materials viz. orange peel, hardwood bark, banana peel and jute in the production media. These agricultural waste materials were washed with distilled water and dried in sunlight and powdered. The lignocellulosic substrates were given pretreatment before using it in production medium. For this purpose, the dried powder was treated with 1.0% w/v NaOH for 2 h, washed with distilled water for several times, and allowed to air dry to use as a medium component (**Gharpuray *et al*., 1983**).

Media optimization for pectinase production

The impacts of 10 independent variables on enzyme activity were tested using Plackett-Burman Design (**Plackett and Burman, 1946**). Each variable was tested at two levels: the high level (+) and the low level (-) as shown in Table 1. The factors investigated in the current study included salts $(NH_4)_2SO_4$, Na_2HPO_4, KH_2PO_4, $CaCl_2.2H_2O$, $MgSO_4.7H_2O$, yeast extract, orange peel pH, incubation temperature and incubation period.Total 13 runs have been performed and actual experimental and predicted values of pectinase production have been measured.

Table 1 Variables included in the PBD design

Code	Independent variables	Level of variables	
		Low level	High level
A	$(NH_4)_2SO_4$ (% w/v)	0.02	2.0
B	Na_2HPO_4 (% w/v)	0.06	6.0
C	KH_2PO_4 (% w/v)	0.03	3.0
D	$MgSO_4.7H_2O$ (% w/v)	0.01	1.0
E	Yeast extract (% w/v)	0.01	1.0
F	Orange peel (% w/v)	0.5	5.0
G	pH	7.0	9.0
H	Incubation temperature (°C)	50	37
J	Incubation Period (h)	24	72
K	$CaCl_2.2H_2O$ (% w/v)	0.001	0.01

RSM using BBD was applied for optimization of pectinase production that includes full factorial experiment and observation of simultaneous, systematic and efficient variation of significant components on the fermentation process. Three important parameters namely Orange peel concentration (X1), incubation temperature (X2) and initial pH (X3) were selected as the independent variables based on the significant model terms obtained by ANOVA analysis of PB Design and the pectinase activity (IU.mL^{-1}) was the dependent response variable. Each of these independent variables was studied at three different levels as per BBD with a total of 15 experimental runs using statistical software package Design Expert 9.0.5, Stat-Ease, Inc., USA. Pectinase activity (IU.mL^{-1}) corresponding to the combined effects of three variables was studied in their specified ranges as shown in Table 2. All the flasks were analyzed for pectinase activity. Three-dimensional curves were generated with the same software.

Table 2 Experimental range and coded levels of process variables for pectinase production

Coded value	Significant process variables	Range and level of variables		
		-1	0	+1
X1	Orange peel (% w/v)	2.5	3.5	5.0
X2	Incubation temperature (°C)	35	40	45
X3	pH	8.0	8.5	9.0

For statistical calculations the independent variables were coded as:

$$xi = (Xi - X0)/ \delta Xi \quad (1)$$

Where Xi is the experimental value of the variable; X0 is the mid-point of Xi, $^{\delta}$Xi is the step change in Xi and xi is the coded value for Xi, i=1–3.
This response surface methodology allows the modeling of a second order equation that describes the process. Pectinase production data was analyzed and response surface model given by Eq. (2) was fitted with multiple regressions through the least squares method.

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_i \quad (2)$$

where β_0, β_i, β_{ii}, and β_{ij} represents, respectively the constant process effect, the linear effect of Xi, quadratic effect of Xi and the interaction effect between Xi and Xj on pectinase activity denoted by Y**(Chapla *et al*., 2010**).

Enzyme characterization

The reaction mixture containing 0.4 mL of 0.1% polygalacturonic acid prepared in 0.1 M Glycine-NaOH buffer (pH 8.5) and 0.1 mL of appropriately diluted enzyme was incubated at 40, 50, 60, 70 and 80˚C temperatures for 10 min for pectinase assay to determine the optimum temperature of the reaction.The pH optima of pectinase at 40°C temperature was determined by measuring activity at various pH values using different buffers, such as sodium citrate (pH 5.5), sodium phosphate (pH 6.0, 6.5, 7.0, 7.5, 8.0), and glycine-NaOH (pH 8.5, 9.0, 9.5) each at 50 mM concentration under standard assay conditions using polygalacturonic acid as substrate.
Rate of polygalacturonic acid hydrolysis was determined, after incubating substrate at various concentrations at optimized conditions, by measuring the enzyme activity at different substrate concentration. The substrate concentration ranged from 2.5 to 25 mg.mL^{-1}. Rate of the reaction was calculated using absorbance at 550nm. Graph of substrate concentration against reaction rate was

plotted. Kinetic parameters such as Km and Vmax were calculated from the Lineweaver-Burk plot.
Effect of various metal salts viz. $AgNO_3$, $CaCl_2$, $CoCl_2$, $CuCl_2$, $FeCl_3$, $HgCl_2$, KCl, $MgCl_2$, $MnCl_2$, NaCl, $NiCl_2$ and $ZnCl_2$ at a final concentration of 1mM for 5 min was determined under standard assay condition. Thermostability of the pectinase was determined by preincubating the enzyme at 35, 45, 50, 60, 70 and 80°C temperature up to 90 mins. After each interval of 30 min, the enzyme was withdrawn and residual enzyme activity was determined by standard assay procedure.Likewise, the pH stability of pectinase was ascertained by preincubating the enzyme in buffers of different 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5 and 10.0 pH. After 30 and 60 min time interval, the enzyme was withdrawn and residual enzyme activity was determined by standard assay procedure.

Pulp sample

Unbleached Kraft pulp used for the present study was a mixture of 82 to 84% mixed hardwood and 16 to 18% bamboo pulp provided by Central Pulp and Paper Research Institute, Saharanpur, U. P., India. The chemical composition of the pulp used was cellulose 75 to 76%, hemicellulose 19 to 20%, lignin 3 to 5% and pectin 0.1%.

Enzymatic pretreatment of Kraft pulp (E_0)

One variable at a time approach (OVAT) was taken to establish the best reaction conditions. Unbleached hardwood pulp was pretreated with the pectino-xylanolytic preparation as per standard laboratory manual of testing procedure of Central Pulp and Paper Research Institute (CPPRI), Saharanpur, U.P. India. Pretreatment of pulp with pectino-xylanolytic enzymes was carried out with different enzyme dosage at 50 °C temperature and pH 8.0 for a retention time of 120 min based on preliminary studies (Table 3). Shredded, screened and unbleached pulp comparable to 100 OD (oven dried) was taken and divided into two parts. One part was kept as control and the second part was treated with a pectino-xylanolytic preparation of crude enzyme in the ratio of pectinase and xylanase of 5.5:1 by adding 5.15 mL and 7.73 mL per gram of OD pulp in set-I and set-II respectively, while maintaining the pulp consistency at 10%. The pulp was thoroughly mixed with enzyme preparation by kneading mechanism and transferred in polyethylene bags to put in a water bath. Control samples were subjected to the same treatment conditions except the enzyme.

Table 3 Pectino-xylanolytic Enzyme Treatment of unbleached pulp (P- stage)

Particulars	Control	Enzyme treated pulp	
Enzyme dose, $IU.gm^{-1}$	-	*Set-I	#**Set-II**
Consistency (%)	10	10	**10**
Temperature (°C)	50	50	**50**
pH	8.0	8.0	**8.0**
Treatment Time (min)	120	120	**120**

*Set-I =P 10.0 +X 1.8, #Set-II = P 15.0 + *X 2.7 where P = Pectinase and X = Xylanase.

Bleaching of Pulp (D_0-E_p-D_1)

The enzyme pretreated pulp was subjected to chemical bleaching through a) D_0-stage (chlorine dioxide treatment - CD), b) E_p-stage (alkali treatment) and c) D_1-stage. Chlorine dioxide dosage was ascertained by estimation of Kappa No. of unbleached pulp. Pulp Bleaching process conditions are briefly summarized in the table 4.

Table 4 Pulp bleaching process conditions

Parameter/stage	D_0	E_p	D_1
Consistency (%)	10%	10%	10%
Temperature (°C)	55	70	80
pH	2-3	>11	3-4
Retention time (min)	45	60	180

a) D_0-stage (chlorine dioxide stage)

In this stage, control pulp was mixed with 6.47 % ClO_2 while the enzyme treated pulp was distributedinto two parts. One part was treated with the same dose of ClO_2 (6.47 %) whereas the other part was treated with a lesser dose of ClO_2 (5.50%). All the three pulp samples (control, same and less dose) were put in the polyethylene bags and placed in the water bath.

b) E_p-stage (alkali extraction stage)

The control and the pulp samples treated with higher and lesser dose of ClO_2 were treated with 2.5% of NaOH and 1.0% of H_2O_2 in different polyethylene bags and put in a water bath. After that, the pulp was washed similarly as done at the end of P-Stage and pulp pad was prepared.

c) D_1-stage (dioxide Stage)

The alkali extracted pulp was treated with Chlorine dioxide in the same way as mentioned in D_0 –Stage. Subsequently the pulp was washed as done after Pretreatment Stage and pulp pad was prepared and tested for kappa number, brightness, whiteness and yellowness as per TAPPI (Technical Association of the Pulp and Paper Industry, Atlanta) protocols.

Analysis of the pulp-free filtrate

Miller's method (1959) and procedure given in Laboratory Manual of Testing Procedure, CPPRI, Saharanpur, U.P. were followed to determine the Total Reducing Sugar (TRS), colour (A_{456}), lignin (A_{280}) and phenolics (A_{237}) of pulp filtrates.Lignin content of the effluents was measured by A_{280}. Samples were diluted if required to acquire the absorbance values within of the range 0.2-0.8. Lignin was calculated using the formula, lignin, $mg.mL^{-1}$ = A_{280} x dilution factor/absorptivity of lignin, where the value 21 is used for hardwood and 20 for agro-based lignin.

Analysis of pulp and paper properties

The enzyme treated pulp was carefully washed and hand sheets were prepared under standardized pressure and air-dried in a room with standardized light, humidity and temperature. The investigation of pulp properties was performed according to TAPPI standard methods. Kappa number, the measure of degree of lignin content of pulp, is estimated by the reaction of pulp samples with acidified potassium permanganate to measure the lignin content **(TM I-D1)**/TAPPI method T236 cm-85). The brightness of the hand sheets was measured as %ISO (International Organization for Standardization, ISO) by reflectance at 457 nm with ISO Colourtech, USA, according to TAPPI protocol (T-452 om-87). The yellowness, whiteness and fluorescence of pretreated pulp were also evaluated by ISO Colourtech, USA at 457 nm (T 1216).

RESULTS AND DISCUSSION

Isolation, Screening and Identification of pectinase and xylanase producer strain

In the present study, 24 bacterial isolates were obtained from the soil contaminated with the effluent of an oil refinery, Digboi, Assam, India. Based on the results of the screening a bacterial strain SDB9 which produced maximum pectinase with concurrent production of mediocre levels of xylanase and negligible cellulase activity was selected for further study. The isolate was identified as ***Bacillus firmus* SDB9** based on 16S rRNA gene sequencing. The sequence was submitted to GenBank with the accession number **KP881618**. The phylogenetic relationship of the isolate SDB9 with other reference taxa is shown in figure 1. The isolate efficiently grew at 30 to 45 °C temperature with an optimum at 40°C (Figure 2a) and optimum pH for the growth was 8.0. *Bacillus firmus* reported from the diverse habitats are well-known for the hydrolases producer. Earlier, *Bacillus firmus* had been reported to be the producer of pectinase and xylanase **(El-Shishtawy *et al*., 2014; Ratanakhanokchai *et al*., 2002; Roosdiana *et al*., 2013**).

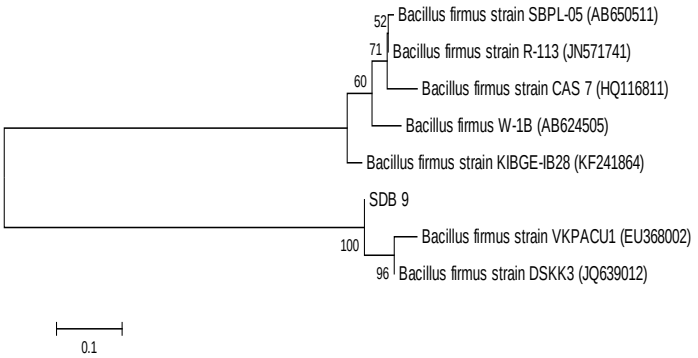


Figure 1 A phylogenetic tree is showing the taxonomic position of culture SDB9 with the GenBank accession numbers in parenthesis. Only bootstrap values greater than N50% are shown at nodes (based on 500 bootstrap resampling).

Concurrent production of enzymes and growth profile

The time course of pectinase production by the isolate was monitored during shake flask fermentation with pectin as a sole source of carbon. Optical density readings at 600nm indicated an active log phase during 6 to 24 h of fermentation. Highest pectinase activity was found 1.94 $IU.mL^{-1}$ during the early stationary

phase at 48 h. The Xylanase activity topped to 0.34 IU.mL^{-1} at 48 h of fermentation with as negligible as 0.086 IU.mL^{-1} cellulase activity at 54 h (Figure 2b). Early production of pectinase was found within 48 h that is desirable for large scale production.Similar rapid pectinase production was reported in *Bacillus licheniformis* (**Rehman *et al*., 2012**), *Bacillus cereus* (**Sanaa *et al*., 2014**) and even in yeast *Saccharomyces cerevisiae* (**Poondla *et al*., 2015**). The use of concurrently produced pectinase and xylanase from the single organism in the pretreatment of kraft pulp can be economically as well as environmentally friendly (**Kaur *et al*., 2010**).

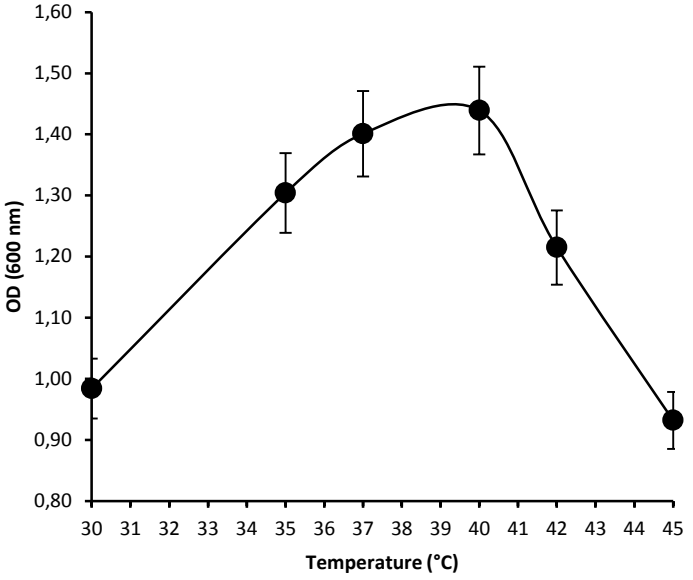


Figure 2a Effect of temperature on growth of isolate SDB9

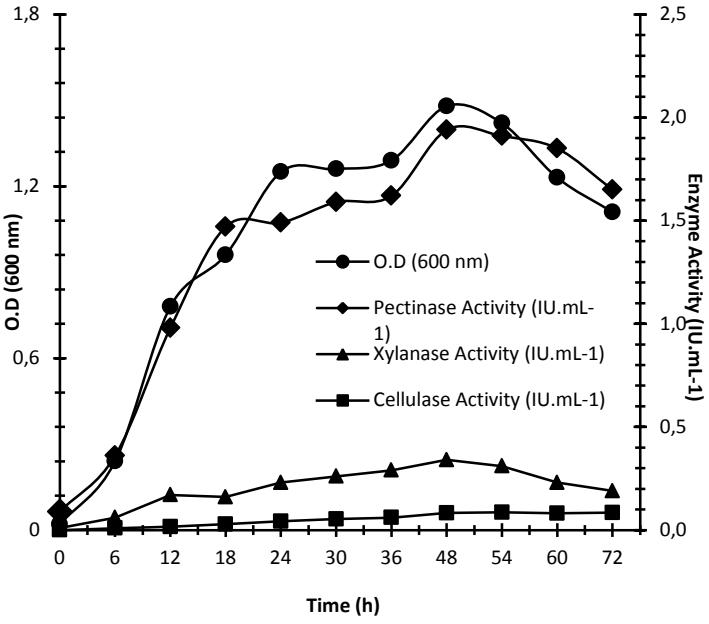


Figure 2b Concurrent production of three enzymes in shake flask condition with growth profile of isolate SDB9

Effect of nitrogen and crude pectin sources on pectinase production

Organic nitrogen sources were superior for the pectinase production than the inorganic nitrogen sources tested in the experiments. A similar pattern is reported in *Bacillus licheniformis* (**Rehman *et al*., 2012**) and *Bacillus* sp. (**Kashyap *et al*., 2003**). Yeast extract was found to be the best source of nitrogen followed by peptone, $NaNO_3$, KNO_3, NH_4Cl and Casein (Figure 3). Yeast extract is more preferentially utilized by the *Bacillus* species for the production of pectinase (**Kashyap *et al*., 2003; Rehman *et al*., 2012**).

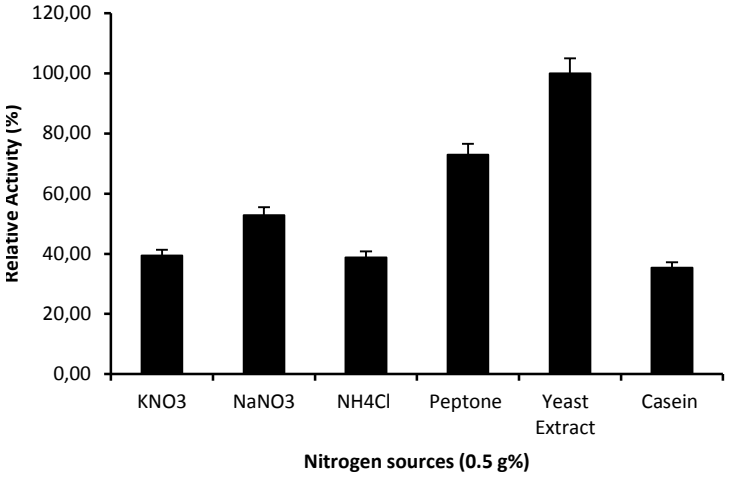


Figure 3 Effect of different Nitrogen sources on pectinase production

The enzyme activity in all the tested crude substrates was found to increase with incubation. On testing different economic waste materials as a substrate for pectinase production, the maximum enzyme output was achieved using an orange peel on 72 h of fermentation. Earlier, orange peel is reported to be the best source for the pectinase production by *Bacillus* sp. NTT33 (**Cao *et al*., 2000**). Though, the initial phase of 48 h did not yield highly differential production data. However after three days the Orange Peel proved to be a most worthy carbon source for the pectinase production followed by hardwood bark, Banana peel, Jute and pectin (Figure 4). The use of agro-residues for the pectinase production is an alternative biotechnological solution for waste valorization (**Martín *et al*., 2013; Rivas *et al*., 2008**) that is highly acceptable for sustainable development.Among the various agricultural waste including oilseed cake, wheat straw, wheat bran, citrus peel/orange peel were well evaluated as a pectin source for the production of pectinase (**Kapoor *et al*., 2001; Sharma and Satyanarayana, 2012**).

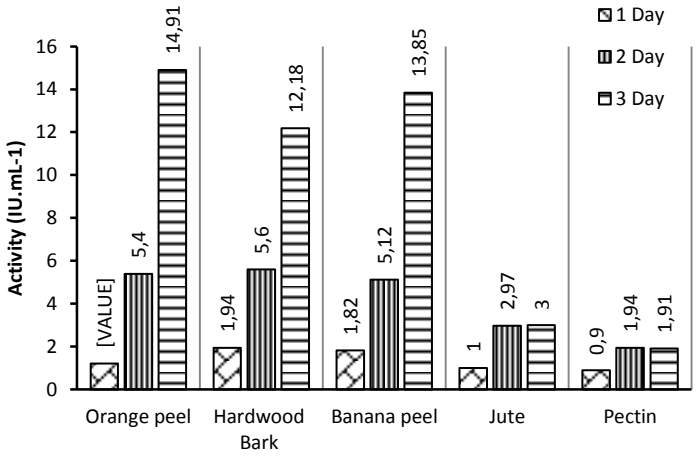


Figure 4 Effect of substrates (Orange peel, Hardwood Bark, Banana peel, Jute and Pectin) on pectinase activity

Media optimization for pectinase production

Pectinase production by SDB9 with media containing 0.5% w/v Pectin was successfully attained with supplementation of 0.5% yeast extract, 0.2% $(NH_4)_2SO_4$, 0.6% Na_2HPO4, 0.3% KH_2PO4 and 0.1% $MgSO_4.7H_2O$. However, in optimization studies pectin was replaced with orange peel that principally consists of as much as 25% to 30% (dry weight) pectins **(Aravantinos-Zafiris *et al*., 1994; Ververis *et al*., 2007**) in order to reduce the cost of production.

Plackett-Burman Design (PBD) for screening significant variables

Manual screening by one variable at-a-time is labor-intensive and time-consuming. Whereas, PBD decreases the number of experiments needed to effectively achieve experimental goals significantly (**Plackett and Burman, 1946**). In all, 13 runs were carried out on ten independent variables and one

dummy variable. The PBD matrix, coded-real values of independent variables studied and the experimental vs. predicted values of pectinase produced are shown in Table 5.

Higher values of studentized effect, the sum of squares and % contribution of orange peel, pH and incubation temperature compared to the rest of the tested variables indicates their pronounced influence on the response (Table 6). Regression analysis and independent variables with significant consequences on pectinase production levels are presented in Table 7. Values of Prob> F less than 0.0500 indicate model terms are significant. In this case, F, G, H are significant model terms. Values greater than 0.1 indicate the model terms are not significant. The Model F-value of 151.69 implies that the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise. The "Pred R-Squared" of 0.8887 is in reasonable agreement with the "Adj R-Squared" of 0.8981; i.e. the difference is less than 0.2. The adequate precision, the signal to noise ratio of 15.7, suggests an adequate signal. Coefficient Estimate for orange peel, media pH and incubation temperature turned out to be 5.50, 1.65 and 1.45. The equation in terms of coded factors is pectinase production= +10.18 +5.50*F+1.65*G+1.45*H. The combined effect of (i) orange peel and incubation temperature (ii) orange peel and media pH and (iii) incubation temperature and media pH on pectinase production are depicted in figure 5.

Table 5 PBD design to search the independent variables (Compositions) that affects the pectinase production (response)

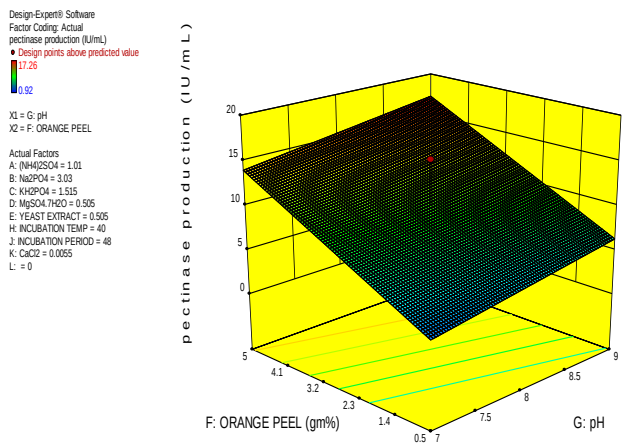
Run	A: $(NH_4)_2SO_4$	B: Na_2PO_4	C: KH_2PO_4	D: $MgSO_4.7H_2O$	E: Yeast Extract	F: Orange Peel	G: pH	H: Incubation Temp.	J: Incubation Period	K: $CaCl_2.2H_2O$	Experimental Pectinase Activity	Predicted Pectinase Activity
	% w/v	% w/v	% w/v	% w/v	% w/v	% w/v		^oC	h	% w/v	$IU.mL^{-1}$	$IU.mL^{-1}$
1	1	1	-1	1	1	1	-1	-1	-1	1	13.5	11.826
2	-1	-1	1	-1	1	1	-1	1	1	1	14.1	14.406
3	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	0.92	1.163
4	0	0	0	0	0	0	0	0	0	0	15.21	15.21
5	1	-1	-1	-1	1	-1	1	1	-1	1	8.31	7.37
6	-1	-1	-1	1	-1	1	1	-1	1	1	15.1	15.453
7	1	-1	1	1	-1	1	1	1	-1	-1	17.26	18.033
8	-1	1	1	-1	1	1	1	-1	-1	-1	15.42	15.45
9	1	-1	1	1	1	-1	-1	-1	1	-1	1.1	1.163
10	-1	1	-1	1	1	-1	1	1	1	-1	8.57	7.37
11	1	1	-1	-1	-1	1	-1	1	1	-1	14.2	14.4
12	1	1	1	-1	-1	-1	1	-1	1	1	3.81	4.79
13	-1	1	1	1	-1	-1	-1	1	-1	1	2.89	3.743

Table 6 Proportionate effects on independent variables on response (Pectinase production)

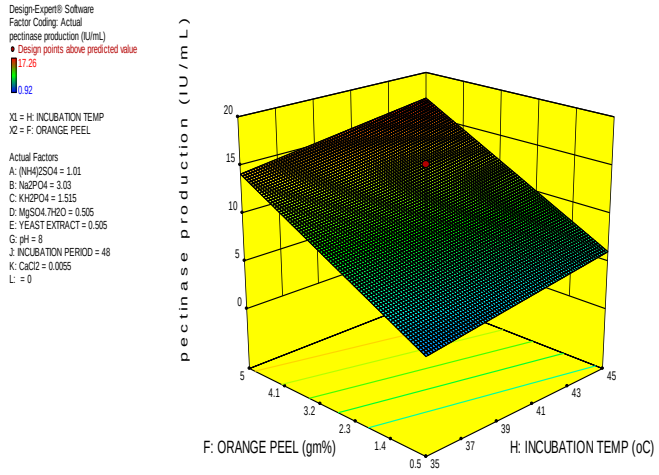
Intercept	Studentized Effect	Sum of Squares	% Contribution
A-$(NH_4)_2SO_4$	0.14	0.062	0.014
B-Na_2PO_4	0.21	0.14	0.030
C-KH_2PO_4	-0.95	2.71	0.59
D-$MgSO_4.7H_2O$	-0.050	7.500E-003	1.648E-003
E-Yeast Extract	1.19	4.25	0.93
F-Orange Peel	10.99	362.34	**79.61**
G-pH	3.30	32.67	**7.18**
H-Incubation Temp.	2.91	25.35	**5.57**
J-Incubation Period	0.090	0.024	5.339E-003
K-$CaCl_2$	0.093	0.026	5.742E-003

Table 7 Regression analysis of PBDesign

Source	Regression Coefficient Estimate	Sum of Squares	Df
Model		420.36	3
F-Orange Peel	5.50	362.34	1
G-pH	1.65	32.67	1
H-Incubation Temperature	1.45	25.35	1
Curvature	-	27.40	1
Residual	-	7.39	8
Cor Total	-	455.15	12



(i)



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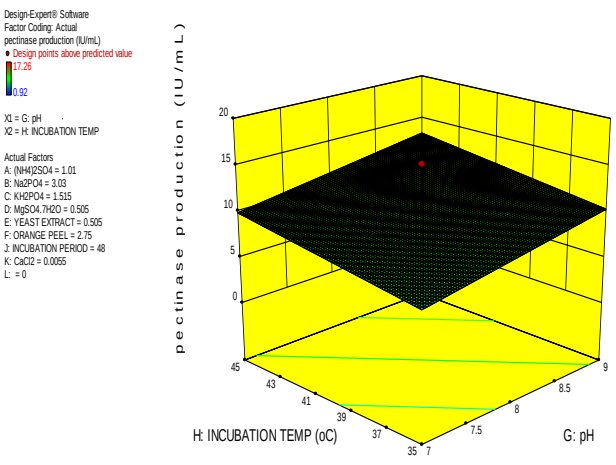

(iii)

Figure 5 The combined effect of (i) orange peel and incubation temperature (ii) orange peel and media pH and (iii) incubation temperature and media pH on the response (Pectinase production)

Response surface methodology for optimization of pectinase production using Box–Behnken design

Three important parameters namely Orange peel concentration (X1), incubation temperature (X2) and initial pH of media (X3) were selected as the independent variables based on PBDesign to determine the optimal values. The plan of BBD in the coded levels of the three independent variables and the obtained results are shown in Table 8. The enzyme activity (Pectinase IU.mL^{-1}) was the only dependent response variable tested.

Table 8 Box-Benkhen Design with coded levels of three independent process variables and experimental vs. predicted pectinase activity

Run	Orange Peel (X1)	Temperature (X2)	pH (X3)	Experimental Pectinase Activity (IU.mL^{-1})	Predicted Pectinase Activity (IU.mL^{-1})
1	0	-1	1	10.89	10.85
2	-1	0	-1	16.55	16.49
3	0	1	-1	14.69	14.761
4	1	0	-1	17.55	17.587
5	0	0	0	12.87	13.17
6	0	0	0	13.23	13.17
7	-1	-1	0	11.81	11.86
8	-1	1	0	11.37	11.36
9	0	-1	-1	14.17	14.137
10	0	0	0	13.41	13.16
11	1	1	0	12.51	12.45
12	-1	0	1	11.01	11.0
13	1	0	1	12.21	12.24
14	0	1	1	10.05	10.06
15	1	-1	0	12.59	12.6

ANOVA (analysis of variance) was employed for the determination of significant effects of variables for pectinase production. The detected Pectinase production ranged between 10.85 to 17.58 IU.mL^{-1} with the conditions tested. To make the model significant the response transformation from the power family with lambda -1.31 and constant k= 0 was applied based on the recommendation of the Box-Cox plot (Table 9).

Table 9 ANOVA for Response Surface Quadratic model

Source	SS	Df	MS	F Statistics	p-value	
Model	6.639E-004	9	7.377E-005	184.65	< 0.0001	Significant
A-Orange Peel	*2.957E-005*	*1*	*2.957E-005*	*74.02*	*0.0004*	
B-Temperature	*4.116E-006*	*1*	*4.116E-006*	*10.30*	*0.0237*	
C-pH	*5.115E-004*	*1*	*5.115E-004*	*1280.28*	*< 0.0001*	
AB	*7.266E-007*	*1*	*7.266E-007*	*1.82*	*0.2353*	
AC	*3.239E-006*	*1*	*3.239E-006*	*8.11*	*0.0359*	
BC	*9.884E-006*	*1*	*9.884E-006*	*24.74*	*0.0042*	
A^2	*2.479E-006*	*1*	*2.479E-006*	*6.21*	*0.0551*	
B^2	*9.381E-005*	*1*	*9.381E-005*	*234.80*	*< 0.0001*	
C^2	*3.207E-006*	*1*	*3.207E-006*	*8.03*	*0.0365*	
Residual	1.998E-006	5	3.995E-007			
Lack of Fit	*2.248E-007*	*3*	*7.494E-008*	*0.085*	*0.9622*	*not significant*
Pure Error	*1.773E-006*	*2*	*8.864E-007*			
Cor Total	6.659E-004	14				

Df- Degree of Freedom, SS- Sum of Squares, MS- Mean of Square

The Model F-value of 184.65 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise. In this case, A, B, C, AC, BC, B^2, C^2 are significant factors or interactions. The Lack of Fit F-value of 0.08 suggests that the model is fit. Analysis of variance for pectinase production shows that quadratic model is significant with F value 184.65 and p-value < 0.0001 as shown in Table 9. The coefficient of determination (R^2) was calculated to be 0.9970 indicating that the model could explain 99% of the variability. The coefficients for the linear effect of Orange Peel, Temperature and pH were highly significant. The interactive effect of Orange Peel and Temperature was less significant than the interactive effect of Orange Peel and pH & Temperature and pH. Also, Temperature and pH were quadratically significant. The fitted Quadratic model for pectinase activity in terms of coded process variables is:

Y= (Enzyme Activity) -1.31 = + 0.034-1.923E-003 * A+7.173E-004* B+7.996E-003* C-4.262E-004* AB-8.999E-004* AC+1.572E-003* BC-8.194E-004 * A2+5.040E-003 * B2 -9.319E-004* C2

The "Adeq Precision" value of 48.759 indicates an adequate signal and therefore, the model is significant for the process. The 3D response surfaces with contour were plotted on the basis of the model equation so as to investigate the interaction of the factors and to measure the optimum values of each factor for maximum pectinase production by *Bacillus* sp. SDB9. The optimum concentration of the respective components is represented by the coordinates of the central point within the highest contour lines in each of the graphics. Contour and 3D plot show the interactions of orange peel and incubation temperature at constant pH 8.0. Pectinase production increases with increasing orange peel concentration (2.5 %, w/v to 5.0%, w/v). The increase in temperature up to 40°C enhanced the production then after it declined (Figure 6a and 6b). Furthermore, moderate increase in pectinase production was seen with increasing orange peel concentration (2.5 %, w/v to 5.0%, w/v) but the rise in pH from pH 8.0 adversely influenced the production (Figure 7a and 7b). Similarly, a modest increase in pectinase production was seen with increasing temperature but the rise in pH from pH 8.0 reduced the production (Figure 8a and 8b).

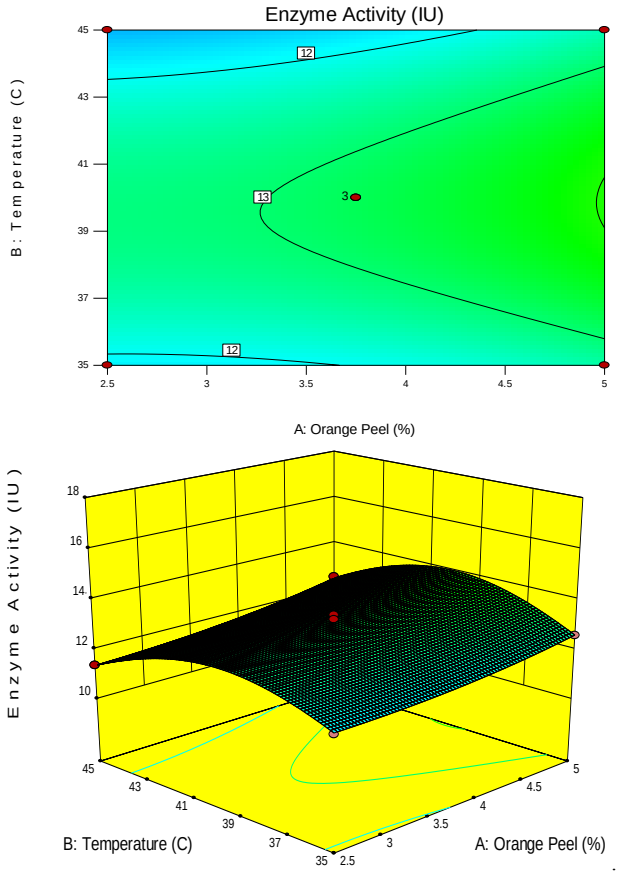

Figure 6 (a) Contour plots and (b) 3D response surface curves shows the interactions of orange peel and incubation temperature on production of pectinase by SDB9 strain at constant pH 8.0

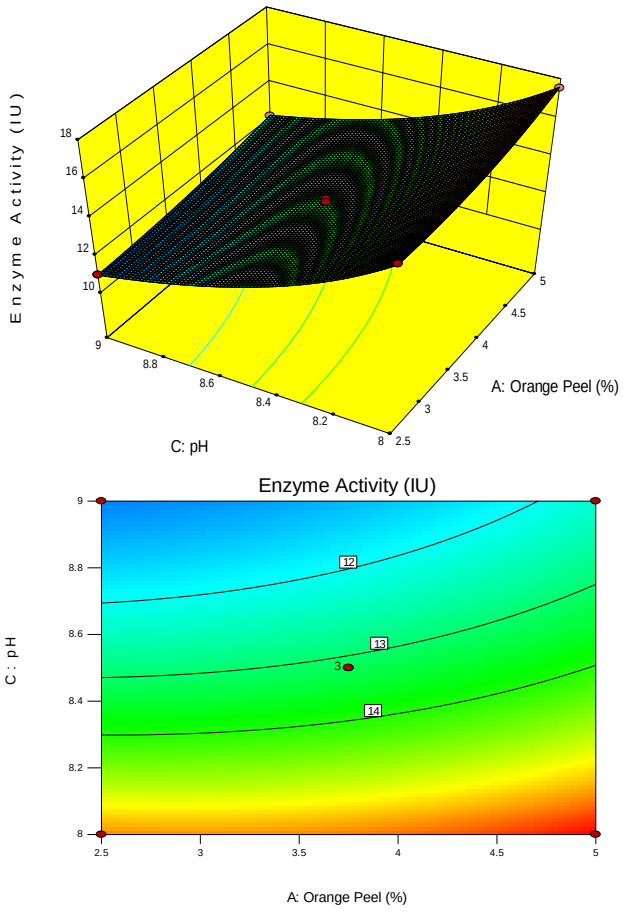

Figure 7(a) Contour plots and (b) 3D response surface curves shows the shows the interactions of orange peel and pH on production of pectinase at constant temperature 40°C

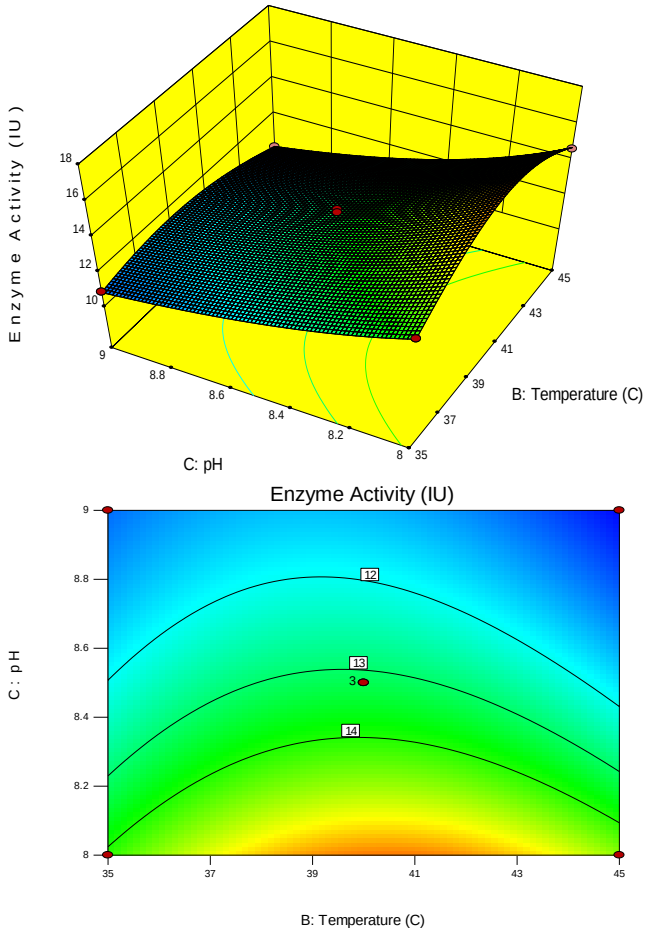

Figure 8 (a) Contour plots and (b) 3D response surface curves shows the interactions of pH and temperature on production of pectinase at constant orange peel concentration (3.75 % w/v)

The maximum predicted pectinase activity was 17.58 IU.mL^{-1}, which was very close to the actual obtained value of 17.55 IU.mL^{-1} corresponding to increasing levels of orange peel (5.0 % w/v), temperature (40°C) and a lower level pH (8.0). (Figure 9). The pectinase yield in the optimized medium was 17% higher than that in the initial medium. The production of pectinase increases with increase in concentration of orange peel, but the incremental production is not proportionate so the lower concentration of 2.5% of orange peel can be considered for practical application and save on the usage of substrate. Production of pectinase from SDB9 at 72 h, 40°C and pH 8.0 on 2.5 % w/v orange peel as agricultural residue material is recommended to achieve higher yield of 16.49 IU.mL^{-1}.Few recent discrete reports on optimization of pectinase production by *Bacillus* species using statistical approach are available in scientific literature that includes the screening by PBDesign and subsequent determination of optimal values of significant model terms C:N ratio, K_2HPO_4 and pH **(Sharma and Satyanarayana, 2006)**.The raw substrate and abiotic parameters dominated the outcome of optimization experiments for pectinase production in *Bacillus licheniformis* SHG10 strains (**Embaby *et al*., 2014**).

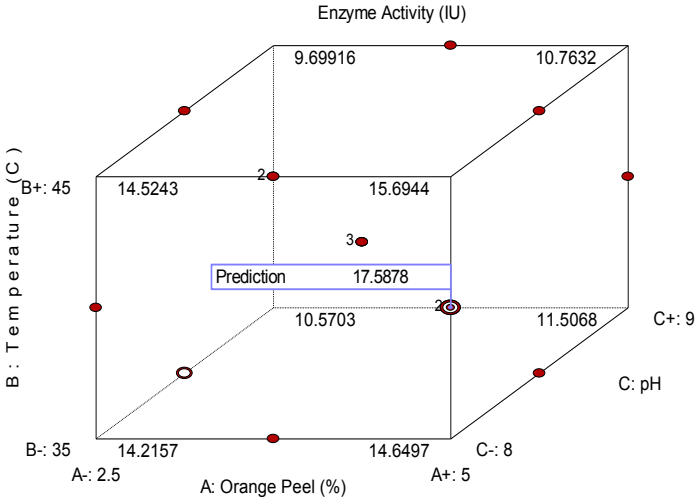

Figure 9 Cube plot showing the influence of factors relevant to the pectinase production A: Orange peel, B: temperature and C: pH

Enzyme characterization

For the precipitation of the enzyme 50% concentration of $(NH_4)_2SO_4$ found to be optimum, followed by dialysis. This partially purified enzyme was used in the characterization.

Using different pH buffers, peak activity of *Bacillus* sp. SDB9 pectinase was observed with assay buffer at pH 8.5 and more than 50% activity was retained at pH 7.5 and 9.5 (Figure 10). The optimum temperature for SDB 9 pectinase activity was found to be 45°C (Figure 11). The catalytic activity of pectinase was reported in neutral to alkaline pH with abroad temperature range that is similar to the pectinase of *Bacillus pumilus* **(Sharma and Satyanarayana, 2006)**. The augmented activity of pectinase in the presence of the divalent cations including Mg^{2+}, Ca^{2+}, Mn^{2+} and Co^{2+} suggest the metalloprotein nature. However K^{+}, Zn^{2+} and Fe^{3+} cause the drastic inhibition of the enzyme activity (Figure 12). Positive modulatory effect of Mg^{2+} and Ca^{2+}, on S-I and S-II pectinase of *B. gibsonii* as well as *Bacillus* sp. KSM-P576 supports our investigation **(Kobayashi *et al*., 2001; Zu-ming *et al*., 2008)**. Zn^{2+} is a well-reported inhibitor of pectinase **(Kusuma and Reddy, 2014; Roosdiana *et al*., 2013)** which in agreement with the present findings.

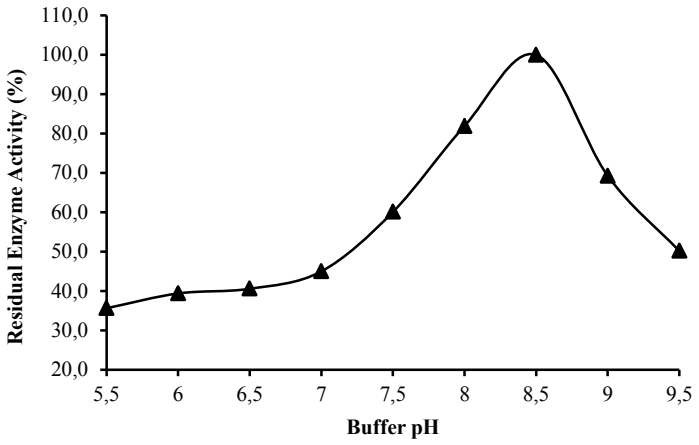

Figure 10 Effect of assay pH on pectinase activity

The enzyme was found to perform optimally at 45°C. However, it works on wide range of temperature ranging from 30 to 50°C. The enzyme was thermo-sensitive beyond 45°C as its activity was decreased drastically (Figure 11).

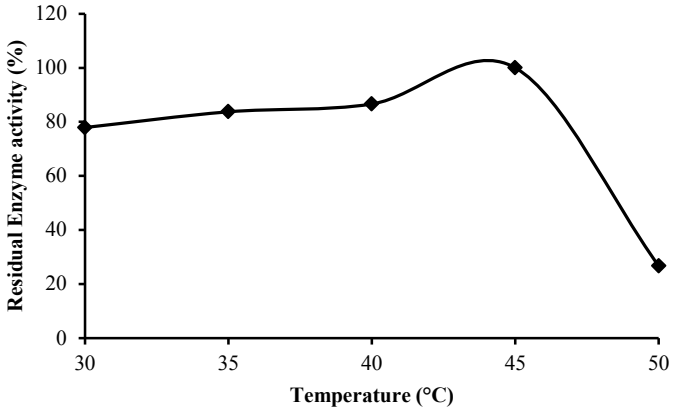

Figure 11 Effect of assay temperature on pectinase activity

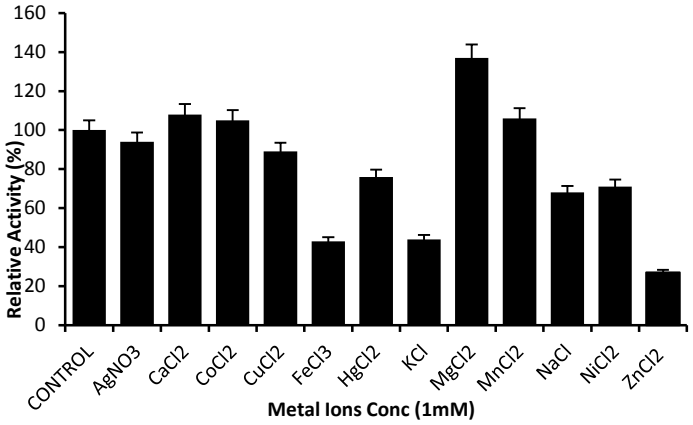

Figure 12 Effect of Metal Ions on SDB9 pectinase activity

The K*m* and V*max* values of the partially purified pectinase from *Bacillus* sp. SDB9 were calculated to be 2.090 $mg.mL^{-1}$ and 1.798 $IU.mL^{-1}$, respectively (Figure 13). The R-value indicates that almost 98% of the variation in v_o^{-1} (y) is due to the variation in S^{-1} (x). The K*m* and V*max* values of the enzyme are harmonious for use at industrial scale.

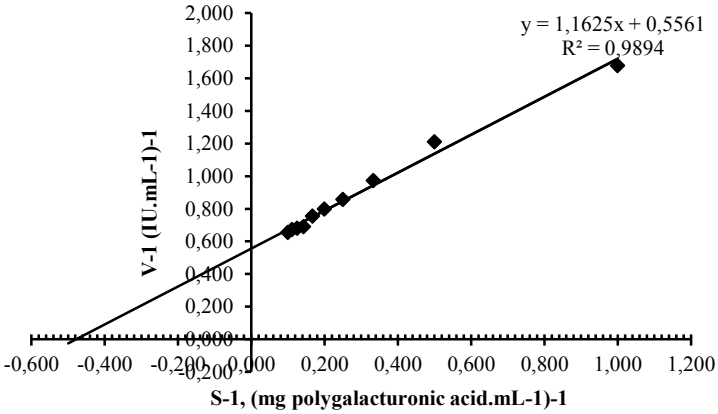

Figure 13 Line Weaver Burk Plot of initial velocity data for pectinase on polygalacturonic acid (2.5 to 25 $mg.mL^{-1}$) measured at 45°C and pH 8.5

An enzyme was found to remain active at elevated temperature, and thus it was thermostable in nature. The enzyme demonstrated highest stability at pH 8.5. The decline in enzyme stability was drastic beyond pH 8.5 whereas it was gradual below this pH. i.e. Pectinase retained only 48.7% and 38.2% activity up to 60 min at pH 7.5 and 9.5 .Furthermore, pectinase from SDB9 retained 50.17% relative activity at pH 9.5 (Figure 14). The enzyme showed thermal stability in a broad range of temperatures. The enzyme retained 86% and 69% activity at 60°C and 70°C for 60 min. Nearly, half activity was conserved up to 90 min at 70°C. Thus, the enzyme is vastly stable thermally up to temperatures as high as 70°C for time up to 90 min. Mere, 12% activity,was recorded at 80°C after 90 min.

(Figure 15). Pectinase of the isolated bacterium is superior in terms of the thermal stability from pectinase of *Bacillus subtilis* CM5 and *Bacillus* sp. MG-cp-2 (**Kapoor *et al*., 2000; Ray, 2010**) ie. 80% at 70 °C for 30 min and 50% for 20 min at 80 °C respectively.

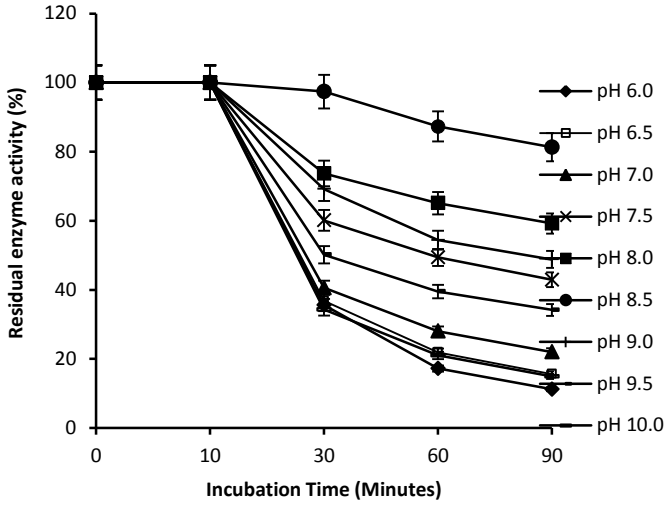

Figure 14 Stability of pectinase in acidic to alkaline pH scale

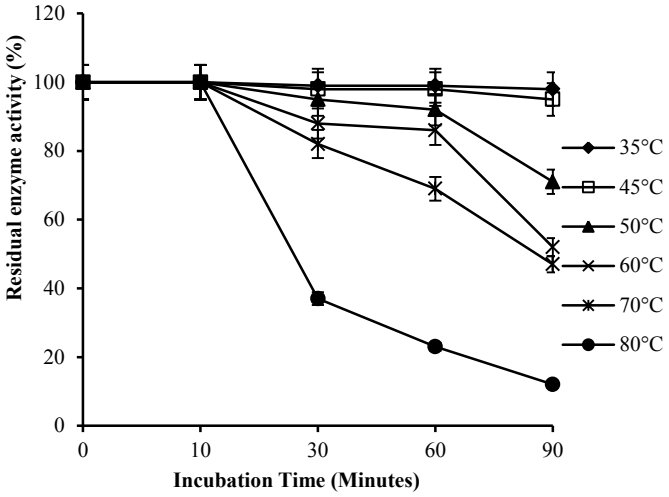

Figure 15 Thermostability of pectinase at various temperature scales

Enzymatic pretreatment of Kraft pulp (E_0)

The drop in kappa number after enzyme pretreatment indicates the inputs of the enzymes in the bleaching of paper pulp. No remarkable changes in kappa number were observed by the treatment with set-I that comprises 10 IU of pectinase and 1.8 IU of xylanase per gram of OD pulp. Whereas, treatment with set-II containing 15 IU of pectinase and 2.7 IU of xylanase per gram of OD pulp reduced the Kappa number by 1.94 unit (Table 10**)** as compared to the control (untreated pulp) and that is nearly double the % reduction than the report of **Thakur *et al*., (2012**). So due to the good result with set-II, it had been channelized for detail study in chemical bleaching process to reduce the load of the chemical bleach agent.

Table 10 Characterization of enzyme (E_0) pretreated and untreated (Control) unbleached pulps

Particulars	Control	Enzyme (E_0) treated pulp	
Enzyme dose (IU.gm^{-1})	Nil	Set-I	Set-II
Kappa no. of pulp	24.55	23.07	22.61
Brightness (% ISO)	24.12	24.24	24.77
Yellowness (%ISO)	40.88	40.91	40.80

Bleaching of Pulp (D_0-Ep-D_1)

The values of improvement in brightness and whiteness of pulp at different sequential stages ranging from ClO_2 stage (D_0) to Alkali Extraction (E_p) Stage and up to ClO_2 stage (D_1) stage of chemical bleaching was determined. Enzyme pretreatment with pectino-xylanolytic preparation showed a reduction in Kappa Number by 1.94 units and increment in % ISO brightness by 1.32 units with set-II at 50˚C temperature and pH 8.0 for 120 min (Table 11). Our result of a reduction in Kappa Number and increment in % ISO brightness is far better than earlier study **(Ahlawat *et al*., 2007**). Moreover, the combined use of pectinase and xylanase are more preferable approach than single enzymes for kraft pulp pretreatment (**Dhiman *et al*., 2009**) because the treatment with the pectinase lowers the cationic demand of the pulp and xylanase predominantly delignify the pulp. Treatment of the kraft pulp with pectino-xylanolytic enzymes extracted from the isolate resulted in 15% less chlorine consumption to obtain the same optical property of the pulp as attained with conventional chemical bleaching. Therefore, this renders the process eco-friendly and sustainable. The outcome of the bleaching after a D_0-E_p-D_1 stage in properties of pulp seemed to be suitable for commercial exploration.

Table 11 D_0-E_p-D_1 bleaching of enzyme treated and untreated pulps

Sr. No.	Particulars	Control	Enzyme treated Set-II	
			Same dose	15% less dose
1	ClO_2 stage (D_0)			
	Applied chlorine, %	6.47	6.47	5.50
	Brightness, % ISO	50.34	50.80	44.91
	Yellowness, %ISO	36.21	32.37	35.18
	Whiteness %ISO	ND	ND	ND
2	Alkali Extraction (E_p) Stage			
	Applied NaOH, %	2.5	2.5	2.5
	Applied Peroxide, %	1.0	1.0	1.0
	Brightness, % ISO	72.46	75.00	69.10
	Yellowness, %ISO	19.41	15.68	19.80
	Whiteness %ISO	38.06	45.00	33.39
3	ClO_2 stage (D_1)			
	Applied chlorine, %	4.31	4.31	4.31
	Brightness, % ISO	88.58	89.90	89.12
	Yellowness, %ISO	7.60	7.77	8.32
	Whiteness %ISO	75.71	76.61	75.18
	Brightness Improvement Unit	-	1.32	0.56

Analysis of treated pulp

The pulp viscosity of enzymes treated Set-II pulp after the D_0-E_p-D_1 stage was 586.67, which was very close to the 581.82 value of enzyme untreated pulp. The viscosity of treated pulp clearly indicated there was no destruction of the cellulosic fibers. Nearly 15 % reduction in post color number (P C Number) of enzyme treated pulp was transpired. Furthermore, after the pulp bleaching process, the chemical analysis of pulp-free filtrate was performed and it divulged the noteworthy enhancement in the release of colour (6.36 Kg.tp^{-1}), phenolics (35.77 Kg.tp^{-1}) and lignin (1.15 Kg.tp^{-1}) as compared to the control. The release of total reducing sugar also supports the investigation **(**Table 12**).** Removal of color, phenolic and lignin with the release of reducing sugars is the indirect assessment to check the efficiency of bleaching using enzymes (**Saleem *et al*., 2009**).

Effects of enzymes treatment on paper quality

Canadian standard freeness enhancement from 280 in untreated to 310 in enzyme treated pulp is the perfect proof of better strength. The gain in Double fold number (3.2%) indicated the improvement in the endurance of paper. Minor variations in Burst index, Tensile index and Tear index of enzyme treated pulp reflected the conservation of pulp properties (Table 13). The result of paper quality testing reflects that the pulp fibrillation, water retention and restoration of fiber bonding are unaffected (**Gupta *et al*., 2000**). Ours is a novel combinatorial approach where the mixture of enzymes is predominated by pectinase rather than xylanase. Nevertheless comparable result was achieved by **Dhiman *et al*., (2009)**.

Table 12 Analysis of pulp filtrates of enzyme treated & untreated pulp samples

Sample Code	TRS Kg.tp^{-1}	Colour at (465nm) Kg.tp^{-1}	Lignin at (280nm) Kg.tp^{-1}	Phenolics at (237 nm) Kg.tp^{-1}
Control	0.15	5.27	0.62	20.88
Enzyme treated (Set-I)	0.17	9.23	1.31	42.75
Enzyme treated (Set-II)	0.20	11.64	1.77	56.65

Table 13 Strength properties of enzyme treated and untreated bleached pulp

Particulars	Control	Enzyme pretreated (Set-II)
Canadian Standard Freeness	280	310
Double Fold	75	77.50
Burst index (Pa.m^2.g^{-1})	3.88	3.44 (11.3%)
Tensile index (Nm.g^{-1})	58.65	56.10 (4.34 %)
Tear index (Nm. m^2.g^{-1})	6.73	6.40

CONCLUSION

The isolated *B. firmus* produced the substantial amount of pectinase and limited xylanase along with negligible cellulase, the enzyme combination worth exploring in pulp pretreatment. Yeast extract and orange peel were screened out as preferred nitrogen source and raw agri-residues respectively using one variable at-a-time approach. More than 17% improvement in pectinase production suggest the successful optimization using RSM and in this way render the process more viable for mass production of pectinase. Enzyme properties concerning the range of optimal pH, temperature and different cationic metals warrant its potential biotechnological practice. The predominant use of pectinase rather than xylanase in combinatorial approach for pulp pretreatment and subsequent analysis of pulp and paper quality yielded an equivalent result to the well-studied approach comprising more xylanase and less pectinase. The lessening of chlorine consumption on account of enzymatic pretreatment eventually leads to a reduction in chlorinated aromatic compounds in the effluent makes it easier to embrace the green technologies in paper industries. Therefore, the bacterial pectinase with thermo-alkalistable nature having the noteworthy ability to reduce the kappa number and increase the brightness of kraft pulp warrants the further investigation for a new horizon.

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EXTRACELLULAR SYNTHESIS OF ZINC OXIDE NANOPARTICLES USING *ACINETOBACTER SCHINDLERI* SIZ7 AND ITS ANTIMICROBIAL PROPERTY AGAINST FOODBORNE PATHOGENS

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ABSTRACT

The present study focuses on the microbial synthesis of zinc oxide nanoparticles (ZnO NPs) and evaluating the antimicrobial property on foodborne pathogens. The bacterial strain, *Acinetobacter schindleri* SIZ7 was isolated from the waste filling area of Sivakasi, Tamil Nadu, India. The biogenic synthesis of ZnO NPs was carried out at room temperature and under suitable, eco-friendly environment using culture supernatant of *A. schindleri*. The physico-chemical properties exhibited by the biogenic ZnO NPs were characterised using UV-Visible Spectrophotometry, Energy dispersive X-ray spectroscopy (EDS), High Resolution Transmission Electron Microscopy (HRTEM), Fourier Transformed Infrared spectroscopy (FTIR) and Thermogravitometric Analysis (TGA). The synthesized ZnO NPs are polydispersed and spherical in shape. The antimicrobial activity of ZnO NPs was investigated against foodborne pathogens, *Staphylococcus aureus* (MTCC 96), *Escherichia coli* (MTCC 739), *Vibrio parahaemolyticus* (MTCC 451) and *Salmonella enterica* (MTCC 9844). The prepared ZnO NPs exhibited strong antimicrobial activity against *E. coli* and *S. enterica* with a minimum inhibitory concentration of 100µg ml^{-1}. Thus, the bacterial strain *Acinetobacter schindleri* SIZ7 could be used for simple, extracellular, non-hazardous and efficient synthesis of antimicrobial ZnO NPs.

Keywords: Metallic Nanoparticle, *Acinetobacter schindleri*, Antimicrobial compounds, 16s rRNA gene, HR-TEM, FTIR, Foodborne pathogens

INTRODUCTION

Metallic nanoparticles are receiving considerable attention in agriculture and medicine due to their unique physical and chemical properties. Nanoparticles (NPs) are used in the fields of drug delivery, imaging, diagnosis, development of antimicrobial compounds and anticorrosive medical devices (**Fayaz *et al*., 2010 and Martinez-Gutierrez, *et al*., 2012**). Metallic NPs synthesis through bacteria, yeast, fungi, plant biomass, live plants, and plant extracts offer several advantages than chemical and physical route of synthesis (**Castro-Longoria *et al*., 2011**). Even though different biotechnological methods and various biological agents can able to synthesize the metallic nanoparticles, bacteria possess distinctive advantage over others, because of their high generation time and can be easily grown in the laboratory. Among the metallic nanoparticles, researchers reported the synthesized silver and gold nanoparticles using bacteria. The culture supernatants of bacteria like *Acetobacter xylinum*, *Aeromonas* sp., *Bacillus cereus*, *Bacillus subtilis*, *Bacillus licheniformis*, *Escherichia coli*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Lactobacillus acidophilus*, *Rhodobacter capsulatus*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* are reported for the synthesis of silver and gold nanoparticles (**Shivaji *et al*., 2011 and Narayanan *et al*., 2010**).

Zinc oxide nanoparticles (ZnO NPs) also have gained much interest owing to their wide applications in the preparation of personal care products, coatings and catalysts in environmental remediation, as antifungal and antibacterial agents (**Kirthi *et al*., 2011**). **Jayaseelan *et al*., (2012)** reported the bacterial mediated synthesis of ZnO NPs using *Aeromonas hydrophila* and its antimicrobial activity was established. Various researchers reported the antibacterial and antifungal properties of ZnO NPs (**Tayel *et al*., 2011 and Xie *et al*., 2011**). In the scientific literature very few reports are available on extracellular synthesis of ZnO NPs using bacteria. Our aim in the present study was to synthesize ZnO NPs using *Acinetobacter schindleri* SIZ7 and to investigate their antibacterial activity against foodborne pathogens.

MATERIAL AND METHODS

Microorganisms

Pure cultures of bacteria were isolated from soil samples, collected from Sivakasi, Tamilnadu, India by serial dilution technique. The bacteria were screened for the ability to synthesize ZnO nanoparticles. The bacterial culture, SIZ7 showed rapid synthesis and was maintained on nutrient agar plates. Morphology of the bacteria was observed under scanning electron microscope (SEM). The bacterial isolate was further identified by 16S rRNA gene amplification and sequencing. The sequence was amplified using 27F forward (AGA GTT TGA TCM TGG CTC AG) and 1492R (TAC GGY TAC CTT GTT ACG ACT T) reverse primers. Phylogenetic tree was constructed using Mega 5.05 software (**Kumar *et al*., 2008**). Sequence of SIZ7 was submitted to the NCBI sequence database under the accession number KR135410.1.

Synthesis of ZnO NPs

The conical flasks (1 litre) containing 500ml of nutrient broth were inoculated with the bacteria SIZ7 and incubated at 37°C for 2 days. The bacterial culture was centrifuged at 1,000 rpm for 10 min and the cell free supernatant was collected. The supernatant was used with the metal ion solution for bioreduction of metals. Typically 100 ml of bacterial supernatant was brought into contact with 5 mM of zinc nitrate solution. The flasks were agitated for 30 min and kept on an orbital shaker at 37°C for 48 h.

Characterization of ZnO NPs

The reaction mixture was observed for visual colour change at different time intervals (4, 8, 12, 16, 24, 32, and 48 h). Subsequently the reaction mixture was monitored by UV–Vis spectroscopy (200-800 nm) as a function of time of reaction. The reaction mixture was subjected to centrifugation at 6,000 rpm and the resultant pellet was collected. The pellet obtained was washed with deionized water for 3 times and the obtained precipitate was dried in a hot air oven at 60°C for 6 h. Elemental composition of the NPs was observed by Energy dispersive X-ray spectroscopy (EDS). The size and shape of synthesized ZnO NPs was

determined by using high resolution transmission electron microscopy (JEOL 3010 TEM-HR) equipped with Gatan digital camera, operated at accelerating voltage of 200kV. The capping agent responsible for the stability of nanoparticles and the functional groups associated with NPs was studied by FTIR spectroscopy. FTIR spectra of the samples were measured on Thermo Scientific Nicolet iN-10, spectrophotometer at the resolution of 4 cm^{-1} in the range of 4000–500 cm^{-1} in KBr pellets. The thermal stability and degradation pattern of NPs was determined by Thermogravitometric analysis (TGA) using Thermogravitometric analyzer (Instrument SDT Q600 V20.9 Build 20 Module DSC-TGA Standard).

Antibacterial activity

The antimicrobial activity of ZnO NPs was tested against the common foodborne pathogenic bacteria, *Staphylococcus aureus* (MTCC 96), *Escherichia coli* (MTCC 739), *Vibrio parahaemolyticus* (MTCC 451) and *Salmonella enterica* (MTCC 9844) procured from Microbial type culture collection (MTCC), IMTECH, Chandigarh. Antimicrobial activity was carried by well diffusion method according to **Jaidev and Narasimha (2010)**. Briefly, 24 h active bacterial culture was seeded into nutrient agar medium. A well of 5 mm was made in the centre of the plate and filled with 150 µg ml^{-1} of ZnO NPS. The plates were incubated at 37°C for 24 h. The zone of inhibition was measured. Minimum inhibitory concentration (MIC) of ZnO NPs against the bacterial strains was determined according to **Behera *et al*., (2010)**. Briefly, test tubes containing 5 ml of Mueller Hinton broth was inoculated with 5 X 10^{-7} CFU ml^{-1} of bacterial culture and various concentrations of ZnO NPs (200, 100, 50, 25, 12.5, 6.25, 3.12 and 1.56 µg ml^{-1}). The tubes were incubated at 37°C for 24 h with 180 rpm and observed for bacterial growth. Optical density was measured for the same at 600 nm using UV-Vis Spectrophotometer. All the experiments were performed in triplicate.

RESULTS

Synthesis and characterization of ZnO NPs

The bacterial isolate SIZ7 showed 99% sequence similarity with *Acinetobacter schindleri* hence named as *Acinetobacter schindleri* SIZ7 (NCBI GenBank accession no: KR135410.1) (Fig. 1). The analysis of SEM photographs revealed that *A. schindleri* SIZ7 was rod shaped bacteria (Fig. 2). Extracellular biosynthesis of ZnO NPs was observed by visual colour change, pale yellow to fluorescent yellow after 48 h of incubation (Fig. 3). UV–Visible spectra of the reaction mixture revealed a characteristic peak between 300 and 360 nm with a maximum at 310 nm, which is due to the surface plasmon resonance of ZnO NPs (Fig. 3) and the result is in good agreement with previous reports (**Bai *et al*., 2011**). Energy dispersive X-ray spectroscopy (EDS) analysis confirmed the presence of zinc and oxygen (Fig. 4). HRTEM image analysis revealed that ZnO NPs were polydispersed and spherical in shape (Fig. 5). The HRTEM micrograph suggested that particle diameter ranged from 20 to 100 nm.

FTIR spectrum analysis of ZnO NPs showed intense absorption bands at 3293, 2926, 2863, 1732, 1660, 1394 and 1058cm^{-1} (Fig. 6). The absorption band observed at 1058cm^{-1} represented the C–O–H bending vibrations. The absorption band observed at wave number 1660cm^{-1} was identified as amide I band. The absorption bands observed at 1394, 2863 and 2926cm^{-1} represents the presence of $–CH_3$ group. The absorption band at 1732cm^{-1} is characteristic of the CO-H stretching. The band around 3293cm^{-1} attributes to the $–CONH_2$ group. FT-IR spectral analysis revealed the biological components of the culture supernatant associated with the formation of ZnO NPs. Thermogravitometric analysis (TGA) measures the weight loss of ZnO NPs as a function of temperature under a controlled atmosphere (Fig. 7). The synthesized nanoparticles showed good thermal stability. Weight loss at higher temperatures 200°C and 400°C was 20.31% and 60.43 % respectively. Water soluble organic compounds secreted extracellularly by bacteria may act both as reducing and stabilizing agents in the green synthesis of ZnO NPs.

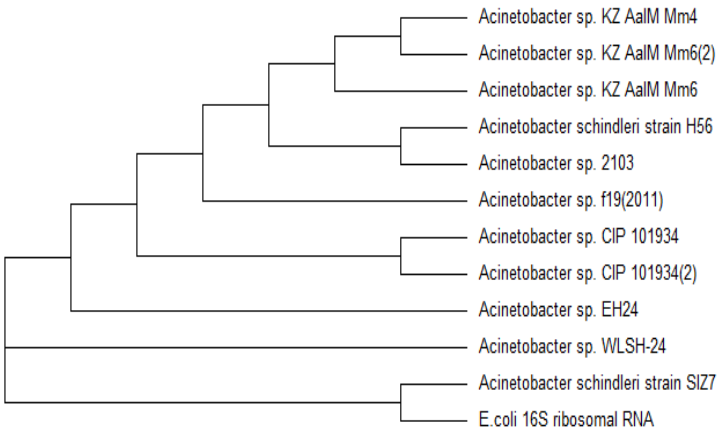

Figure 1 Phylogenetic tree showing genetic relationship between the isolate *A. schindleri SIZ7* and other closely related reference bacteria.

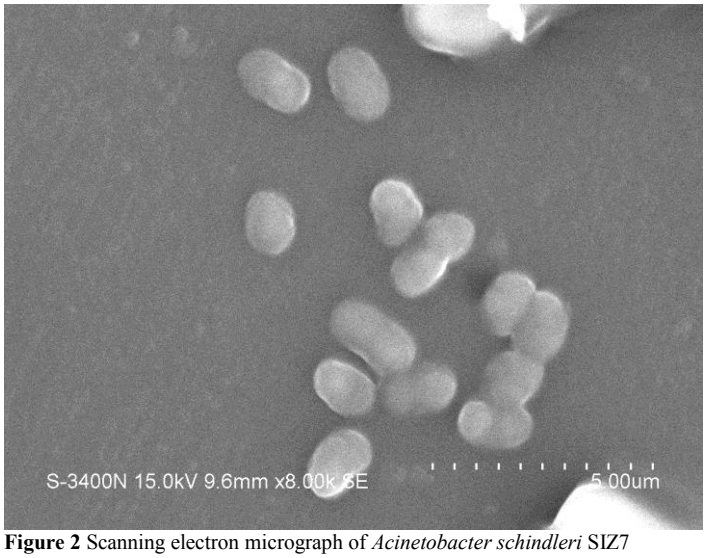

Figure 2 Scanning electron micrograph of *Acinetobacter schindleri* SIZ7

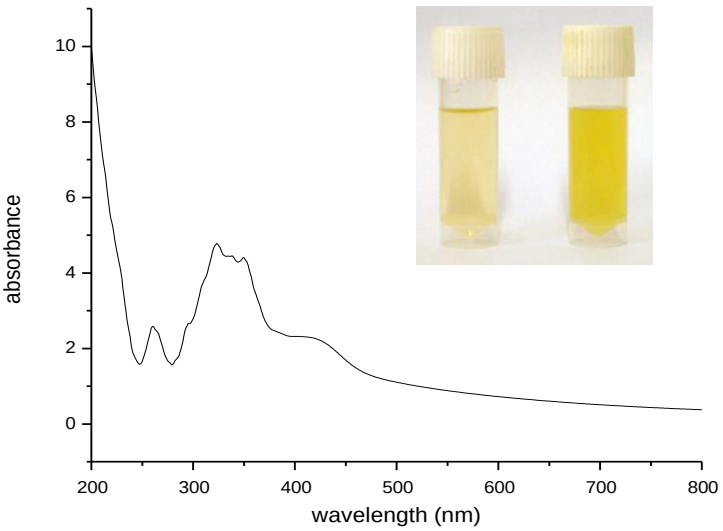

Figure 3 UV–visible spectra of bacterial filtrate as a function of time.. The peak at 310 nm corresponds to the surface plasmon resonance of

ZnO NPs. Inset shows the corresponding colour change.

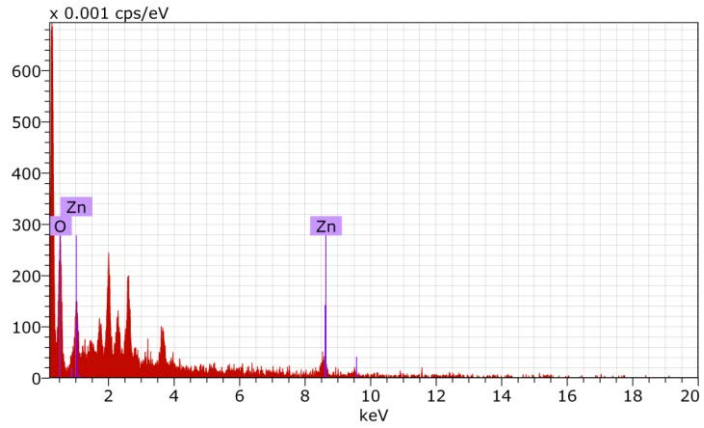

Figure 4 Energy dispersive X-ray (EDS) spectrum of ZnO NPs.

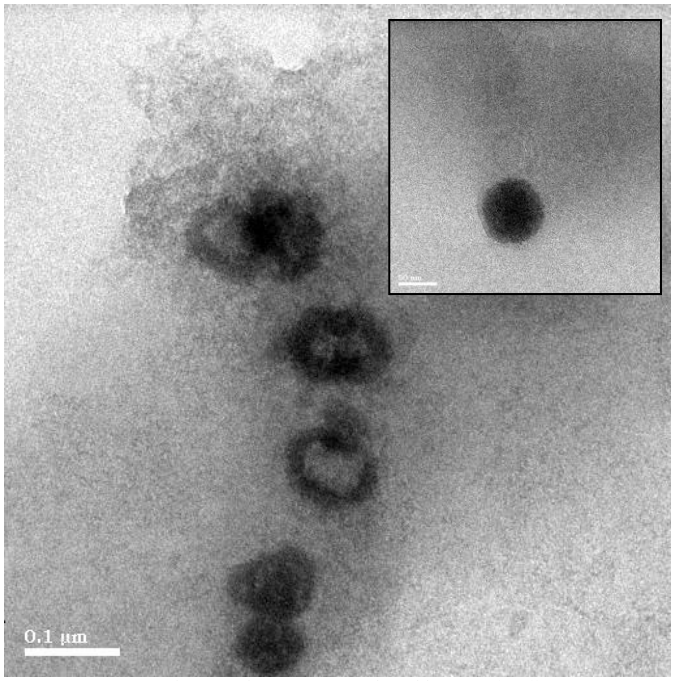

Figure 5 HR-TEM image of ZnO NPs produced by *A. schindleri* SIZ7

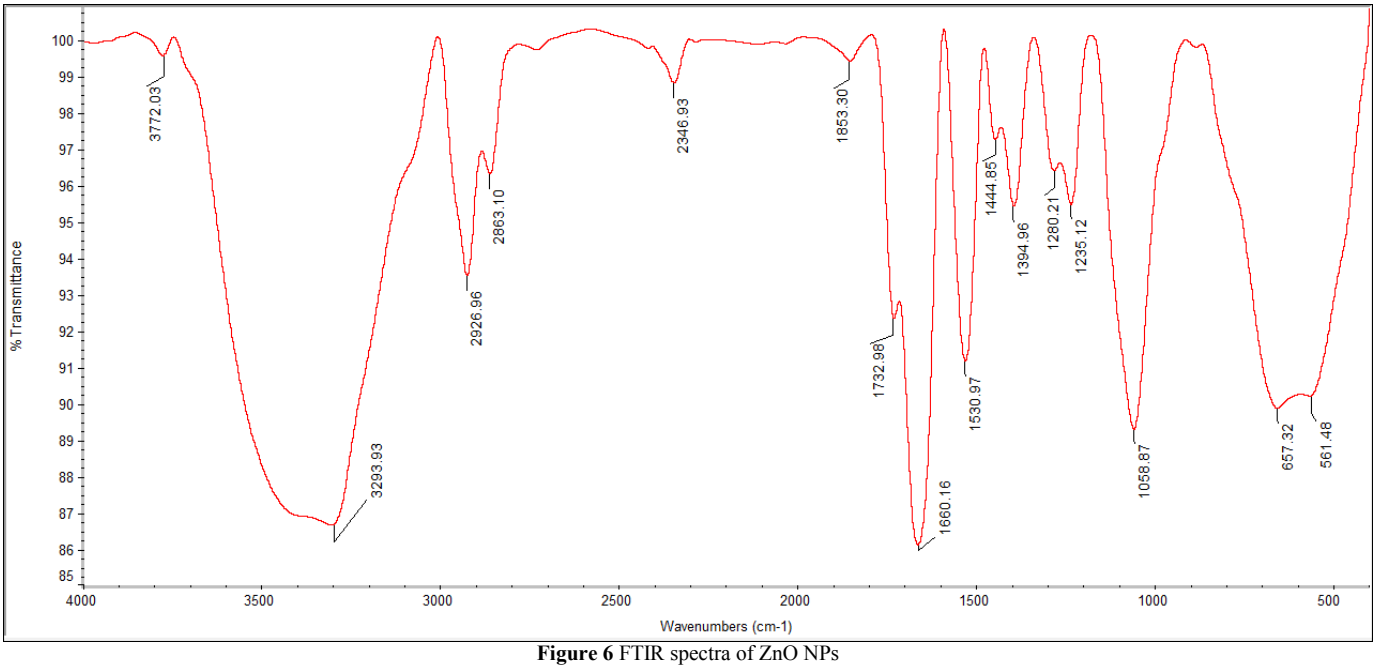

Figure 6 FTIR spectra of ZnO NPs

Antibacterial activity

The ZnO NPs exhibited the antibacterial activity against the common foodborne bacteria tested. At a concentration of 150µg ml^{-1} the zone of inhibition diameters were 16, 18, 16 and 20 mm against *Staphylococcus aureus* (MTCC 96), *Escherichia coli* (MTCC 739), *Vibrio parahaemolyticus* (MTCC 451) and *Salmonella enterica* (MTCC 9844) respectively. The MIC values of ZnO NPs against tested pathogens were in the range of 100 - 200 µg ml^{-1}. The MIC value of ZnO NPs against *E. coli* and *S. enterica* was 100 µg ml^{-1}. The bacterial strains, *S. aureus* and *V. parahaemolyticus* are less sensitive to ZnO NPs, MIC value of 200 µg ml^{-1} (Table 1).

Table 1 Antimicrobial activity of ZnO NPs synthesized by *A. schindleri* SIZ7.

Bacteria	Zone of inhibition (in mm)	MIC (µg ml^{-1})	MBC (µg ml^{-1})
S. aureus	16	200	400
E. coli	18	100	200
V. parahaemolyticus	16	200	400
S. enterica	20	100	200

DISCUSSION

Zinc oxide possesses antibacterial activity and it depends on the size of the particle (**Osamu Yamamoto, 2001**). ZnO NPs exhibits significant antimicrobial activity over a wide spectrum of bacteria and hold good biocompatibility with human cells (**Sirelkhatim *et al*., 2015**). ZnO NPs can be used as antimicrobial agents in food or in the development of packaging materials to prevent the microbial contamination of food and to extend the shelf life of food (**Xie *et al*., 2011**). **Sangeetha *et al*., (2011)** reported the green synthesis of ZnO NPs by *Aloe barbadensis* miller leaf extract and its structure and optical properties were studied. **Nagarajan Sangeetha and Kumaraguru Arumugam Kuppusamy (2013)** demonstrated the extracellular synthesis of ZnO NPs using seaweed *S. myriocystum*. **Sarkar *et al*., (2014)** reported the extracellular mycosynthesis of ZnO NPs by *Alternaria alternate*. Zinc oxide nanoparticles (ZnO NPs) were synthesized using bacteria, *Aeromonas hydrophila* and its antibacterial and antifungal activity was determined (**Jayaseelan *et al*., 2012**). In the present study, *Acinetobacter schindleri* was employed for the extracellular synthesis of ZnO NPs.

The antibacterial potential of ZnO NPs against foodborne pathogens were evaluated and reported against *Salmonella typhimurium* and *Staphylococcus aureus* (**Tayel *et al*., 2011**). Similarly in the present investigation antimicrobial activity of ZnO NPs against foodborne pathogens was reported. The result clearly indicates that *E. coli* and *S. enterica* are more sensitive to ZnO NPs. **Xie *et al*.,**

(2011) investigated antibacterial effect of zinc oxide (ZnO) nanoparticles on *Campylobacter jejuni* and suggested that the antibacterial mechanism of ZnO nanoparticles is most likely due to disruption of the cell membrane and oxidative stress in *Campylobacter*. Anticandidal activity of ZnO NPs against *C. albicans* is also correlated with reactive oxygen species (ROS) production (**Shoeb *et al*., 2013**). The mechanism of the inhibitory effects of ZnO NPs on foodborne pathogens might be similar to previous reports.

Due to their potent antimicrobial activity against bacterial strains, researchers focused their investigations more on the synthesis and evaluation of mechanism of antimicrobial activity of ZnO NPs against an array of pathogenic bacteria.

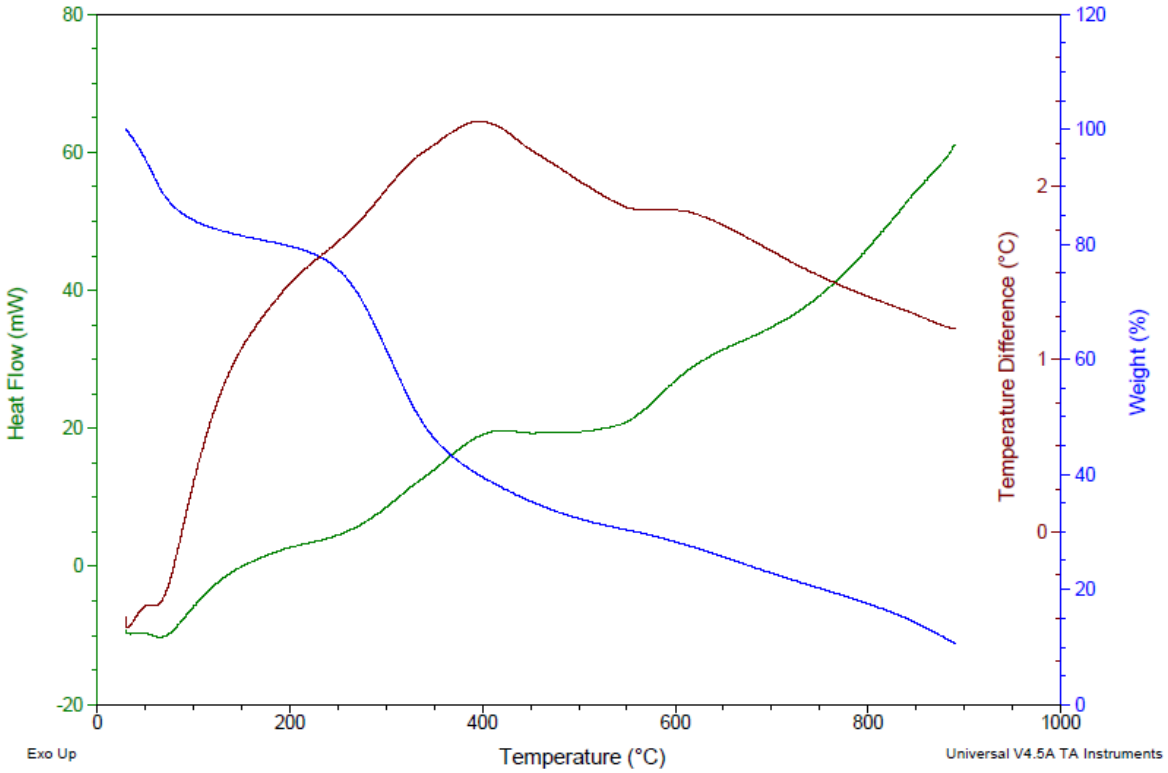

Figure 7 Thermogravitometric analysis of synthesized ZnO NPs

CONCLUSION

Antimicrobial ZnO NPs were produced with culture supernatant of *A. schindleri* SIZ7. The formation of ZnO NPs was observed, achieved after 48 h of incubation with culture supernatant. The ZnO NPs formed were spherical in shape and polydispersed with diameters of 20–100 nm. The properties of ZnO NPs were confirmed by HR-TEM, EDS, FTIR and TGA analysis. The ZnO NPs exhibited potential antimicrobial activity against foodborne pathogens, *E. coli* and *S. enterica* with a MIC of 100 µg ml^{-1}. The bacterial system, therefore, has the potential for low-cost and environmentally friendly production of antimicrobial ZnO NPs.

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ANTIMICROBIAL ACTIVITY OF SOME ESSENTIAL OILS ALONE AND IN COMBINATION WITH AMIKACIN AGAINST *ACINETOBACTER* SP

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ABSTRACT

Acinetobacter sp. as gram negative bacilli is one of the most problematic bacteria in hospital environments. The emergence of multi-drug resistant isolates of *Acinetobacter* sp. encourages the scientists to find the new antimicrobial agent with less side effects. The aim of this study was to evaluate the antibacterial activity of *Cymbopogon olivieri, Heracleum persicum, Juniperus comminus, Azillia eryngioides, Dacus carrota, Ferula gummosa, Acorus calamus, Mentha pulegium, Achillea biebersteinii,* and *Chaerophyllum macropodum* essential oils against clinical trials of *Acinetobacter* sp. by disc diffusion and micro broth dilution assays. The synergistic effect of these essential oils and amikacin (AMI) were determined. The higher inhibition zone diameters were for 2 µl of *C. macropodum* (15.3±0.48 mm). The lower MIC and MBC values were for *C. olivieri* (1.4 and 1.9 µl/ml) and *J. comminus* (1.9 and 2.6 µl/ml), followed by *C. macropodum* (2.01 and 3.2 µl/ml), *D. carrota* (2.1 and 3.8 µl/ml), *A. eryngioides* (2.3 and 3.1 µl/ml) essential oils and *F. gummosa* (2.4 and 4 µl/ml). AMI showed synergistic effect with all of the essential oils. *D. carrota* and *A. eryngioides* showed the best synergistic effect with AMI, followed by *C. macropodum*, *A. biebersteinii*, *J. comminus* and *F. gummosa* essential oils.

Keywords: *Acinetobacter* sp., essential oil, synergistic effect, amikacin

INTRODUCTION

Acinetobacter sp. isolates are problematic pathogens in intensive-care units and other hospital units in recent years. They are the causes of health care associated pneumonia, surgical site infections, bloodstream infections, urinary tract infections **(Tolbat *et al.*, 2006).** *Acinetobacter* sp. isolates with multi drug resistance (MDR) are markedly increasing and treatment of *Acinetobacter* sp. infections have been limited to few broad spectrum antibiotics, including carbapenems, amikacin, doxycycline, minocycline, and ampicillin/sulbactam **(Van Looveren and Guossens, 2004)**. As resistance to antibiotics has emerged, the mortality rates in *Acinetobacter* sp. infected patients have increased. Therefore, the popularity of natural essential oils as alternative treatment has increased **(Sienkiewicz *et al.*, 2011; Mikaili *et al.*, 2011; Candan *et al.*, 2003; Damjanovic- Vratnica *et al.*, 2011)**.
In this research, we isolated 35 clinical isolates of *Acinetobacter* sp. and determined the sensitivity of these isolates to different antibiotics; then we evaluate the anti *Acinetobacter* sp. activity of ten essential oils alone against clinical isolates of *Acinetobacter* sp. The combination of ten different essential oils with amikacin (AMI) was evaluated against one AMI resistant isolates by measuring the FIC and FIC indexes.

MATERIAL AND METHODS

Essential oils and their analysis

10 different essential oils including *Cymbopogon olivieri, Heracleum persicum, Juniperus comminus, Azillia eryngioides, Dacus carrota, Ferula gummosa, Acorus calamus, Mentha pulegium, Achillea biebersteinii* and *Chaerophyllum macropodum* were prepared from Barij Essence Pharmaceutical Company. The essential oils were analyzed using GC-FID and GC-MS. The GC-FID and GC-MS apparatus were conducted on an HP 6890 GC system coupled with 5973 network mass selective detectors with a capillary column of HP-5MS (30 m × 0.25 mm, film thickness 0.25 µm). The oven temperature program was initiated at 60 °C, held for 1 min, then raised up to 245 °C at a rate of 3 °C/min held for 10 min. Helium was used as the carrier gas at a flow rate 1.5 ml/min. The detector and injector temperatures were 250 and 230 °C, respectively. The compounds of the essential oil were identified by comparison of their retention indices (RI), mass spectral fragmentation with those in the stored Wiley 7n.1 mass computer library **(Adams, 2001)**.

Antibiotics

The antibiotic discs that were used in this study including ciprofloxacine (CIPR 5 µg), cefepime (FEP 30 µg), ceftazidime (CAZ 30 µg), levofloxacin (LEVOF 5 µg), amikacin (AMI 30 µg), amoxicillin (AMOXY 30 µg), Imipenem (IMI 10 µg), tobramycin (TOB 10 µg), cefotaxim (CTX 30 µg), norfloxacin (NOR 10 µg), ampicillin+sulbactam (SAM 20 µg (10+10)), meropenem (MRP 10 µg), gentamicin (GEN 10 µg), piperacillin+tazobactum (PI 100+ IZ 10 µg), amoxicillin+clavulonate (AMC 30 µg; (20+10)) were purchased from Rosco (Diagnostica A/S, Taastrupgaardsvej 30 DK-2630 Taastrup).

Acinetobacter isolates and antimicrobial susceptibility testing

A total of 35 clinical isolates cultured from different samples of wounds, trachea, blood, CSF, catheter and other samples of patients at hospitals from Tehran were the subject of this investigation. Antimicrobial susceptibility testing was evaluated using disc diffusion **(NCCLS, 2012)** and micro broth **(CLSI, 2009)** dilution assays. This inoculate of microorganism was adjusted to 0.5 McFarland (1×10^7-1×10^8 CFU/ml) and using a sterile cotton swab, the microbial suspensions were cultured on appropriate media. Subsequently, sterile blank discs (6 mm in diameter) were saturated with 0.5, 1 and 2 µl of essential oil and were put on the cultured media. The plates were incubated at 37 °C for 24 h. The inhibition zones (IZ) diameters were measured in millimeters (mm) and average of IZ was recorded as means ± SD (Standard Deviation).
The minimal inhibitory concentration (MIC) and minimal Bactericidal Concentration (MBC) values of essential oils were determined by micro broth dilution assay. The essential oil was twofold serially diluted (8 - 0.0125 µl/ml of essential oil). Cation adjusted Muller Hinton broth was used as broth media. After shaking, 100 µl of essential oil was added to each well. The above microbial suspensions were diluted to 1×10^6 and then 100 µl were added to each well and incubated at 35±2 °C. MIC was defined as the lowest concentration of essential oil that inhibits bacteria after 24 h. MBC value was the first well that

showed no growth on suitable media. All experiments were done in triplicates.Statistical data analysis was performed by SPSS software (version 17, Chicago, Illinois, USA). Statistical analysis (ANOVA) was applied to determine the differences ($P<0.05$). Significant differences between the essential oils and microorganisms were determined by Tukey test.

Checkerboard titer test

AMI were purchased from Sigma-Aldrich Co. LLC. and dissolved in water. The dilutions were prepared in water in concentration 64-0.0125 µg/ml and the antimicrobial susceptibility testing was performed as CLSI procedure (CLSI, 2009).

Eight serial twofold dilutions of essential oils and AMI were used. Fifty µl of each dilution of essential oil was added to the wells of 96-well plates in vertical orientation and 10 µl of AMI dilution was added in horizontal orientation. 50 µl of AMI resistant *Acinetobacter sp* (10^6 CFU/ well) was added to each well and incubated for 24 h. Fractional inhibitory concentrations (FICs) were calculated as the MIC of the combination of essential oil and AMI divided by the MIC of essential oil or AMI alone. The FIC index (FICI) was interpreted as a synergistic effect when it was ≤0.5, as additive or indifferent when it was >0.5-2 and as antagonistic when it was>2.0 **(Rosato *et al*., 2007)**.

Table 1 Antibiotic resistant profile of clinical isolates of *Acinetobacter* sp.

Antibiotics	Resistance (%)
CIPR	(12/35) **65.7**
FEP	(14/35) **60**
CAZ	(9/35) **74.2**
LEVOF	(18/35) **48.6**
AMI	(20/35) **42.9**
AMOXY	(1/35) **97.1**
IMI	(12/35) **65.7**
TOB	(26/35) **25.7**
CTX	(3/35) **91.4**
NORFX	(9/35) **74.3**
SAM	(14/35) **60**
MRP	(12/35) **74.3**
GEN	(10/35) **71.4**
PI+IZ	(13/35) **62.8**
AMC	(9/35) **74.2**

CIPR= ciprofloxacine; FEP= cefepime; CAZ= ceftazidime; LEVOF= levofloxacin; AMI =amikacin; AMOXY= amoxicillin; IMI= Imipenem; TOB= tobramycin; CTX= cefotaxim; NORFX= norfloxacin; SAM= ampicillin+sulbactam; MRP= meropenem; GEN= gentamicin; PI+IZ= piperacillin+Tazobactum; AMC= amoxicillin+clavulonate

RESULTS AND DISCUSSION

Resistance of *Acinetobacter* sp to antibiotics

As the tab 1 is shown, the resistant profile of 35 clinical isolates were included: CIPR (65.7%), CTX (60%), CAZ (74.2%), LEVOF (48.6%), AMI (42.9%), AMOXY (97.1%), IMI (65.7%), TOB (25.7%), CTX (91.4%), NORFX (74.3%), SAM (60%), MRP (74.3%), GEN (71.4%), PI+IZ (62.8%), AMC (74.2%). The higher sensitivity was for TOB and AMI (tab 1).

Chemical composition and antibacterial screening

The antibacterial evaluation of essential oils against clinical isolates of *Acinetobacter* sp. by disc diffusion method showed that the activity was increased dose dependently. Increasing in the amount of essential oils increased the inhibition zone diameter of essential oils (tab 3). The higher inhibition zone diameters were for 2 µl of *C. macropodum* (15.3±0.48 mm), and *H. persicum* (13.3±0.48 mm).

The main components of *H. persicum* was n-octyl acetate (72.3%), 2-methyl-octyl ester butanoic acid (5.5%), 1-octanol (4.2%) while the chemical composition of *C. macropodum* showed the presence of *trans*-ocimene (49.2%), cis-ocimene (23.6%), γ-terpinene (7.7%), β-myrcene (4.4%), p-cymene (5.5%), and fenchyl acetate (2.7%) as the main components (tab 2).

Table 2 Chemical attributes of essential oils

Essential oil	Main components
Cymbopogon olivieri	Piperitone (72.8%), 4-carene (11.8%), β-himachalene (7.6%)
Heracleum persicum	n-octyl acetate (72.3%), 2-methyl-octyl ester butanoic acid (5.5%), 1-octanol (4.2%)
Juniperus comminus	Camphene (37.7%), β-pinene (15.7%), γ-terpinene (12%), murola-4(14),5-diene (trans) (11.8%), α-terpinene (1.89%)
Azillia eryngioides	α-pinene (63.8%), bornyl acetate (18.9%), β-pinene (2.6%), linalool (2.1%), z-citral (1.3%)
Dacus carrota	Carotol (46.1%), 3-octen-5-yne,2,7-dimethyl-(z) (15.7%), α-pinene (10.7%), trans caryophyllene (4.6%), trans-β-farnesene (4.5%), α-bergamotene (2.53%)
Ferula gummosa	β-pinene (62.7%), α-pinene (9.5%), δ-carene (7.5%)
Acorus calamus	Cis-asarone (27.5%), acorenone (17.4%), elemene (8.9%), α-salinene (7.2%), camphor (3.1%), camphene (2.6%)
Mentha pulegium	Piperitone (38.1%), piperitenone (33.1%), α-terpineol (4.8%), 1,8-cineole (4.1%), piperitenone oxide (3.4%), menthone (3.0%)
Achillea biebersteinii	Germacrene-D (46.6%), camphor (6.2%), 1,8-cineole (5.2%), bicyclogemacrene (4.8%), spathulenol (3.8%)
Chaerophyllum macropodum	*trans*-ocimene (49.2%), cis-ocimene (23.6 %), γ-terpinene (7.7 %), β-myrcene (4.4 %), p-cymene (5.8 %), and fenchyl acetate (2.7 %)

As we mentioned before, the higher sensitivity of antibiotics against clinical isolates of *Acinetobacter* sp. was for tobramycin, AMI and levofloxacin. The inhibition zone diameter of these antibiotics was 14.1, 10.5 and 12.5 mm respectively and was lower than *C. macropodum* essential oil. The MIC and MBC evaluation of these essential oils showed the different results with disc diffusion method.

The lower MIC and MBC values were for *C. olivieri* essential oil (1.4 and 1.9 µl/ml) and *J. comminus* (1.9 and 2.6 µl/ml) followed by *C. macropodum* (2.01 and 3.2 µl/ml), *D. carrota* (2.1 and 3.8 µl/ml), *A. eryngioides* (2.3 and 3.1 µl/ml) and *F. gummosa* (2.4 and 4 µl/ml) essential oils. Piperitone (72.8%), 4-carene (11.8%), β-himachalene (7.6%) were found in *C. olivieri* essential oil. Camphene (37.7%), β-pinene (15.7%), γ-terpinene (12%), murola-4(14), 5-diene (trans) (11.8%), α-terpinene (1.89%) were the main components of *J. comminus* essential oil. The MIC values for *H. persicum* (3.5 µl/ml), *A. biebersteinii* (3.6 µl/ml), *A. calamus* (3.9 µl/ml) essential oils were almost the same but the MBC values were 4.8, 5.7 and 6.5 µl/ml, respectively. Therefore, there is no correlation between the inhibition zone diameter and MIC values ($P>0.05$).

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Table 3 The antimicrobial activity of essential oils against clinical isolates of *Acinetobacter* sp.

Essential oil	Inhibition Zone (Means±SE mm)				Microbial Concentrate (µl/ml)	
	0.5 µl	1 µl	2 µl	µg	MIC	MBC
C. olivieri	6.2±0.1	8.7±0.23	11.9±0.21	-	1.4±0.07	1.9±0.11
H. persicum	6.2±0.07	8.2±0.22	13.3±0.48	-	3.5±0.07	4.8±0.15
J. comminus	6.3±0.09	8.2±0.19	11.5±0.17	-	1.9±0.1	2.6±0.14
A. eryngioides	6.1±0.03	7.3±0.16	10.2±0.24	-	2.3±0.1	3.1±0.14
D. carrota	6.1±0.03	6.8±0.15	10.6±0.24	-	2.1±0.1	3.8±0.19
F. gummosa	6.1±0.03	6.5±0.74	10.7±0.19	-	2.4±0.09	4±0.25
A. calamus	6.1±0.04	7.7±0.17	11.2±0.19	-	3.9±0.11	6.5±0.25
M. pulegium	6.7±0.12	9.1±0.34	13.6±0.25	-	2.3±0.09	4.1±0.24
A. biebersteinii	6.1±0.04	7.7±0.16	11.4±0.19	-	3.6±0.11	5.7±0.25
C. macropodum	8.4±0.3	10.8±0.36	15.3±0.48	-	2.01±0.15	3.2±0.35
TOB	-	-	-	14.1±1.1		
AMI	-	-	-	10.5±1.2		
LEVOF	-	-	-	12.5±1.2		

LEVOF= levofloxacin; TOB= tobramycin; AMI = amikacin; MIC= Minimal Inhibitory Concentration; MBC= Minimal Bactericidal concentration

Todays, interest in essential oils or extracts as alternative treatment due to their loss or no adverse effects and multifunctional properties such as anti-inflammatory, analgesic, immune enhancing and antimicrobial activities are increasing. There are many investigations that evaluate the antibacterial activities of plant derivatives against *Acinetobacter* sp. as a main human pathological agent. The antibacterial activity of *Foeniculum vulgare* Miller essential oil **(Jazani *et al.*, 2009)**, garlic chloroform extract and allicin **(Jazani *et al*., 2007)**, green tea aqueous extract **(Hosseini Jazani *et al.*, 2007)**, thyme essential oil **(Lysakowska *et al*., 2011)**, *Cassia fistula* extract **(Aneja *et al*., 2011)**, *Satureja hortensis* essential oil **(Mihajilov-Krstev *et al*., 2009)** were confirmed.

Table 4 Fractional Inhibitory Concentration (FIC) and FIC indices (FICI)

	FIC	FICI
Cymbopogon olivieri AMI	0.5 0.003	0.503
Heracleum persicum AMI	0.5 0.006	0.506
Juniperus comminus AMI	0.25 0.003	0.253
Azillia eryngioides AMI	0.015 0.05	0.065
Dacus carrota AMI	0.0004 0.05	0.0504
Ferula gummosa AMI	0.25 0.2	0.45
Acorus calamus AMI	0.5 0.003	0.503
Mentha pulegium AMI	0.003 0.5	0.503
Achillea biebersteinii AMI	0.004 0.2	0.204
Chaerophyllum macropodum AMI	0.0004 0.2	0.2004

AMI= amikacin; **FIC of essential oil**=MIC in combination with AMI; **FIC of AMI** =MIC in combination with essential oil, MIC of essential oil alone, MIC of AMI alone, **FICI**= FIC of essential oil+ FIC of AMI

This study evaluates the antibacterial activity of new essential oils against clinical isolates *Acinetobacter* sp. Other studies showed the chemical composition of essential oils play an essential role in their antimicrobial activity. It is shown, different chemotypes of basil essential oil including estragol, linalool-estragol, methyl eugenol-anethol, anethol chemotypes had different antibacterial activity against *Acinetobacter* sp. and exhibited more sensitivity to methyl eugenol chemotype than lonalool or estragol chemotypes **(Koba *et al*., 2009)**.
Therefore, the different antibacterial activity of essential oils is related to the composition of essential oils. Among the 11 different essential oils, *C. olivieri*, *J. comminus* and *C. macropodum* showed the best antibacterial activity against clinical isolates of *Acinetobacter* sp. Piperitone as the first main component of *C. olivieri* showed antimicrobial activity **(Cardenas-Ortega *et al*., 2005, Shahverdi *et al*., 2004)**. Camphene **(Gerige and Ramjaneyulu, 2007)**, β-pinene**(Andrew *et al*., 1980)**, γ-terpinene **(Cristani *et al*., 2007)** is responsible for antibacterial activityof *J. comminus* essential oil.

Synergistic evaluation

The synergistic evaluation of essential oils and AMI showed synergistic effect (the FICI was lower than 0.5). *D. carrota* and *A. eryngioides* showed the best synergistic effect with AMI, followed by *C. macropodum*, *A. biebersteinii*,*J. comminus* and *Ferula gummosa* essential oils (tab 4). The results of synergistic evaluation showed that all of the essential oils decreased the MIC value of AMI. The lower FICIs were for *D. carrota* and *A. eryngioides* essential oils. Therefore, it does not mean that the essential oil with higher antibacterial activity has the higher synergistic effect. It is showed that piperitone has increased the antimicrobial activity of Furazolidone and nitrofurantoin **(Shahverdi *et al*., 2004)**.
Furtheremore, the synergistic effects of AMI with lemon essential oil **(Guerra *et al*., 2011)**, ciprofloxacin, gentamycin, piperacillin, tetracycline, cefoprazone with *Coriandrum sativum* essential oil **(Duarte *et al*., 2012)** were reported. Therefore, *C. olivieri*, *J. comminus* and *C. macropodum* essential oils can be used as alternative treatment for controlling of *Acinetobacter* sp. Therefore, *D. carrota* and *A. eryngioides* can be used along with AMI for decreasing the effective dose of this antibiotics. More clinical studies are used for exhibiting the efficacies in clinical trials.

CONCLUSION

This study evaluate the antibacterial activity of ten essential oils against clinical isolates of *Acinetobacter* sp. The results of antibacterial screening showed different essential oils with different chemical composition has different antibacterial activity. Among ten essential oils,*C. macropodum*, *C. olivieri* and *J. comminus* (1.9 and 2.6 µl/ml) has the higher antibacterial activity against *Acinetobacter* sp. AMI showed synergistic effect with all of the essential oils. *D. carrota* and *A. eryngioides* showed the best synergistic effect with AMI, followed by *C. macropodum*, *A. biebersteinii*,*J. comminus* and *F. gummosa* essential oils.Therefore, these essential oils can be as alternative treatment for lowering dose of AMI. More clinical studies are required to providing these essential oils in clinical.

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CHEMICAL PROFILE AND ANTIMICROBIAL ACTIVITIES OF TWO EDIBLE MUSHROOMS *(Termitomyces robustus* and *Lentinus squarrosulus)*

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ABSTRACT

The methanol extracts of two edible mushrooms; *Termitomyces robustus* (ewe) and *Lentinus squarrosulus* (erirokiro) were screened for phytochemicals of medicinal importance and the chemical profile investigated using standard analytical methods with the aim of assessing their health promoting properties. Both mushrooms tested positive to flavonoids, saponin, tannin and terpenoid but negative to steroid, anthraquinone and phlobatannin. The results of proximate compositions in % were; moisture contents (7.22 ± 0.07; 11.03 ± 0.21), crude protein (31.34 ± 0.01; 42.77 ± 0.57), ash (7.07 ± 0.04; 10.45 ± 0.43), crude fibre (4.07 ± 0.18; 9.48 ± 0.04), crude fat (3.71 ± 0.16; 6.76 ± 0.22), carbohydrate by difference (24.90 ±0.11; 41.27 ± 0.19), calorific value in kcal (331.55±3.41; 342.35±3.09), and total dietary fibre (10.21 ± 0.00; 11.68 ± 0.00). The anti-nutrient factors in mg/g were; tannin (3.25 ± 0.80; 7.40 ± 0.14) oxalate (1.53 ± 0.00; 1.71 ± 0.07), and phytate (1.48 ± 0.06; 1.94 ± 0.05). Mineral elements, vitamins, essential and non-essential amino acids in substantial quantities were detected in the mushrooms. The phenolic compounds identified and quantified were gallic acid, catechin,chlorogenic acid, caffeic acid, ellagic acid, epicatechin, rutin, isoquercitrin, quercitrin, quercetin and kaempferol.The mushrooms exhibited various antifungi and antimicrobial activities. The two mushrooms possessed good nutritional and chemical qualities and could be sources of many different nutraceuticals.

Keywords: Phytochemicals, proximate, flavonoids, Polyphenols, *Termitomycesrobustus, Lentinussquarrosulus*

INTRODUCTION

Mushrooms are the higher fungi which have long been used for food and medicinal purposes. Fresh and preserved mushrooms are consumed in many countries as a delicacy, particularly for their specific aroma and texture **(Pavel , 2012).**They have rich nutritional value with high protein content (up to 44.93%), vitamins, minerals, fibers, trace elements and low calories and lack cholesterol **(Hrudayanath and Sameer, 2014; Agahar- Murugkar and Subbulakshmi, 2005; Wani *et al*., 2010).** Wild mushrooms are becoming more and more important in our diet due to their value as food as well as their medical and nutraceutical values (**Chang and Miles, 2004**). Their global economic value is now increasing as a result of their nutritional, organoleptic, and pharmacological characteristics **(Diez and Alvarez, 2001; Solak *et al.*, 2006).**The leading countries in mushroom production are China, US, Netherlands, India and Vietnam, according to recent FAO report **(FAO, 2014; Yaoqi *et al*., 2014**). Mushrooms production can alleviate poverty in rural communities and improve the diversification of agricultural production **(Godfrey *et al.*, 2010)** as well as national economy if given its proper place especially now that mushrooms are being considered as an alternative food source to provide adequate nutrition for world's increasing population. Several researches have established some edible mushroom species as sources of physiological agents for medicinal applications, possessing antitumour, cardiovascular, antiviral, antibacterial and other activities **(Halpern and Miller, 2002; Wasser, 2002; Chang, 1996).** Each mushroom type produces a specific set of metabolites capable of dealing with the set of microbes that coexist in that specific environment (**Dembitsky, 2010**). It was reported by **Bobek *et al*., (1991)** that the consumption of a mushroom-containing diet prevented serum cholesterol increase at the end of the four week period and lowered by almost 40 % as compared with control groups which have not had mushroom in their diet. **Kabir and Kimura (1989)** reported that dietary mushrooms have reduced the blood pressure in rats. *Termitomyces robustus* is called 'Ewe'meaning "expand" among the Yorubas in western Nigeria. The mushroom caps, globular at first, expands and opens out to become almost flat. It has blackish brown colour. It is the most popular edible mushroom in Nigeria but remains underutilized due to traditional believes, myths or dietary habit. *Lentinus sqarrosulus,*called 'erirokiro' to describe its tough, leathery texture is whitish in colour and also common in the genus *Lentinus* **(Oso, 1976)**. Over the last decade, mushrooms have been studied as novel functional food, there has been many studies on the nutritional contents of different mushroom species globally, little or no work has been carried out on chemical qualities of so many species and there is a dearth of information about polyphenolic components of the species in Nigeria. The objective of this study was to investigate the chemical profile of two edible mushrooms, screen them for phytochemicals and use Reverse-Phase HPLC-DAD to quantify the polyphenols present.

MATERIAL AND METHODS

Samples preparation

The two species obtained from local markets in Nigeria were scraped and thoroughly cleaned with water to remove sand, cut into smaller pieces (both the pileus and stipes), oven dried at 60 ^{o}C, then ground and sieved to give 40 mm mesh size powder.

Preparation of extracts

The powdered mushrooms were subjected to a cold maceration process for 72 h with methanol and ethanol separately to obtain the alcoholic extracts and for 24 h to obtain the aqueous extracts and filtered. The extracts were concentrated under vacuum and evaporated using a rotary evaporator at low temperature (45^{o}C).

Phytochemical screening of the extracts

Phytochemical screening of the methanol extracts was carried out using qualitative tests for analyses of different constituents of plant materials according to the common phytochemical methods described by **Harborne (1973); Trease and Evans (1983)** and **Sofowora, (1993).**

Chemical analyses

Proximate composition (fat, crude fibre, and ash) was determined on dry basis by the standard method of Association of Official Analytical Chemist **(AOAC, 2006)**, the protein content was determined using the micro-Kjedahl method (N x 6.25) and the carbohydrate determination by difference **(AOAC, 2006).** Total dietary fibre (TDF) was determined to dried, fat-free sample according to Megazyme TDF Assay procedure, K-TDFR 05/12 (MEGAZYME INTERNATIONAL, IRELAND). The mineral elements were determined using Atomic Absorption Spectrophotometer **(Pearson, 1976),** vitamins by spectrophotometric methods (**Biesalski *et al*., 1986; Benderitter *et al*., 1998; Okwu and Josiah, 2006**), and antinutrients by titrimetry and spectrophotometric methods **(Makkar and Goodchild, 1996; Day and Underwood, 1986).**

Determination of antibacterial activity

The antibacterial activity of aqueous, methanolic and ethanolic extracts of the mushrooms against *Staphylococcus aureus, Pseudomonas aeruginosa* and *Salmonella typhi* bacteria was evaluated by using agar well diffusion method **(Ahmad and Beg, 2001; Srinivasan *et al*., 2001)**. Plate count agar (PCA) plates were inoculated with 100 µl of standardized inoculum ($1.5x10^8$ CFU/ml) of each selected bacterium and spread with sterile swabs. Wells of 8 mm size diameter were made with sterile borer into agar plates containing the bacterial inoculum and the lower portion was sealed with a little molten agar medium. About 0.5 ml of each of the extracts was poured into a well of inoculated plates. Chemical antibiotics, Streptomycin sulphate (10 $ugml^{-1}$) was used as a positive control which was introduced into a well instead of plant extract. The solvents; deionized water, methanol or ethanol were used as negative controls, which were introduced into the wells instead of the extracts. The plates thus prepared were left at room temperature for ten min allowing the diffusion of the extracts into the agar **(Rios *et al.,* 1988)**. After incubation for 24 h at 37 ^oC, the plates were observed. If antibacterial activity was present on the plates, it was indicated by an inhibition zone surrounding the well containing the plant extract. The zone of inhibition was measured and expressed in millimetres.

Determination of antifungal activity

The antifungal activity of plant extracts was evaluated against food-associated fungi by using poisoned food technique. Potato dextrose agar (PDA), was weighed (39g) and dispersed in a litre of deionised water sterilized at 121 ^oC for 15 min, allowed to cool (45 ^oC) before pouring (20 ml) into separated dishes. The fungi; *Trichoderma rubrum* and *Aspergillus fumigatus* were inoculated on potato dextrose agar (PDA) plates and incubated for 25 ^oC for 72 h, to obtain young, actively growing colonies of moulds. 0.2 ml of each of the extract was mixed with 20 ml of cooled (45^oC) molten PDA medium and allowed to solidify at room temperature for 30 min. Thereafter 10 µl of fungal spores in distilled water was added at the centre of the solidified PDA plates. PDA plates with 10 $ug.ml^{-1}$ of bonlate were used as positive controls. PDA plates with the solvents; deionized water, methanol or ethanol were used as negative controls **(Georgii and Korting, 1991, McCutcheon *et al*., 1994)**. The inoculated plates were incubated at 25 ^oC and colony diameter measured and recorded after 3 days. Percentage mycelial growth inhibition (% MGI) was calculated as given below:

$$\% \text{ MGI} = \frac{\text{Mean dia. of fungal colony in control - Mean dia. of fungal colony in plant extract x 100}}{\text{Mean dia. of fungal colony in control}}$$

Determination of amino acid profile

About 20 g of each frozen fresh mushroom sample was weighed properly and minced by using 100 ml phosphate buffer supplemented with 2 % SDS (sodium dodecyl sulfate) by using philip household blender. The homogenate was filtered through double layered cheese cloth. The filtrate was subjected to ammonium sulphate salt precipitation method at 65 % saturation. The proteins were pelleted by centrifugation, concentrated by dialysis and then freez-dried for amino acid analysis. The 4.0 g protein isolate was hydrolyzed and evaporated in a rotary evaporator. The amino acid profile in the samples were determined using Technicon sequential multi-sample amino acid analyzer (TSM) **(Benitez, 1984).**

Quantification of phenolic compounds by high-performance liquid chromatography with diode-arraydetection

Reverse phase chromatographic analysis was carried out under gradient conditions using C_{18} column (4.6 mm x 150 mm) packed with 5µm diameter particles; the mobile phase was water containing 2 % acetic acid (A) and methanol (B), and the composition gradient was: 5 % of B until 2 min and changed to obtain 25 %, 40 %, 50 %, 60 %, 70 % and 100 % B at 10, 20, 30, 40, 50 and 60 min, respectively, following the method described by **Amaral *et al*., (2013).** Each sample extract was analyzed at a concentration of 20 mg/ml. The presence of eleven compounds was investigated for, *T. robust* and ten for *L.*

Phytochemical components

The screen of the methanol extracts of the samples showed the presence of some bioactive compounds (Tables 1). It is noteworthy that both mushrooms contained flavonoids, saponin and tannin but steroid, anthraquinone and phlobatannin were absent. Only *T.robustus* tested positive to alkaloid. The presence of flavonoids in both is well understood as it is in agreement with the previous literature that plants are major sources of phenolic compounds, which are synthesized as secondary metabolites during normal development in response to stress conditions, such as wounding and UV radiation among others **(Sies, 1997).** The alkaloids, saponnis and tannins play important roles in various antibiotics used in treating common pathogenic strains as reported by **Kubmarawa *et al.,* (2007).**

Chemical composition

The result of the proximate analysis of the mushrooms on dry weight basis is presented in Table 2. The moisture content of *T. robustus* was significantly higher ($p \leq 0.05$) than that of *L. squarrosulus*. Fresh wet mushrooms have higher moisture contents which make them easily perishable. Excessive moisture content in raw materials favours the microbial growth and the decomposition of active compounds by hydrolysis. The moisture content of dried plants varies, depending on the prevailing local environment and length of storage. The protein content of *T. robustus* was significantly higher ($p \leq 0.05$) than that of *L. squarrosulus* and do not agree with the study carried out by **Jose and Kayode (2009)** which indicated that *L. squarrosulus* had higher protein than *T. robustus*. **Adejumo and Awosanya, (2005)** however, reported a higher percentage of crude protein and moisture for *Termitomyces mammiforms* than *Lentinus tigrinus.* Percentage of crude protein obtained in this study for *T. robustus* was higher than (33.8%) reported by **Aletor (1995)** for this same species. **Oboh and Shodehinde (2009)** obtained 28.60 % and 24.80 % for the pileus and stipes of *T. robustus* respectively. It has been reported that the protein content of mushrooms is affected by a number of factors. The development stage of mushroom is a significant factor affecting the protein content. In addition, the type of mushroom, the part sampled, the location as well as the substrates affect protein content **(Barros*et al*., 2007; Kalmiş *et al*., 2011)**. The whole mushrooms (pileus and stipes) were analysed in this work. Proteins are the building blocks of life. The body needs protein to repair and maintain itself. Since it was present in appreciable quantity in both mushrooms, nutritional power of these fungi as protein supplements cannot be ignored.

Both mushrooms contained higher ash, than crude fat, the same trend obtained by **Oboh and Shodehinde (2009),** and the ash content of the two compared favorably with percentage ash of 7.8 ± 0.6, 8.3 ± 0.0, and 7.3 ± 0.3 reported earlier for *Ganoderma spp., Omphalotus olearius* and *Hebeloma mesophaeum* respectively (**Aremu *et al.,* 2009),** also with some other vegetables such as *Occimumgraticimum* (8.00 %) and *Hibiscus esculentus* (8.00 %) **(Akindahunsi and Salawu, 2005).** High ash content is an indication of high mineral content, a reflection of the mineral contents preserved in the food materials **(Antia *et al*., 2006)**. Mineral elements are essential for tissue functioning and a necessity in daily requirement for human nutrition. The result therefore suggests a high deposit of mineral elements in the mushrooms**.** *T. robustus* has higher crude fat than that of *L. squarrosulus,* the values obtained for the two however fell within the range of percentage crude fat obtained for some wild mushrooms in Turkey, which was 1.40 ± 3.00 – 10.58 ± 0.30 for *Cantharellus cibarius* Fr and *Lycoperdon perlatum* Pers., respectively **(Ahmet *et al*., 2009).** Dietary fats help in absorbing and retaining flavours, thus, increase the palatability of food **(Antia *et al*., 2006).** Although, a diet providing 1-2 % of its caloric of energy as fat is said to be sufficient to human beings; as excess fat consumption is implicated in certain cardiovascular disorders such as atherosclerosis, cancer and aging **(Antia *et al*., 2006), Ononugbu (2002**) reported that vegetable fats and oil lower blood lipids thereby reducing occurrence of disease associated with damage of coronary artery. Plant lipids are useful as essential oils, spice, oleoresins and natural food colors and have been developed to products that work with diverse requirements, as culinary, medicinal and cosmetics **(Yadav and Tyagi, 2006).**

The *L. squarrosulus* in this study had significantly higher crude fibre than *T.robustus,* both had higher values than percentage crude fibre of 3.5 ± 0.2, 2.8 ± 0.5 and 3.2 ± 1.0 reported for *Ganoderma spp., Omphalotus olearius* and *Hebeloma mesophaeum* respectively by **Aremu *et al.* (2009).** The total dietary fibre values were higher in both mushrooms than crude fibre as expected. Carbohydrate by difference of *L. squarrosulus* was significantly higher ($p \leq 0.05$) than that of *T. robustus,* the lower carbohydrate level can not affect energy contribution due to the compensation from higher protein values in *T.robustus.* Therefore, there was no significant differences in calorific values of the two mushrooms. Carbohydrates are one such group of carbon compounds, which are essential to life. Almost all organisms use carbohydrates to exploit their rich supply of potential energy to maintain life. Calculated energy values of the *T.robustus* and *L. squarrosulus,* here were found to be lower than previous data

obtained for eight edible wild mushrooms in Turkey which varied from 367.88 kcal/100 g to 450.20 kcal/100 g on dry matter basis **(Ahmet *et al*., 2009).**
The macro elements; calcium, phosphorus, potassium, sodium and magnesium as well as the trace elements iron, copper and zinc were all in higher concentrations in *T. robusts* than *L. squarrosulus*. Lead, mercury and manganese were not detected in any of the mushrooms. Calcium is a major factor sustaining strong bones; it plays a part in blood clotting, muscle contraction and relaxation. **Muhsin, (2006)** reported Ca levels of 124 ppm in 1 g of dried *Lactarius delicious,* calcium and phosphorus are the minerals are abundant in the the bones. *Agaricus bisporus* was found to contain 110 mg/100g phosphorus **(Peter *et al.,* 2012).** Potassium is the most abundant element in both mushrooms, edible wild mushroom species have an average potassium content of 34,350 mg/kg on a dry basis, making them an important and valuable potassium source for the human diet **(Kalmiş *et al.,* 2011).** Studies have revealed that the potassium concentration of mushrooms is relatively constant **(Vetter, 1994).** The sodium content of *Tricholoma terreum* was reported as 92.6-325 mg/kg on a dry basis **(Demirbaş, 2001; Vetter, 2003; Kalmiş *et al*., 2011),Vinhal,*et al*., 2012** also reported 255.34 and 613.03 mg/100g for sodium and potassium respectively in *Agaricus sylvaticus.* Sodium and potassium are important intracellular and extracellular cations respectively. Values obtained for magnesium in both mushrooms were lower than what was reported by **Ezeibekwe, *et al*., (2009)** for *Plerotus tuber-regium* (0.24 %) and *Auricularia auricular* (0.36 %). Magnesium functions as a co-factor of many enzymes involved in energy metabolism, protein synthesis, RNA and DNA synthesis. **Ahmet, (2009)** obtained the range 30.20 ± 2.90 - 550.00 ± 15.00 in mg/kg for Fe in *Ramaria flava* and *Lycoperdon perlatum* Pers; and 15.20 ±1.10 - 330.00 ± 91 for Cu in *Craterellus cornucopioides* and *Armillaria mellea* respectively. Iron plays important role in the formation of haemoglobin **(Latunde-Dada, 2006)** and hence recommended for anaemic convalescence, copper is an essential micronutrient which functions as a biocatalyst required for body pigmentation in addition to iron. The trace metal contents of mushrooms are related to species of mushroom, collecting site of the sample, age of fruiting bodies and mycelium, as well as distance from sources of pollution **(Kalac *et al*., 1991)**. They are mainly affected by acidic and organic matter contents of the soil. This may be responsible for the wide difference in the Zn contents of the two mushrooms in this study. Zn content of 370.00 mg/kg dry weight was found in *R. flava* and 47.00 mg/kg dry weight in *L. perlatum* **(Ahmet, 2009).** Zinc is widespread among living organisms due to its biological significance and mushrooms are known as zinc accumulators **(Mendil, 2004).** Manganese is part of enzyme involved in urea formation, pyruvate metabolism and the galactotransferase of connective tissue biosynthesis **(Chandra, 1999),** but it was not detected in any of the mushrooms under study.
Vitamins A (carotene equivalent) and tocopherol E were detected in the two mushrooms investigated (Table 2). Vitamin A was found in significantly higher ($p \leq 0.05$) concentration in *T. robustus* but E in lower concentration than *L. squarrosulus.* Vitamin A is involved in immune function, vision, reproduction, and cellular communication **(Johnson and Russell, 2010; Solomons, 2006).** According to Mushroom and Health Report, common *Agaricus bisporus* contained 13.0 µg/100 Beta-carotene equivalent vitamin A (2 µg RE) **(Peter *et al.,* 2012)** and *Agaricus sylvaticus* was also found to contain α-tocopherol of 0.020 mg/100 g **(Vinhal *et al*., 2012).**Vitamin E is an important vitamin required for the proper function of many organs in the body. It is also an antioxidant. This means it helps to slow down processes that damage cells **(Yun-Zhong et al., 2002)**. Water soluble vitamins; thiamine (B_1) and ascorbic acid (C) were also detected in the two mushrooms in appreciable quantities compared with vitamin C of 12.65 mg/100 g reported for *Agaricus sylvaticus* **(Vinhal *et al*., 2012)** and B_1 of 0.025 mg/100 g fresh weight found in *Agaricus bisporus* **(Peter *et al*., 2012).** All B vitamins help the body convert food (carbohydrates) into fuel (glucose), which is used to produce energy and also help the body metabolize fats and protein.

Tannin, oxalate and phytates were found in higher concentrations in *T.robustus* than *L. Squarrosulus.* phytate and oxalate concentrations obtained in the present study were lower compared to phytic acid content range of 160 mg/100 g in *T. robustus* to 360 mg/100 g in *C. cyathiformis* with a CV of 28.4 % and oxalate content range of 80 mg/100 g in *T. robustus* to 220 mg/100 g in *A. auricula* with a CV of 3.8 % obtained by **Aletor (1995)** from a research on some edible tropical species of mushrooms. Antinutrients are substances that bind enzymes or nutrients and inhibit the absorption of the nutrients. Tannins even at low levels inhibit digestive enzymes activities making their presence in food undesirable from a nutritional point of view **(El-Adawy *et al*., 2000).** However, tannins have been shown to give substantial protection against cancer of the lungs and stomach when ingested orally **(Yavelow *et al.,* 1983).** Tannin and other phenols may play a role in fighting tooth decay by inhibiting the growth of bacteria that cause tooth decay **(Moles and Waterman, 1985).** Phytates, like tannins have also been found to interact with digestive processes in a beneficial way, slowing down the absorption of sugars and regulate insulin levels when present in small amounts in food, this is beneficial in the prevention and treatment of diabetes and hyperlipidemia (high blood fats) and phytic acid also acts as antioxidant (**Hawkins *et al*., 1993; Phillippy and Graf, 1997)**. Many researchers believe that dietary restriction cannot significantly reduce risk of stone formation because dietary oxalate was found to accounts for only 10-15% of the oxalate that was detected in the urine of individuals who formed calcium oxalate stones, **(Assimos and Holmes, 2000; Curhan, 1999; Parivar *et al.*, 1996; Hanson *et al*., 1989).** This result therefore suggests that the mushooms are safe for consumption.

Amino acid composition of the mushrooms

The result of the amino acid analysis of the protein isolates from the two mushrooms is presented in Table 3. All the essential and non-essential amino acids detected were in significantly higher ($p \leq 0.05$) concentrations in *T.robustus* than *L.squarrosulus* except leucine which was the highest amino acid found in *L. squarrosulus*. Both mushrooms contained most of the essential amino acids in substantial quantities and even exceeded protein requirement pattern **(FAO/WHO, 1991)** in some cases. Aspartic acid (0.37 %), arginine (0.21 %), alanine (0.09 %), proline (0.06 %), and tyrosine (0.19 %) content obtained for *L. squarrosulus* by **Sharma *et al.,* (2012)** were lower than the quantities found in the same species in this study. Glutamic acid was the highest amino acid in *T.robustus* in conformity with the work of **Nakalembe and Kabasa, (2013)** on *Termitomyces* species, followed by leucine while glutamic acid was second to leucine in *L. squarrosulus* contrary to the findings of **Dembitsky *et al.,* (2010)** that aspartic acid dominate other amino acids in *Boletus* species. The most important benefit of leucine is its capacity to maintain blood sugar level. It can also help in producing growth hormones, healing bones and skin, sustaining nitrogen balance and maintaining mental ability **(Anthony *et al.,* 2002).** Overall, the amino acid profiles were similar in both mushroom species though they belong to different families, also similar to the profile obtained from *P. leurotus* species except for norvaline and tryptophan found in small amounts in *P. ostreatus* and *P. Sajorcaju* **(Pornariya and Kanok-Orn, 2009).**

Phenolic compounds in the mushrooms

HPLC fingerprinting of *T. robustus* extract revealed the presence of the gallic acid (t_R = 11.57 min; peak 1), catechin (t_R = 16.09 min; peak 2), chlorogenic acid (t_R = 23.51 min; peak 3), caffeic acid (t_R = 25.06 min; peak 4), ellagic acid (t_R = 32.14 min; peak 5), epicatechin (t_R = 35.98 min; peak 6), rutin (t_R = 38.25 min; peak 7), isoquercitrin (t_R = 42.56 min; peak 8), quercetin (t_R = 45.01 min; peak 9), quercitrin (t_R = 48.93 min; peak 10) and kaempferol (t_R = 54.39 min; peak 11) (Fig. 1 and Table 4) while that of *Lentinus squarrosulus* extract revealed the presence of the gallic acid (t_R = 10.52 min; peak 1), catechin (t_R = 16.03 min; peak 2), chlorogenic acid (t_R = 22.53 min; peak 3), caffeic acid (t_R = 25.11 min; peak 4), ellagic acid (t_R = 32.15 min; peak 5), epicatechin (t_R = 35.09 min; peak 6), rutin (t_R = 39.64 min; peak 7), isoquercitrin (t_R = 42.95 min; peak 8), quercitrin (t_R = 45.10 min; peak 9) and quercetin (t_R = 48.72 min; peak 10) (Fig. 2 and Table 4).

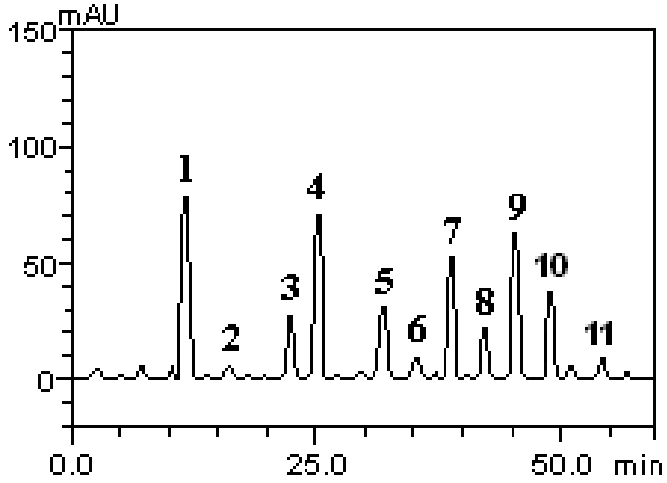

Figure 1 Reverse-phase high performance liquid chromatography profile of *Termitomyces robustus* extract. Gallic acid (peak 1), catechin (peak 2), chlorogenic acid (peak 3), caffeic acid (peak 4), ellagic acid (peak 5), epicatechin (peak 6), rutin (peak 7), isoquercitrin (peak 8), quercitrin (peak 9), quercetin (peak 10) and kaempferol (peak 11).

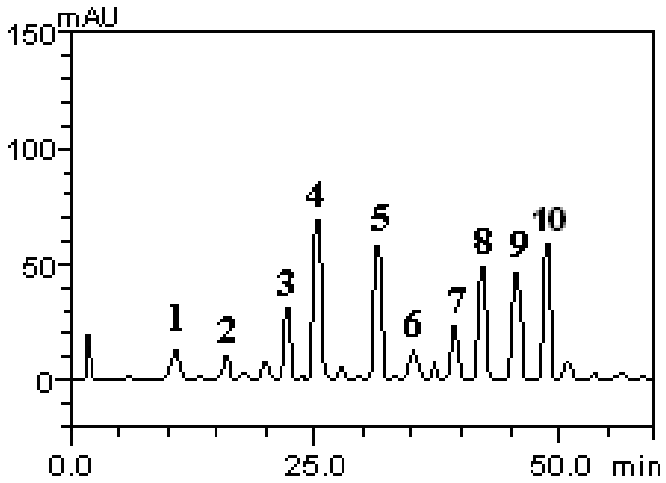

Figure 2 Reverse-phase high performance liquid chromatography profile of *L. squarrosulus* extract. Gallic acid (peak 1), catechin (peak 2), chlorogenic acid (peak 3), caffeic acid (peak 4), ellagic acid (peak 5), epicatechin (peak 6), rutin (peak 7), isoquercitrin (peak 8), quercitrin (peak 9) and quercetin (peak 10).

Gallic acid, caffeic acid, rutin, quercetin and isoquercitrin were present in both at different concentrations. Gallic acid, a benzoic acid derivative and caffeic acid, a cinnamic acid derivative belong to a phenolic acid class. Gallic acid concentration is significantly higher ($p \leq 0.05$) in *T. robust* than *L. squarrosulus,* ellagic and chlorogenic acids are significantly higher ($p \leq 0.05$) in *L. squarrosulus* but no significant difference in the caffeic acid concentrations of the two mushrooms. According to the studies published by **Kaur *et al*., (2009)** and **Rasool *et al*., (2010),** gallic acid possesses significant antioxidant activity and may protect the liver from the harmful effects of free radicals that are formed as a result of various metabolic processes in the body and inhibit the growth of human prostate cancer cells. Caffeic acid (3, 4-Dihydroxycinnamic Acid) is a naturally occurring substance found in many plants, including coffee beans but is entirely unrelated to caffeine and shares no stimulant activity with that of caffeine. Ellagic acid is a type of chemical found in a variety of fruits, berries and plants. Foods high in ellagic acid include raspberries, pomegranates, blackberries, pecans and walnuts. Structurally, chlorogenic acid is a combination of two molecules. It is a caffeic acid molecule bound to a quinic acid moiety, the combination is referred to as chlorogenic acid, and all three molecules can be bioactive after chlorogenic acid ingestion **(Jin *et al*., *2012).*** Catechin, epicatechin, isoquercitrin and quercetin were in higher concentrations in *L.squarrosulus,* while rutin and quercitrin in lower concentrations than in *T.robustus.* Quercetin and kaempferol are in flavonol 3-O-glycosides class, quercetin, is a flavonol occurring in fruit and vegetable in food component with proven beneficial impact on health. Kaempferol was presnt in *T. robustus* but not found in *L.squarrosulus.* Isoquercitrin (2-(3, 4-dihydroxyphenyl)-5, 7-dihydroxy-3-oxy-chromen-4-one) is quercetin 3-glucoside, while quercetin, is present in many plants, but for practical purposes it is derived from rutin (quercetin-3-O-rhamnoglucoside). Quercetin is one of the most potent antioxidants among polyphenols and has also been demonstrated to display the antiviral, antibacterial, anticarcinogenic and antiinflammatory effects **(Walle, 2004; Naidu *et al*., 2012).** Chemically, quercetin is closely related to rutin and quercitrin, two other flavonoids. Quercetin lacks a sugar molecule that is attached to these other flavonoids. When rutin and quercitrin are digested, intestinal bacteria remove the sugar molecule.

Antimicrobial Activities of Mushrooms

The antibacterial activities of aqueous solutions and alcoholic extracts of *T. robustus* (ewe) and *L. squarrosulus* (erirokiro) at concentrations 0.05 g/ml are presented in Figures 3- 5 with Streptomycin sulphate used as positive controls for antibacterial. None of the extracts showed activity against *P. aeruginosa* except ethanol extract of *L. squarrosulus. S. typhi* was also susceptible to an aqueous and methanol extracts of *L. squarrosulus*. Only alcoholic extracts of *T. robustus* and *L. Squarrosulus* showed inhibitory activities against *S. aureus*. The antimicrobial activities of the extracts against *P. aeruginosa* and *S. typhi* were similar, methanol extracts showed more effectiveness than ethanol, this may be due to the fact that they are gram negative organisms and they are physiologically related, this was noticed to be related to the work of **Abosi and Raseroka (2003)**.The extracts showed better antimicrobial activities against *S. aureus*, this may be linked with the composition of their cell wall as they are gram positive. Aqueous extract of *L. squarrosulus* was active against *S. typhi* but that of *T. robustus* was not. Both mushrooms showed activities against *S. aureus* but *T. robustus* showed no activity against *P. aeruginosa* and *S. typhi*. Generally, all the extracts of the two mushrooms exhibited weak antibacterial activities compared with the chemical antibiotics; streptomycin sulphate. The antifungal activities of the mushrooms against the selected pathogens are presented in Figures 6 - 7. These present the percentage mycelia growth inhibition of the sample extracts against filamentous fungi, *T. rubrum* and *A. fumigatus*. The results revealed methanol extracts of the samples had no inhibitory effects against any of the fungi in contrast with bonlate which was used as the positive control. *A. fumigatus* showed higher susceptibility to aqueous and ethanol extracts. The aqueous and ethanol extracts of the mushrooms demostrated quite appreciable antifungal activities when compared with bonlate. The high susceptibility displayed by the fungi to the ethanolic extracts of the samples suggests that the ethanolic extracts may be developed as antifungal drugs to treat infections caused by these organisms and preservatives for stored products like grains.

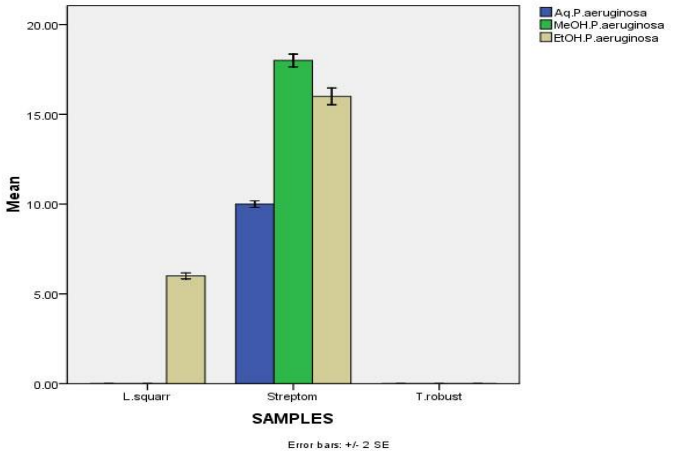

Figure 3 Antibacterial activities of extracts (0.05 g/cm^3) with Streptomycin sulphate as positive control against *Pseudomonas aeruginosa* at 24 h incubation

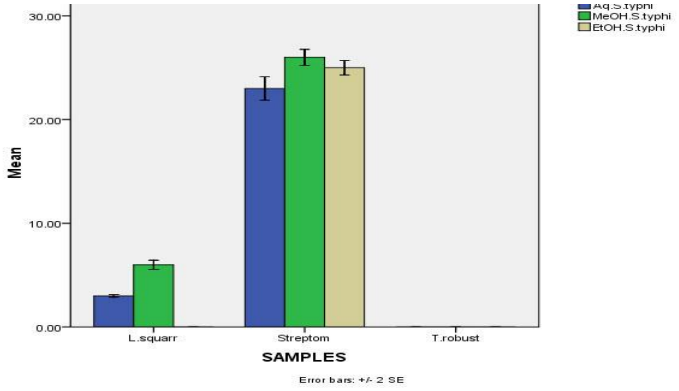

Figure 4 Antibacterial activities of extracts (0.05 g/cm^3) with Streptomycin sulphate as positive control against *Salmonella typhii* at 24 h incubation

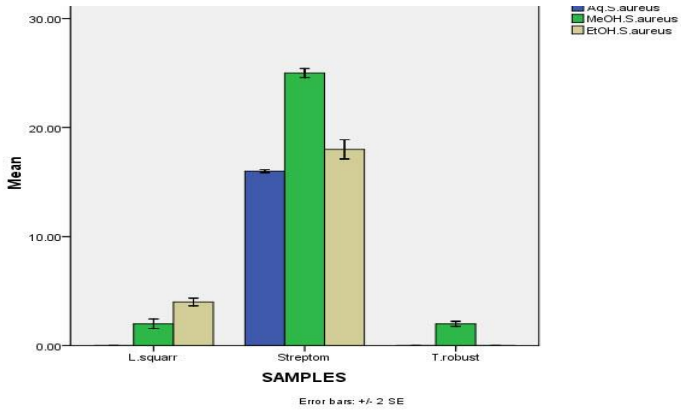

Figure 5 Antibacterial activities of extracts (0.05 g/cm^3) with Streptomycin sulphate as positive control against *Staphylococcus aureus* at 24 h incubation

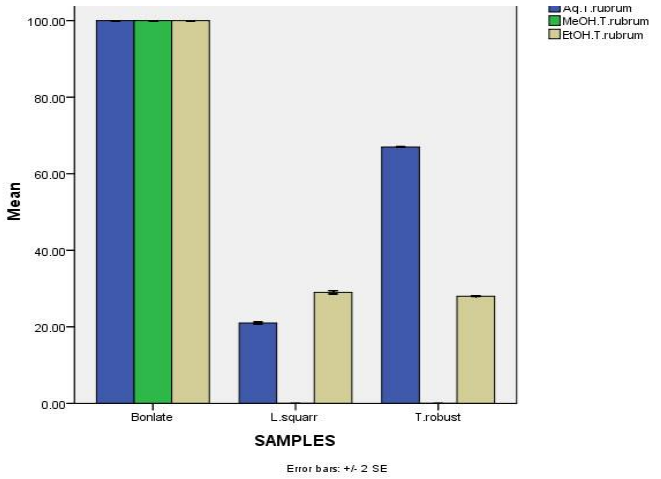

Figure 6 Antifungal activities of extracts (0.05 g/cm^3) with bonlate as positive control against *Trichoderma rubum* at 24 h incubation

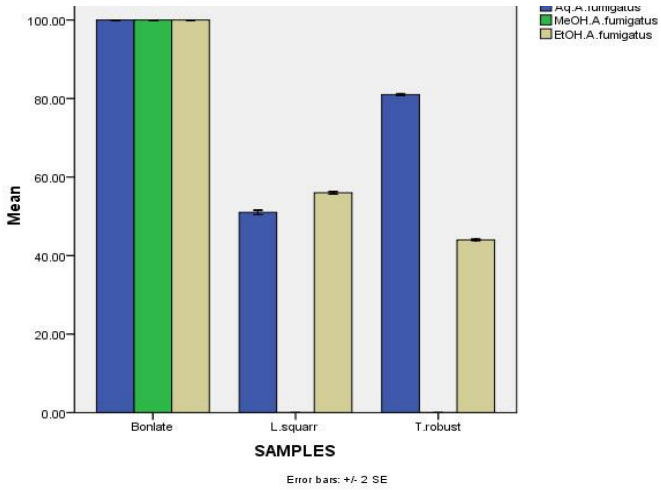

Figure 7 Antifungal activities of extracts (0.05 g/cm^3) with bonlate as positive control against *Aspetrillus fumigatus* at 72 h incubation

Figure 8 *Termitomyces robustus*

Figure 9 *Lentinus squarrosulus*

Table 1 Phytochemicals screening result

Phytochemicals	Mushrooms species	
	T.robustus	*L. squarrosulus*
Alkaloid	+	-
Saponin	+	+
Tannin	+	+
Flavonoid	+	+
Steroid	-	-
Terpenoid	+	+
Anthraquinone	-	-
Phlobatannin	-	-

\- = Absent
\+ = present

Table 2 Chemical compositions of the mushrooms

Compositions	Mushroom species	
	T. robustus	*L. squarrosulus*
Moisture	11.03±0.22b	7.22±0.07a
Crude protein	42.77±0.57b	31.24±0.02a
Ash	10.45±0.43b	7.07±0.04a
Crude fibre	4.07±0.18a	9.48±0.04b
Crude fat	6.76±0.22b	3.71±0.16a
Carbohydrate	24.90±0.11a	41.27±0.19b
Calorific Value (kcal)	331.55±3.41a	342.35±3.09a
Total dietary fibre	10.21±0.00a	11.68±0.00b
Sodium (mg/100g)	270.00 ± 0.08b	200.00 ± 0.03a
Potassium (mg/100g)	1460.00 ± 0.11b	800.00 ± 0.33a
Calcium (mg/100g)	60.00 ± 0.01b	40.00 ± 0.03a
Magnesium (mg/100g)	106.00 ± 0.05b	98.00 ± 0.04a
Zinc (mg/100g)	81.00 ± 0.03b	19.00 ± 0.02a
Copper (mg/100g)	0.90 ± 0.02b	0.20 ± 0.02a
Iron (mg/100g)	2.70 ± 0.01b	0.40 ± 0.01a
Phosphorus(mg/100g)	30.80 ± 0.01b	22.50 ± 0.02a
Manganese (mg/100g)	ND	ND
Lead (mg/100g)	ND	ND
Mercury (mg/100g)	ND	ND
Vitamin A(β-Carotene Equivalent, µg/g)	13.33 ± 0.02b	12.80 ± 0.01a
Thiamine (µg/g)	324.23 ± 0.02a	328.42 ± 0.00b
Ascorbic acid (µg/g)	14.22 ± 0.01b	11.61 ± 0.00a
Tocopherol (µg/g)	383.12 ± 0.02a	1191.39 ± 0.01b
Tannin (mg/g)	7.40 ± 0.14b	3.25 ± 0.80a
Oxalate (mg/g)	1.71 ± 0.07b	1.53 ± 0.00ab
Phytate (mg/g)	1.94 ± 0.05b	1.48 ± 0.06a

Values represent means of triplicate readings ± S.D. Values with the same superscript along the row are not significantly different ($p \geq 0.05$).

Table 3 Amino acid composition

Amino acids	Concentrations in the Mushroom species (g/100g)		
	T.robustus	*L.squarrosulus*	**RP %*
Histidine	2.33	1.96	1.9
Isoleucine	3.77	2.85	2.8
Leucine	9.07	16.99	6.6
Lysine	5.02	2.67	5.8
Methionine	1.28	0.73	
Phenylalanine	4.39	3.37	6.3a
Threonine	3.48	2.53	3.4
Valine	3.91	3.01	3.5
Arginine	5.01	3.62	
Aspartic acid	8.62	7.17	
Serine	2.39	2.03	
Glutamic acid	10.98	10.00	
Proline	2.75	2.14	
Glycine	4.33	3.00	
Alanine	3.33	3.23	
Cysteine	0.99	0.60	2.5b
Tryosine	3.02	2.06	

a = Phenylalanin with Tyrosine, b = Cystein with Methionine, **RP %*= Requirement Pattern in % protein (FAO/WHO 1991)

Table 4 Phenolic compounds in the mushrooms

Phenolic compounds	Concentrations in the Mushroom species (mg/g)	
	T.robustus	*L.squarrosulus*
Gallic acid	47.91±0.02b	7.93±0.02a
Chlorogenic acid	18.47±0.01a	20.61±0.03b
Caffeic acid	41.80±0.05a	41.09±0.02a
Ellagic acid	20.52±0.02a	38.67±0.02b
Catechin	3.64±0.01a	6.58±0.01b
Epicatechin	7.28±0.01a	8.23±0.01b
Rutin	36.94±0.01b	15.34±0.01a
Isoquercitrin	13.85±0.02a	30.71±0.02b
Quercitrin	40.96±0.02b	29.55±0.03a
Quercetin	26.11±0.01a	38.96±0.01b
Kaempferol	7.83±0.03b	0±0.00a

Values represent means of triplicate readings ± S.D. Values with the same superscript along the row are not significantly different ($p \geq 0.05$).

CONCLUSION

The nutritional quality of the mushrooms in terms of protein, vitamins and mineral elements revealed their potentials as nutritious foods. The reported health benefits of *Termitomyces robustus* and *Lentinus squarrosulus* could be related to the presence of their natural phytochemicals like phenolic acids, flavonoids, amino acids and vitamins which are known to possess antioxidant properties. They could be developed as functional foods for the prevention of degenerative diseases. Increase consumption of these mushrooms would seem to be of great health benefit. Large scale cultivation should therefore be encouraged not only for the nutraceutical potentials but food security as well as economic sustainability. Research into favourable conditions to domesticate the above mentioned mushrooms and the numerous wild species as well as government policies to promote production and trade are essential in order to maximise the poential of mushrooms as valuable natural resources for food, medicine and biochemicals for industrial purpose.

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NUTRITIONAL PROFILES OF PROCESSED *Spondias mombin* FOLIAGE AND PHYSIOLOGICAL RESPONSE OF RUMEN MICROORGANISMS TO THE EXTRACTS

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ABSTRACT

Spondias mombin foliage was processed into fresh (as control), air-dried and sun-dried samples and were analyzed for proximate, fibre, minerals, vitamins and antinutrients. Also, Identified rumen microorganisms were exposed to non-reconstituted ethanol extract of the foliage. The results showed that crude protein, crude fibre and gross energy were greatly improved by drying from 4.9% to 15.1%, 2.1% to 18.4% and 0.9 kcal/g to 2.9kcal/g in that order. Fibre constituents improved from 11.5% to 67.9%, 7.2% to 53.6% and 2.9% to 9.3% for neutral detergent fibre, acid detergent fibre and acid detergent lignin. Similarly, all the mineral components were improved from 0.083% to 0.21%, 0.193% to 0.533% and 0.073% to 0.23% for Calcium, Phosphorus and Sodium respectively by drying. Drying decreased the Ascorbic acid, Riboflavin and Niacin contents from 27.8mg/100g to 9.1mg/100g, 0.083mg/100g to 0.033mg/100g and 0.323mg/100g to 0.143mg/100g in that arrangement. Above all, it was observed that drying tremendously improved the nutritional value of *S. mombin* foliage by reducing tannin from 2.2% to 1.64%, oxalic acid (2.1% to 1.38%), phytic acid (1.15 to 0.45%), saponin (1.18% to 0.72%) and trypsin inhibitor from 39.74% to 16.57%. However, drying did not influence the toxic potential of the foliage as indicated by susceptibility of all the rumen microorganisms except the mould species. The nutritional quality potentials observed in the present study suggested that *S. mombin* foliage may be efficiently utilized by ruminants for optimal performance.

Keywords: Feed processing, novel feed resource, nutritional quality, rumen microorganisms susceptibility, ruminant nutrition

INTRODUCTION

Spondias mombin commonly called yellow mombin belongs to the *Anacardiaceae* family. It is well distributed across Mexico, South America and widely cultivated in Paleotropics and native range in West Indies. It is a multipurpose shrub of enormous economic values as food (i.e. the fruits), fence (i.e. boundary demarcation), environmental ornament and antitumour agent (**Idu *et al*., 2002; Wiersema and Leon, 1999**). Yet, there is insufficient reports on its possible utilization as fodder in ruminant nutrition particularly in Nigeria, where it grows everywhere in the rainforest belt. Although it is a deciduous shrub, it has a very high aftermath capability indicating high biomass fodder availability at all times. Since grasses and agricultural waste have been speculated to be inadequate for optimum ruminant productivity, shrubs and trees with potential feeding values should be evaluated for diet supplementation, especially during off season when grasses and agro byproducts may not be readily available.

Several researches have shown that feed resource evaluation is an important tool that provides useful information necessary for diet formulation in order to optimize performance. In series of experimental studies, it was established that rumen microorganisms are responsible for feedstuff degradability, digestibility and utilization by the host ruminant. But the earliest research frontiers however dwelled so much on feedstuff quality testing using chemical constituent technique (**Midkiff, 1984**), gas production technique (**Menke *et al*., 1979**) and cell-free fungal cellulose technique (**De-Boever *et al*., 1986**). Also, nylon bags technique and feed resource digestion technique with unidentified/mixed rumen microorganisms were used to determine the nutritional quality of feedstuff meant for ruminants (**Mehrez and Orskov, 1977; Tilley and Terry, 1963**). Unfortunately, some of these techniques were somewhat not adopted in animal agriculture probably due to some forms of complexities. Above all, feed resource quality evaluation using a mixed culture of rumen microorganisms may not have actually indicated which rumen microorganism was actively involved in the gas production or the fermentation processes (**Makkar, 2004; Sullivan and Martin, 1999**). Consequently *in vitro* exposure of pure culture rumen microorganisms to feedstuff sample could be a novel and better technique of feed quality evaluation. Hence, the present study examined the nutritional profile and susceptibility of rumen microorganisms to ethanol extract of *S. mombin* foliage.

MATERIALS AND METHODS

Samples preparation

Fresh foliage of *S. mombin* was collected from the University of Ibadan Campus and authenticated at the Herbarium, Department of Botany and Microbiology at the same University. Two hundred and fifty grams (250g) of the foliage without leaf stalk was weighed and processed as fresh, air-dried and sun-dried samples. The fresh sample (which served as the control) was crushed in a mortar (Pyrex®) and stored in the freezer. The sample for air-drying (i.e. indoor drying) was spread on the bench in a well-ventilated laboratory at the Institute of Agricultural Research and Training, Ibadan, at a mean room temperature of 28.5°C for 48hrs. Sample for sun-drying (i.e. outdoor drying) was spread on a special drying platform at a mean temperature of 33.2°C for 2 days between 08.00 and 16:00hr GMT each day. The air-dried and sun-dried samples were crispy dried yet retained the green colour. Thereafter, they were ground using hammer mill (Arthur Thomas Co. USA) to a mesh size of 2mm and stored in a cool dry shelf in the laboratory. In a cold extraction, 1g of each sample was thoroughly mixed with 40ml of ethanol (80%). The mixture was left overnight and was filtered using Whitman No. 1 filter paper®. Although concentration of the bioactive ingredients of the filtrate was not determined, it was heated for 2mins to get rid of the ethanol while the pure extract which was not also quantified was stored in McCartney bottles kept in the refrigerator.

Nutrients and antinutrients determination

While the fresh *Spondias mombin* foliage samples were analyzed as wet basis (control treatment), the dried samples were analyzed on dry matter basis for proximate compositions according to AOAC (2000). The fibre constituents were determined following the description of van Soest *et al.* (1991). The mineral and vitamin contents were estimated adopting the methods of **Wiseman and Cole**

(1990) while the gross energy was measured with an adiabatic bomb calorimeter (IKA C7000, Staufen - Germany) standardised with benzoic acid as prescribed by **Witt (1987)**.

Haemagglutinin was estimated as described by **Liener (1955)**, mimosine (**Megarrity, 1978**), cyanide (**Bradbury, 1999**), trypsin inhibitor (**Smith *et al*., 1980**), Oxalic acid (**Bateman and Beer, 1965**) while the alkaloid content was evaluated according to **Henry (1973)**. The phytic acid was determined as demonstrated by **Vaintraub and Lapteva (1988)** and the total saponin and tannin contents were analyzed using the methods of **Makkar *et al*. (1993)**.

Isolation and identification of rumen microorganisms

Samples of rumen liquor were collected in sterile bottles from cattle and goats just slaughtered at Bodija abattoir, Ibadan. Meanwhile, what the animals were fed prior to liquor collection was not taken into cognizance. The samples were pooled and 1g was thoroughly mixed with 9ml of distilled water in a serial dilution procedure given by Black (1986). In an aseptical condition, 1ml of the serial dilution was mixed with 5ml of sterile Eosin Methylene Blue – EMB (idg Lab M^{TM}, UK) and Plate Count Agar – PCA (idg Lab M^{TM}, UK) for rumen bacteria isolation. The mixture was incubated using Temperaturregler (GmbH, Germany) at 37ºC for 48hrs under anaerobic condition recommended by **Levett (1990)**. Subculture of the growth observed was done by flaming and streaking followed by incubation at 37ºC overnight to obtain pure isolates.

Using flamed inoculating wire loop, samples were collected from the pure isolates in an Oxygen free atmosphere and further subcultured in a liquid substrate "Peptone Water Broth" (idg Lab M^{TM}, UK) and stored in an incubator at 35ºC. Similarly, 1ml of the serial dilution was mixed with 5ml of sterile Potato Dextrose Agar – PDA (DM215, Micro Master Laboratories®) and an antibiotic (Vanclox, Evans®) was added to prevent bacterial growth and then incubated for 4 days at 34ºC according to **Kudo *et al.* (1990)**. The rumen fungal isolates were purified and transferred into liquid broth (Malt Extract: Difco Laboratories, USA) stored in an incubator at 35ºC. The rumen bacteria pure isolates were subjected to morphological and biochemical tests as reported by Yokoyama and Johnson (1993). Meanwhile, the procedure was modified to include antibiosis (i.e. Ionophore and Avopacin) for detailed characterization of the rumen microbial strains. Originally, Avopacin and Ionophore (monesin, lasalocid) were used to depress certain rumen microbial strains in order to improve feed utilization efficiency (**Aderinboye and Onwuka, 2010; Yokoyama and Johnson, 1993**). The rumen fungi isolates were identified according to **Kudo *et al.* (1990)**. The identification features were modified to include some structural characteristics like reproductive stage, sporangiophore, mycelium and rhizoid for proper characterization of the rumen fungal strains. Although concentration of the *S. mombin* foliage extract was not estimated, susceptibility of the rumen microorganisms was examined by subjecting each of the identified rumen bacteria and fungi to the *S. mombin* foliage non-reconstituted pure extract. Each of the 12-punched-well agars was filled with the non-reconstituted foliage extract, streaked with each of the identified rumen microorganisms at a time and incubated overnight in the case of rumen bacteria and 4days for rumen fungi (**Levett, 1990**).

Experimental design, data collection and analysis

Randomized Completely Block Design where the feedstuff processing technique was blocked was adopted and all the parameters were determined in triplicates. Data were collected on proximate compositions, fibre components, mineral and vitamin contents as well as antinutritional constituents of the fresh, air-dried and sun-dried *S. mombin* foliage. Also, information on the suspected rumen microorganisms following morphological examination and biochemical tests were recorded. The growth pattern around each of the wells was observed and recorded as minimum inhibition concentration after overnight and 4 days incubation periods for the identified rumen bacteria and fungi respectively **(Black, 1986)**. The sets of data obtained were subjected to analysis of variance procedure of **SAS (1999)** and the means were separated as given by Duncan's multiple range test of the same software package.

RESULTS AND DISCUSSION

Nutritional compositions

The nutrient compositions of processed *S. mombin* foliage are given in Table 1. All the parameters measured were significantly improved ($P<0.05$) by air-drying and sun-drying compared to the control treatment. Meanwhile the sun-dried values were superior in all the cases except in gross energy and cellulose, where there were no statistical differences ($P<0.05$) in the air-dried and sun-dried values. The crude protein value ranged from 4.9% in control to 10.3% (air-dried) and 15.1% in sundried. Ether extract, crude fibre and ash values were 1.3%, 2.1% and 2.8% in control compared to 13.3%, 18.4% and 13.8% in sun-dried. The energy value varied from 2.5kcal/g in air-dried to 2.9kcal/g in sundried compared to as low as 0.9kcal/g in control. The neutral detergent fibre, acid detergent fibre and acid detergent lignin values which were as high as 67.9%, 53.6% and 9.3% respectively in sun-dried, was slightly followed by air-dried (43.8%, 21.9% and 6.8%) with the least values (11.5%, 7.2% and 2.9%) in control.

The observed nutritional values contradicted the report of Ikhimioya and Oriakhi (2004) where fresh *S. mombin* leaves had 10.06% crude protein and dry leaves 6.41%. Although the crude protein contents (4.9 to 15.1%) observed in the present study were less than 18.83% reported by Mecha and Adegbola (2006), they were within the reported values 3.6 – 22.5% **(ESGPIP, 2008)**, 5.08 – 10.06% (Ikhimioya and Oriakhi, 2004) and recommended range value of 1.61 – 7.76g per kilogram metabolic weight ($W^{0.75}$) (NRC, 1981) for ruminant nutrition. The ether extract, crude fibre and ash contents observed were within 0.7 – 9.3%, 7.8 – 40.7% and 1.0 – 12.5% respectively recorded in some forages (**Mecha and Adegbola, 2006**). Meanwhile, the energy value was less than 0.7 – 6.88Mcal recommended by **NRC (1981)** for optimal ruminant productivity. However, all the values were in agreement with the nutritional requirements recommended by **Givens *et al*. (2000)**. The range values of neutral detergent fibre (11.5 – 67.9%), acid detergent fibre (7.2 – 53.6%) and cellulose (4.3 – 44.3%) were within 18.31 – 68.87% recorded in some other ruminant feed resources (**Idahor *et al*., 2012; Rosiji and Iposu, 2002**).

Also, drying was observed to significantly improved ($P<0.05$) all the minerals determined. Calcium, Iron, Magnesium, Potassium and Phosphorus values were highest (0.21%, 0.003%, 0.353%, 0.463% and 0.533%) in sun-dried followed by air-dried (0.17%, 0.002%, 0.193%, 0.343% and 0.153%) and control (0.083%, 0.001%, 0.093%, 0.153% and 0.193%). Furthermore, Sodium, Zinc, Copper and Manganese values were highest (0.233%, 0.0034%, 0.0009% and 0.004% respectively) in sun-dried sample and slightly followed by air-dried sample (0.153%, 0.0029%, 0.0005% and 0.003%) with the least (0.73%, 0.002%, 0.0002% and 0.001%) in fresh sample (control). The Magnesium, Phosphorus, Potassium and Sodium values were within the ranges 0.04 – 0.25%, 0.16 – 0.4%, 0.5 – 0.8% and 0.06 – 0.18% respectively recommended for ruminant animals **(NRC 1981)**.

However, the Calcium level was less than 0.21 – 0.58% recommended for optimal ruminant productivity (**Church, 1993; NRC, 1981**).

In all the vitamins evaluated, it was observed that dry processing significantly depressed ($P<0.05$) the values compared to the control. The Ascorbic acid, Riboflavin and Niacin values varied from 9.1 to 15.4mg/100g, 0.033 to 0.053mg/100g and 0.143 to 0.213mg/100g) in sun- dried and air-dried samples but were quite high (27.8mg/100g, 0.083mg/100g and 0.323mg/100g) in control. The levels of Riboflavin and Niacin observed were less than 4.5 – 32mg/100kg BW and 26 – 182mg/100kg BW respectively recommended for ruminant animals. This observation concurred with the earlier report by **Wiseman and Cole (1990)** that vitamins were insufficient in processed feed hence their inclusion in ruminant nutrition is essential. The observed depression in the vitamin values could be largely due to their nature as biologically active biochemical compounds that are generally sensitive to their physical and chemical environments. According to **Coelho, (1999)**, several vitamins contain unsaturated carbon atoms or have double bonds that make them highly susceptible to oxidation. Although, feed processing tend to improve the distribution and digestibility of nutrients, it could be harmful to heat labile nutrients such as vitamins that can easily oxidized **(Gadient, 1986; Schneider, 1986)**.

It was observed that all the fresh sample values were seemingly lower, suggesting feed drying (particularly in hay form) superiority over fresh form (fodder). The observed distinct disparities in all the values recorded in the nutritional parameters, could be largely due to the moisture contents and possibly due to denaturation of heat-labile nutrients thereby enhancing the concentration of others. While, the disparities with other reported values could be due to the agro climatic differences, stage of growth, plant species and the laboratory protocols adopted.

Table 1 Effect of processing on nutritional profiles of *S. mombin* foliage

Parameters (††)	Processing techniques			
	Fresh	Air-dried	Sun-dried	SEM
Proximate				
Organic matter (%)	97.3^{b}	91.0^{ab}	86.2^{a}	0.01
Crude protein (%)	4.9^{a}	10.3^{b}	15.1^{c}	0.01
Ether extract (%)	1.3^{a}	7.5^{b}	13.3^{c}	0.01
Crude fibre (%)	2.1^{a}	8.8^{b}	18.4^{c}	0.01
Ash (%)	2.8^{a}	9.1^{b}	13.8^{c}	0.01
Gross energy (kcal/g)	0.9^{a}	2.5^{b}	2.9^{b}	0.07
Fibre constituents (%)				
Neutral detergent fibre	11.5^{a}	43.8^{b}	67.9^{c}	0.01
Acid detergent fibre	7.2^{a}	21.9^{b}	53.6^{c}	0.01
Acid detergent lignin	2.9^{a}	6.8^{b}	9.3^{c}	0.01
Cellulose	4.3^{a}	15.1^{b}	44.3^{c}	0.02
Hemicellulose	4.3^{a}	22.0^{b}	14.3^{c}	0.02
Minerals (%)				
Calcium	0.083^{a}	0.17^{b}	0.21^{b}	0.01
Iron	0.001^{a}	0.002^{ab}	0.003^{b}	0.02
Magnesium	0.093^{a}	0.193^{b}	0.353^{c}	0.01
Potassium	0.153^{a}	0.343^{b}	0.463^{c}	0.01
Phosphorus	0.193^{a}	0.343^{b}	0.533^{c}	0.01
Sodium	0.073^{a}	0.153^{b}	0.233^{b}	0.01
Zinc	0.002^{a}	0.0029^{b}	0.0034^{b}	0.02
Copper	0.0002^{a}	0.0005^{b}	0.0009^{c}	0.02
Manganese	0.001^{a}	0.003^{b}	0.004^{b}	0.02
Vitamins (mg/100g)				
Ascorbic acid	27.8^{c}	15.4^{b}	9.1^{a}	0.01
Riboflavin	0.083^{c}	0.053^{b}	0.033^{a}	0.01
Niacin	0.323^{c}	0.213^{b}	0.143^{a}	0.01

^{a,b,c}: Means along the row with different superscripts differ significantly at P<0.05; ^{SEM}: Standard error of means; ††: All values were expressed on DM basis except fresh sample on wet basis.

Toxic factor components

The antinutrients determined in *S. mombin* foliage are shown in Table 2. It was discovered that drying drastically reduced (P<0.05) all the antinutrients concentrations. The determined phytic acid, oxalic acid, tannin and saponin levels that were as high as 1.15%, 2.1%, 2.21% and 1.18% respectively in the fresh sample, were reduced to as low as 0.45 – 0.54%, 1.38 – 1.53%, 1.64 – 2.04% and 0.72 – 0.93% by drying. Also, the alkaloid, cyanide, mimosine, haemaglutinin and trypsin inhibitor concentrations declined from 2.37 %, 84.74mg/kg, 3.92mg/100g, 9.66HIU/mg protein and 39.74% accordingly in the fresh sample to as low as 1.1 – 1.55%, 36.04 – 66.92 mg/kg,1.55 – 2.13mg/100g, 4.14 – 7.69HIU/mg protein and 16.57 – 21.32% after drying.

Table 2 Effect of processing on antinutrients components of *S. mombin* foliage

Antinutrients (††)	Processing techniques			
	Fresh	Air-dried	Sun-dried	SEM
Phytic acid (%)	1.15^{b}	0.45^{a}	0.54^{a}	0.01
Oxalic acid (%)	2.1^{b}	1.53^{a}	1.38^{a}	0.02
Tannin (%)	2.21	2.04	1.64	0.01
Saponin (%)	1.18	0.93	0.72	0.01
Alkaloid (%)	2.37^{b}	1.55^{a}	1.1^{a}	0.01
Cyanide (mg/kg)	84.74^{c}	66.92^{b}	36.04^{a}	0.01
Mimosine (mg/100g)	3.92^{b}	2.13^{a}	1.55^{a}	0.02
Haemaglutinin(HIU/mg protein)	9.66^{c}	7.69^{b}	4.14^{a}	0.02
Trypsin inhibitor (%)	39.74^{c}	21.32^{b}	16.57^{a}	0.02

^{a,b,c}: Means along the row with different superscripts differ significantly at P<0.05; ^{SEM}: Standard error of means. ††: All values were expressed on DM basis except fresh sample on wet basis.

The phytic acid and saponin concentrations were less than 3.1% and 5.0% respectively reported in *Moringa oleifera* leaves which Makkar and Becker (1996) described as innocuous. However, the tannin concentration was higher than 0.62% detected in Sesbania (**Reed 1986**) and 2.05% found in Gliricidia (**Ahn *et al*., 1989**) but was lower than 3.0 – 14.0% reported in Leucaena (**D'Mello and Fraser 1981**). Also, the mimosine and saponin concentrations were observed to be less than 12.0% and 11.0% respectively discovered in Leucaena (**Tangendjaja *et al*., 1990**). Since **Barry and McNabb (1999)** reported that tannin concentration greater than 4.0% could depress feed intake and there are several reports that ruminants can tolerate and utilize some levels of antinutrients (**Hoskin *et al*., 1997**), *S. mombin* foliage could be a suitable feed resource in ruminant production.

More significantly, the oxalic acid, trypsin inhibitor, alkaloid, haemaglutinin and cyanide values were less than the threshold levels reported in livestock (**Olomu, 2011**). The relatively safe levels estimated in all the antinutrients determined, could be probably due to the part collected and maturity of the foliage. The observed supremacy of feedstuff drying technique could explain the need for proper feed processing prior to utilization in ruminant nutrition.

Susceptibility of the identified rumen microorganisms

The morphological examinations and biochemical tests of the rumen microorganisms are shown in Tables 3 and 4. The identified rumen bacteria were *Bacteroides ruminicola, B. succinogenes, Butyrivibrio fibrisolvens* and *Lactobacillus ruminus*. Others were *Ruminococcus albus, R. flavefaciens, Selenomonas ruminantium and Streptococcus bovis*. The rumen fungi strains were *Neocallimastix frontalis, Orpinomyces joyonii, Saccharomyces cerevisiae, Mucor species, Caecomyces communis and Rhizopus species*. The kinds of rumen microorganisms that were recorded in the rumen liquor could be possibly due to the nature of feed the hosts (cattle and goats) were fed and probably due to their age.

Table 3 Morphological and biochemical tests of the rumen bacterial isolates

Isolate code No.	Morph. Exams		Biochemical tests								Suspected microorganism
	Gram stain		Proteolysis		Amylolysis				Antibiosis		
	Gr	Shape	Gel	Cas	L.mil	Star	Cellu	Sucr	Iono	Avop	
PCA C_1^a	-	Cocci	-	-	+	-	-	-	R	R	*Selenomonas ruminantium*
PCA C_1^b	+	Cocci	-	-	-	+	+	+	S	S	*Ruminococcus albus*
PCA C_4	-	Rods	+	+	+	+	-	-	S	S	*Butyrivibrio fibrisolvens*
PCA C_5	+	Cocci	-	-	-	-	+	-	S	S	*Ruminococcus flavefaciens*
PCA C_7	+	Cocci	-	-	+	-	-	-	R	R	*Selenomonas ruminantium*
PCA C_8	+	Cocci	-	-	+	-	-	-	R	R	*Selenomonas ruminantium*
PCA C_9	+	Cocci	-	-	+	-	-	-	R	R	*Selenomonas ruminantium*
PCA C_{10}^a	+	Cocci	-	-	-	-	+	-	S	S	*Ruminococcus flavefaciens*
PCA C_{10}^b	+	Cocci	-	-	-	+	+	+	S	S	*Ruminococcus albus*
PCA G_1	-	Rods	+	+	+	+	+	+	R	R	*Bacteriodes ruminicola*
PCA G_2	-	Rods	+	+	+	+	+	+	R	R	*Bacteriodes ruminicola*
PCA G_3	+	Rods	+	+	+	+	-	-	S	S	*Butyrivibrio fibrisolvens*
PCA G_4	+	Rods	+	+	+	+	-	-	S	S	*Butyrivibrio fibrisolvens*
PCA G_6	-	Rods	-	-	-	+	+	+	R	S	*Bacteroides succinogenes*
PCA G_8	-	Rods	+	+	+	+	+	+	R	R	*Bacteroides ruminicola*
PCA G_9	-	Rods	+	+	+	+	+	+	R	R	*Bacteroides rumincola*
EMB C_1	-	Rods	+	+	+	+	+	+	R	R	*Bacteroides rumincola*
EMB C_2^a	-	Rods	+	+	+	+	+	+	R	R	*Bacteroides rumincola*
EMB C_2^b	+	Cocci	+	+	+	+	+	+	S	R	*Streptococcus bovis*
EMB C_6	+	Cocci	-	-	-	-	+	-	S	S	*Ruminococcus flavefaciens*
EMB C_{10}	-	Rods	-	-	-	+	+	+	R	S	*Bacteroides succinogenes*
EMB G_1	+	Cocci	-	-	-	+	+	+	S	S	*Ruminococcus albus*
EMB G_2^a	+	Rods	+	+	+	+	+	+	S	S	*Lactobacillus ruminus*
EMB G_2^b	-	Rods	-	-	-	+	+	+	R	S	*Bacteroides succinogenes*
EMB G_4^a	+	Cocci	-	-	-	+	+	+	S	S	*Ruminococcus albus*
EMB G_4^b	+	Rods	+	+	+	+	+	+	S	S	*Lactobacillus ruminus*
EMB G_7	-	Rods	-	-	-	+	+	+	R	S	*Bacteroides succinogenes*

EMB G_8	-	Rods	-	-	-	+	+	+	R	S	*Bacteroides succinogenes*
EMB G_9	+	Cocci	+	+	+	+	+	+	S	R	*Streptococcus bovis*
EMB G_{10}	+	Cocci	-	-	-	-	+	-	S	S	*Ruminococcus flavefaciens*

Morph. Exams = Morphological Examinations; EMB = Eosin methylene blue; PCA = Plate count agar; $C_1 – C_{10}$ = Isolates from cattle rumen liquor; $G_1 – G_{10}$ = Isolates from goats rumen liquor; Gel = Gelatin; Cas = Casein; L.mil = Litmus milk; Star = Starch; Cellu = Cellubiose; Sucr = Sucrose; Iono = Ionophore; Avop = Avopacin; Gr = Gram stain response; + = Positive response; - = Negative response; R = Resistant; S = Susceptible.

Table 4 Morphological and biochemical test of the rumen fungal isolates

Isolate code No.	Morphological examination						Biochemical Test						Identification
	Structure						Starch fermentation						Suspected microorganism
	Col	Cell	Myc	Rhi	Rep	Spo	Pec	Malt	Glu	Gal	Lac	Hcell	
PDA C_1^a	ND	Poly	ND	ND	ND	ND	+	+	+	-	+	+	*Orpinomyces joyonii*
PDA C_1^b	ND	Poly	ND	ND	ND	ND	+	+	+	-	+	+	*O. joyonii*
PDA C_3	ND	Mono	ND	ND	ND	ND	-	+	+	-	+	+	*Neocallimastix frontalis*
PDA C_4	ND	Mono	ND	ND	ND	ND	-	-	+	-	-	-	*Caecomyces communis*
PDA C_5^a	ND	ND	ND	ND	Bud	ND	+	+	+	+	+	+	*Saccharomyces cerevisiae*
PDA C_5^b	ND	ND	ND	ND	Bud	ND	+	+	+	+	+	+	*S. cerevisiae*
PDA C_6	ND	Mono	ND	ND	ND	ND	-	+	+	-	+	+	*N. frontalis*
PDA C_7^a	ND	Mono	ND	ND	ND	ND	-	+	+	-	+	+	*N. frontalis*
PDA C_7^b	White/brown/yellow	CAM	Asep	Abs	Hyp	Bran	ND	ND	ND	ND	ND	ND	*Mucor spp*
PDA C_7^c	White/brown/yellow	CAM	Asep	Abs	Hyp	Bran	ND	ND	ND	ND	ND	ND	*Mucor spp*
PDA C_7^d	White/gray	DCAM	Asp	Pre	Hyp	Unbra	ND	ND	ND	ND	ND	ND	*Rhizopus spp*
PDA C_8	ND	Poly	ND	ND	ND	ND	+	+	+	-	+	+	*O. joyonii*
PDA C_9	ND	Mono	ND	ND	ND	ND	-	+	+	-	+	+	*N. frontalis*
PDA C_{10}	ND	Poly	ND	ND	ND	ND	+	+	+	-	+	+	*O. joyonii*
PDA G_1	ND	Mono	ND	ND	ND	ND	-	-	+	-	-	-	*C. communis*
PDA G_2	ND	Mono	ND	ND	ND	ND	-	+	+	-	+	+	*N. frontalis*
PDA G_3	ND	Mono	ND	ND	ND	ND	-	-	+	-	-	-	*C. communis*
PDA C_4	ND	Mono	ND	ND	ND	ND	-	+	+	-	+	+	*N. frontalis*
PDA C_6^a	ND	Poly	ND	ND	ND	ND	+	+	+	-	+	+	*O. joyonii*
PDA C_6^b	White/brown/yellow	CAM	Asep	Abs	Hyp	Bran	ND	ND	ND	ND	ND	ND	*Mucor spp*
PDA C_7^a	ND	Poly	ND	ND	ND	ND	+	+	+	-	+	+	*O. joyonii*
PDA C_7^b	White/brown /yellow	CAM	Asep	Abs	Hyp	Bran	ND	ND	ND	ND	ND	ND	*Mucor spp*
PDA G_7^c	White/gray	DCAM	Asep	Pre	Hyp	Unbra	ND	ND	ND	ND	ND	ND	*Rhizopus spp*
PDA G_8	ND	ND	ND	ND	Bud	ND	+	+	+	+	+	+	*S. cerevisiae*
PDA G_9	ND	ND	ND	ND	Bud	ND	+	+	+	+	+	+	*S. cerevisiae*
PDA G_{10}^a	ND	Mono	ND	ND	ND	ND	-	+	+	-	+	+	*N. frontalis*
PDA G_{10}^b	White/gray	DCAM	Asep	Pre	Hyp	Unbra	ND	ND	ND	ND	ND	ND	*Rhizopus spp*

ND = Not determined; Col = Colour; Myc = Mycelium; Rhi = Rhizoid; $C_1 – C_{10}$ = Isolates from cattle rumen liquor; - = Negative response; $G_1 – G_{10}$ = Isolates from goats rumen liquor; + = Positive response; Rep = Reproductive stage; PDA = Potato Dextrose Agar; Spo = Sporangiophore; Pec = Pectin; Malt = Maltose; Glu = Glucose; Gal = Galactose; Lac = Lactose; Hcell = Hemicellulose; Poly = Polycentric; Mono = Monocentric; Hyp = Hyphae; Abs = Absent; Pre = Present; Asp = Aseptate; Unbra = Unbranched; Bran = Branched; CAM = Cottony aerial mycelium; DCAM = Dense cottony aerial mycelium.

The minimum inhibition concentration of *S. mombin* foliage extract on the rumen microorganisms is expressed in Table 5. All the treatments were observed to absolutely inhibited (2mm MIC) *Butyrivibrio fibrisolvens, Ruminococcus flavefaciens, Bacteroides ruminicola, Streptococcus bovis* and *Lactobacillus ruminus* and mildly inhibited (1.60 – 1.62mm MIC) *Bacteroides succinogenes*. Air-dried and sun-dried samples were also observed to absolutely inhibited (2mm MIC) *Selenomonas ruminantium* and mildly inhibited (1.51 – 1.52mm MIC) *Ruminococcus albus*. Similarly, sun-dried and fresh samples absolutely inhibited (2mm MIC) *Caecomyces communis, Saccharomyces cerevisiae* and *Neocallimastix frontalis* but *Orpinomyces joyonii* was mildly inhibited (0.31 – 1.31mm MIC). On the other hand, it was discovered that *Mucor* and *Rhizopus species* were absolutely insusceptible (0.0mm MIC) to all the treatments.

It was shown that all the rumen microorganisms were susceptible to the processed *S. mombin* foliage pure extract except, *Mucor* and *Rhizopus species* that were absolutely not inhibited, indicating that dry processing technique may not improve rumen microbial degradation and subsequent nutrient utilization of feedstuff resource by the host as reported by some scientists **(Olomu, 2011; Hoskin *et al*., 1997; Makkar and Becker (1996**). The observed toxic potential of *S. Mombin* foliage to rumen microorganisms, agreed with the reports in extracts of *Vernonia amygdalina, Chamaecytisus palmensis, Sesbania sesban, Acacia angustissima* and *Leucaena leucocephala* that affected the growth of pure culture of *Cellulolytic bacteria* (**Osuji *et al*., 1995**). However, it contradicted the report of **Makkar and Becker (1996)** in *Moringa oleifera* leave extract where uninhibited microbial growth was recorded.

The cause of high degree susceptibility observed in the present study is somewhat not clear because the antinutrients concentrations in browse plants reported by several scientists to be a possible cause of rumen microbial toxicity vis-à-vis host ruminant were tremendously reduced by the feedstuff processing techniques evaluated in the present study. Meanwhile, it could be due to the growth media used, number of punched-well, foliage extract concentration and probably due to the medium of extraction. The absolute insusceptibility of the mould species could simply be due to their characteristics mode of growth.

Table 5 Physiological response of rumen microorganisms to ethanolic extract of *S. mombin* foliage

Rumen microorganisms	Minimum inhibition concentration (mm)			
	Fresh	Air-dried	Sun-dried	SEM
Bacteria				
S. ruminantium	1.04^a	2.00^b	2.00^b	0.35
R. albus	2.00^b	1.51^a	1.51^a	0.16
B. fibrisolvens	2.00	2.00	2.00	0.003
R. flavefaciens	2.00	2.00	2.00	0.003
B. ruminicola	2.00	2.00	2.00	0.003
B. succinogenes	1.61	1.62	1.60	0.006
S. bovis	2.00	2.00	2.00	0.003
L. ruminus	2.00	2.00	2.00	0.003
Fungi				
O. joyonii	1.31^b	0.31^a	1.03^b	0.29
N. frontalis	2.00^b	1.51^a	1.52^a	0.16
C. communis	1.31^a	1.30^a	2.00^b	0.24
S. cerevisiae	1.51^b	1.01^a	2.00^c	0.29
Mucor species	0.0	0.0	0.0	0.03
Rhizopus species	0.0	0.0	0.0	0.003

^{a,b,c}: Means along the row with different superscripts differ significantly at $P<0.05$; ^{SEM}: Standard error of means.

The approach used in this study where pure culture rumen microorganisms were exposed to feedstuff sample *in vitro* could be a novel and better technique of feed quality evaluation. Besides, it may be more economical compared to the digestion technique, nylon bags technique, gas production technique and cell-free fungal cellulose technique described by other scientists. This is because the outcome from the mixed rumen microbial culture as adopted in other techniques does not actually reflect which microorganism is actively involved in the fermentation and degradation processes in the feedstuffs as shown in **Sullivan and Martin (1999)**. More so, the experimental outcome takes lesser time, cannulation is not required and above all, many feed resources can be assessed at the same time. Although *in vitro* results may not conform to *in vivo* trial, the present study is targeted at encapsulating myriads of known rumen

microorganisms that would be made available in commercial quantity for feed resource quality rapid test anywhere in the world.

CONCLUSIONS

The results revealed that drying technique significantly improved the proximate, fibre and mineral compositions of *S. mombin* foliage. In contrast, all the vitamins determined were considerably reduced by drying. Similarly, the concentrations of all the antinutrients were significantly lowered by drying. As a result, drying of feed resources may be necessary prior to utilization in ruminant feeding. On the other hand, all the rumen microorganisms exposed to the *S. mombin* foliage extract were susceptible except the mould species. This apparently indicated that drying may not be proficient in reducing the toxic potential of *S. mombin* foliage. However, the observed nutritional quality potentials suggested that *S. mombin* foliage may be efficiently utilized by ruminants for optimal performance. Although, it has been established that rumen fermentation is the net result of different microorganisms interactions in the rumen ecosystem, the present findings may not conform to *in vivo* feedstuff trial. Thus, ruminant feeding trial to elucidate *S. mombin* foliage suitability for prompt adoption and utilization or otherwise is required.

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EVALUATION OF ANTIOXIDANT ACTIVITY, TOXICITY AND ANTIBACTERIAL POTENTIAL OF EXTRACTS OF *SENSEVIERIA AETHIOPICA* (THUNB) AGAINST BACTERIA ASSOCIATED WITH OTITIS

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ABSTRACT

Otitis is highly prevalent infection caused mainly by bacteria and frequently prone to secondary infections. It may lead to other permanent dysfunctions in children and multidrug-resistance has been reported among implicated bacterial pathogens. The aim of this study is to evaluate the phytochemicals, antioxidants and antibacterial potential of acetone and methanolic extracts of *Sansevieria aethiopica* (Thunb.) leaf against bacterial pathogens responsible for otitis. The phytochemical analyses of the extracts were determined using standard assay methods and the antioxidant activity was assessed using 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic-acid (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), hydrogen peroxide (H_2O_2) and ferric reducing power. Brine shrimp lethality test was used to determine the cytotoxicity effect of the extract while macrobroth dilution method was used for the determination of minimal inhibitory concentrations (MICs) of the extracts. The phenolic contents of the extracts were 57.13 and 19.06 mg tannic acid/g in acetone and methanolic extracts respectively. Flavonols and proathocyanidin recorded the least values in methanolic and acetone extracts respectively. The extracts have good antioxidant properties although lower than the standard chemicals used as controls. The extracts expressed antibacterial effects on both Gram negative and Gram positive bacteria however, their activity was more pronounced on Gram negative organisms. Although, slightly toxic, the extracts have both bacteriostatic and bactericidal effects on the selected bacteria associated with otitis, especially Gram negative.

Keywords: Otitis, *Sensevieria aethiopica*, antioxidant, phytochemicals, antibacterial

INTRODUCTION

Otitis (ear infection) is a spectrum of infections of the ear usually associated with microbial infections of macerated skin and subcutaneous cellular tissue (**Aneja *et al.*, 2010**). It is more common in children than adults, approximately 75 % of children experience at least three or more ear infections during the first three years of life (**Gates, 1996**). Acute otitis is one of the most common childhood infections and is the leading indication for prescription of antimicrobials in children and it is highly prevalent worldwide (**Ifante and Fernandez, 1993**). Over 50 % of the cases of otitis are caused by bacteria and it has been reported to be the most common infection in young children (**Gunnsteinn, 2004**), with the average toddlers having two to three episodes a year (**Richard and Roberts, 1996**) and with mortality rate of more than 50,000 children (under 5 years) per year (**Rovers *et al.*, 2006a**). Children below the age of seven are much more susceptible to otitis media (**Weiner and Collison, 2003**).
Ear infection is mainly caused by bacteria and fungal pathogens (**Roland and Stroman, 2002**). Antimicrobial resistance in these pathogens has become widespread and highly prevalent globally (**Doern *et al.*, 2001**). This has led to treatment failures which have become more frequent (**Kristinsson *et al.*, 2005**). Otitis is frequently associated with secondary bacterial and/or fungal infections (**Hungria, 1991**). The clinical manifestations of otitis include pain, pruritus and erythema also as the disease progresses, edema, otorrhea and conductive hearing loss may also develop (**Damoiseaux, 2005**). In childhood it leads to significant hearing loss in pediatric patients, resulting in developmental problems in speech, language, and the acquisition of social skills (**Bluestone and Klein, 1988; Topcuoglu *et al.*, 2012**). Acute cases of bacterial otitis does not only come with earache but also severe pain, fever (39 °C or more) and febrile seizures (**Damoiseaux *et al.*, 2000; Damoiseaux, 2005; Rovers *et al.*, 2006b**).
Indiscriminate use of commercial antimicrobial drugs has led to the development of multiple drug resistant strains of bacteria and fungi. This on the other hand informs the search for new antimicrobials from natural sources (**Fagbohun *et al.*, 2010**). The use of medicinal plants, especially in public health care programs in developing countries is gaining recognized globally. *Sansevieria* is a genus of xerophytic perennial herbs that occur mostly in dry tropical and subtropical habitats. About 70 species are known with a distribution range from Africa, Asia to Burma and the islands of the Indian Ocean (**Purseglove, 1972; Alfani *et al.*, 1989**).
Sansevieria aethiopica (Thunb.) is a species of the genus *Sansevieria* in the family *Asparagaceae*. It grows as a perennial, stemless, succulent plant with about 1 centimeter thick rhizomes . The 13-30 and succulent rosettes arranged closely together standing leaves are spread out in ascending order. The concave upper surface and the base sometimes narrowed easily (**Mansfeld, 2012**). *S. aethiopica* is commonly found in Kenya and southern Africa. In South Africa it is found in the provinces of Northern Cape, North West , Eastern Cape and Gauteng spread to dry, open places or in the bush on permeable soils (**Hutchings *et al.*, 1996; Newton, 2001)** where it is used for the treatment of ear infections, dental caries and ulcers (**Hutchings *et al.*, 1996**).
The aim of this study is to evaluate the antioxidant activity, toxicity and antibacterial potential of acetone and methanolic leaf extracts of *S. aethiopica* against bacterial pathogens responsible for otitis.

MATERIALS AND METHODS

Plant material

Fresh leaves of *S. aethiopica* were collected in February, 2012, at the Alice Township in Nkokobe Municipality, Eastern Cape Province (32°58'58"S, 26°54'28"E and Altitude 589 m). The plant was identified in the Department of Botany, University of Fort Hare, Alice, Eastern Cape, South Africa. A voucher specimen (DavMed, 2012/2) was prepared and deposited in the Giffen Herbarium of the University. Plant sample was dried in the oven at the temperature (40°C).
The dried plant sample was pulverized and 40 g was separately extracted in acetone and methanol for 48 h on an orbital shaker (Stuart Scientific Orbital Shaker, Greater Manchester UK). The extracts were filtered through Whatman No. 1 filter paper. The extracts were evaporated to dryness under reduced

pressure at 40°C using a rotary evaporator (Laborota 4000-efficient, Heldolph, Germany). The extract of the plant was kept in the refrigerator until used.

Phytochemical screening of the plant

Determination of total phenolics content

The total phenolics content of the extract were determined by Folin-Ciocalteu method described by **Wolfe *et al.* (2003)** with little modification. To 5.0 ml of plant extract with concentration of 0.1 mg/mL, 5.0 mL of 10% Folin-Ciocalteu reagent and 4.0 mL of sodium carbonate (75% w/v) was added. The mixture was vortexed for 15 s and incubated at 40 ^{o}C for 30 min for colour appearance. The absorbance was measured at 765 nm using spectrophotometer. Samples of the extract were evaluated at the final concentration of 0.1 mg/mL. The amount of total phenolic content was expressed as mg/g tannic acid equivalent using the expression obtained from the calibration curve: $Y = 0.1231x$, $R^2 = 0.9742$, where x is the absorbance and Y is the tannic acid equivalent in mg/g.

Determination of total flavonoids content

The total flavonoids were determined using the method of **Ordonez *et al.* (2006)**. A volume of 0.5 mL of 2% $AlCl_3$ ethanol solution was added to 0.5 mL of extract solution. The mixture was incubated for 1 h at room temperature for yellow color appearance; the absorbance was measured at 420 nm. Plant extracts were evaluated at a final concentration of 0.1 mg/mL. Total flavonoids content was calculated as quercetin equivalent (mg/g) using the equation obtained from the curve: $Y = 0.0263x$, $R^2 = 0.9701$, where x is the absorbance and Y is the quercetin equivalent.

Determination of total flavonols content

The total flavonols content were determined using the method of **Kumaran and Karunakaran (2007)**. Two milliliter (2.0 mL) of the sample was mixed with 2.0 mL of $AlCl_3$ prepared in ethanol and 3.0 mL of 50 g/L sodium acetate solution were added. The mixture was incubated at 20 °C for 2.5 h after which the absorption was read at 440 nm using spectrophotometer. Plant extracts were evaluated at a final concentration of 0.1 mg/mL. Total flavonoids contents were calculated as quercetin (mg/g) using the following equation based on the calibration curve $Y = 0.0263x$, $R^2 = 0.9824$, where x is the absorbance and Y is the quercetin equivalent.

Determination of proanthocyanidins content

The total proanthocyanidin were determined using the procedure reported by **Sun *et al.* (1998)**. A volume of 0.5 mL of 0.1 mg/mL of extract solution was mixed with 3.0 mL of 4% vanillin-methanol solution and 1.5 mL hydrochloric acid, the mixture was allowed to stand for 15 min at room temperature, the absorbance was measured at 500 nm. Total proanthocyanidin contents were expressed as catechin (mg/g) using the following equation of the curve: $Y= 0.5902x$, $R^2 = 0.9714$, where x is the absorbance and Y is the catechin equivalent.

Antioxidant screening of the extract

ABTS radical scavenging activity

The method of **Re *et al.* (1999)** was adopted for the determination of ABTS activity of the plant extract. The working solution was prepared by mixing two stock solutions of 7 mM ABTS and 2.4 mM potassium persulphate in equal amounts and allowed to react for 12 h at room temperature in the dark. The resulting solution was further diluted by mixing 1 ml of freshly prepared ABTS solution to obtain an absorbance of 0.706 ± 0.001 units at 734 nm after 7 min using spectrophotometer. The percentage inhibition of $ABTS^{-+}$ by the extract was calculated and compared with that of BHT and rutin using the following equation:

$ABTS^{-+}$ scavenging activity = $[(A_0 – A_1)/ (A_0)] \times 100$

Where; A_0 is the absorbance of $ABTS^{-+}$ + methanol; A_1 is the absorbance of $ABTS^{-+}$ + sample extract or standard.

Determination of diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

The method of **Liyana-Pathiranan and Shahidi (2005)** was adopted for the determination of scavenging activity of DPPH free radical in the solution of the extract. A solution of 0.135 mM DPPH in methanol was prepared and 1.0 ml of this solution was added into 1.0 ml of the extract prepared in methanol with concentrations ranging between 0.025 and 0.400 mg/ml and standard drugs BHT and Rutin. The reaction mixture was vortexed thoroughly and was left in the dark at room temperature for 30 min. The absorbance of the mixture was measured in the spectrophotometer at 517 nm. The ability of the plant extract to scavenge DPPH radical was calculated by the equation: DPPH radical scavenging activity = $[(A_0 – A_1)/(A_0)] \times 100$; where A_0 is the control and A_1 is the absorbance of the extract.

Determination of hydrogen peroxide inhibition activity

The H_2O_2 inhibition effect of the extract was determined by the method of **Gulcin (2006)**. A 1.0 ml of the sample was added to a 0.6 ml of 40 mM hydrogen peroxide solution prepared in phosphate buffer (pH 7.4). The absorbance of the hydrogen peroxide at 230 nm was determined after 10 min at room temperature against a blank solution containing phosphate buffer solution alone. BHT and ascorbic acid were used as positive controls. The percentage scavenging of hydrogen peroxide of the samples was calculated as follow

H_2O_2 inhibition capacity (%) = (1 - (H_2O_2 concentration of sample /H_2O_2 concentration of control)) × 100.

The ferrous reducing antioxidant power (FRAP) Assay

The FRAP assay was carried out according to **Benzie and Strain (1999)** with a slight modification. FRAP reagent solution consisted of 300 mmol/l acetate buffer (pH 3.6), 10 mmol/l of 2,4,4-Tri(2-pyridyl)-s-triazine (TPTZ) in 40 mmol/l hydrochloric acid, and 20 mmol/l iron (III)-chloride hexahydrate. The fresh working solution was prepared by mixing 25 ml acetate buffer, 2.5 ml TPTZ, and 2.5 ml $FeCl_3 \cdot 6H_2O$. The temperature of the solution was raised to 37 °C before using. Plant extracts (150 µL) were allowed to react with 2850 µL of the FRAP solution for 30 min in the dark condition. Readings of the colored product (ferrous tripyridyltriazine complex) were taken at 593 nm. The standard curve was linear between 200 and 1000 µM FeSO4. Results are expressed in µM Fe (II)/g dry mass and compared with that of BHT, ascorbic acid and catechin.

Brine shrimp lethality test

Toxicity was studied using the larvae of brine shrimp nauplii, *Artemia salina* L (**Meyer *et al.*, 1982**). Shrimp eggs were allowed to hatch and mature as nauplii in two days in a hatching tank filled with seawater. The free-swimming nauplii were attracted by a light to a compartment from which they could be collected for the assay proper. Vials containing 4 to 20 µg ml-1 samples were prepared by dissolving the extracts in distilled water and transferring the solution to each vial. The crude extract was initially dissolved in dimethyl sulfoxide (DMSO) to make the extracts hydrophilic. Then distilled water was added to get final concentrations. In the control tube the same volume of DMSO and sea water were taken. After 24 h of exposure the mortality was determined using this formula:

$$C_m = [(O_m - C_m)/(100 - C_m)] \times 100$$

Where C_m = Corrected mortality, O_m = Observed mortality, C_m = Control mortality.

The observed data was the subject to Probit analysis was calculated according to **Finney (1974)** to get the dose that will kill 50% of the brine shrimps (LD_{50}).

Antimicrobial properties of the extract

Source and standardization of test bacteria

Eight bacterial isolates were used in this study are *Pseudomonas aeruginosa* ATCC 19582, *Klebsiella pneumoniae* ATCC 10031, *Enterobacter cloaca* ATCC 13047, *Esherichia coli* ATCC 25922, *Bacillus cereus* ATCC 10702, *Staphylococcus aureus* ATCC 6538, *Staphylococcus aureus* OK1 and *Bacillus pumilus* ATCC 14884. The isolates were collected from the Department of Biochemistry and Microbiology, University of Fort Hare, Alice. Each of the bacteria was maintained on nutrient agar (Biolab No. 2, Wadeville, Gauteng, South Africa) plates. The grown cultures were used for preparation of bacterial suspensions in sterile distilled water with densities adjusted to 0.5 McFarland Standard. A 0.25ml of the standardized suspension was added to the 25ml Mueller Hinton Broth and used for the inoculation of the tubes.

Determination of minimum inhibitory concentration (MIC)

Macrobroth dilution method was used for the determination of minimal inhibitory concentration (MIC) of the extract as described by **CLSI (2012)**. Nutrient broth medium was used to prepare different concentrations ranging from 0.0977 to 25 mg/ml by serial dilutions. Each prepared concentration in tubes was inoculated with 100 µl of each of the standardized culture of the test bacteria. Tube containing nutrient agar without extract was used as negative control. The tubes were incubated aerobically at 37 °C for 18 h. The first tube in the series with no sign of visible growth was taken as the MIC.

Determination of minimum bactericidal concentration (MBC)

MBC was determined by taken one standard loopful of culture from each of the first three broth tubes that showed no growth in the MIC tubes and inoculated on fresh nutrient agar plates. After incubation for 24 h, the least concentration of the extracts that showed no colony formation on the agar was taken as the MBC. The ratio of MBC to MIC was determined to predict effect of the extract on the test bacteria. MBC/MIC ratios greater than 1 was considered bacteriostatic while other MBC/MIC ratios were considered bactericidal (**Shanmughapriya *et al.*, 2008**).

RESULTS AND DISCUSSION

The phytochemical screening of the extracts of the plant shows that the solvents extracted different components of the plant. The phenolic was 57.13 and 19.06 mg tannic acid/g in acetone and methanolic extracts respectively. Flavonols and proathocyanidin recorded the least value in methanolic and acetone extracts respectively. Apart from flavonoids, acetone extracted other phytochemicals in larger quantity than methanol. The amount of the phenolic in the acetone extract was three times more than that of methanolic as shown in Table 1. Acetone performed better than methanolic in extracting the phytochemicals. Plants play a major roles development of new chemotherapeutic agents. The first step towards this goal is the *in vitro* antibacterial activity assay. Polyphenols have been reported to be the major plant compounds with antioxidant activity due to their redox properties (**Roginsky and Lissi, 2005**). They adsorbed, neutralize free radicals and quenching singlet and triplet oxygen (**Ilias and Carlos, 2001**). Flavonoids have been reported to possess strong antioxidant properties (**Diouf** *et al.,* 2009; **Oyedemi *et al.*, 2010**).

Table 1 Phytochemical analyses of extracts of *S. aethiopica* leaf

Phytochemicals	Extract	
	Methanolic	Acetone
Phenolic (mg/g)*	19.06±3.92	57.13±13.05
Flavonoids (mg/g)**	6.84±2.14	5.92±1.82
Flavonols (mg/g)**	2.78±0.38	15.20±4.27
Proathocyanidin (mg/g)***	6.42±2.74	14.52±3.11

Data are expressed as means ± SD of triplicate determinations; *Expressed as mg tannic acid/g of dry plant materials, **Expressed as mg quercetin/g of dry plant materials and ***Expressed as mg catechin/g of dry plant material

The rate of ABTS radical scavenging was determined for the two extracts of *S. aethiopica*. The activity of the plant extracts was lower than the phenolic-derivative standards: BHT and rutin. As shown in Figure 1, the ABTS radical scavenging activity of the extracts at 0.8 mg/mL compared with the standard is in the decreasing order: BHT (94.95%) > Rutin (87.52%) > Acetone extract (83.07%) > methanolic extract (76.79%). This showed that the plant has good ability to prevent oxidative damages (**Benzie and Strain, 1999**). ABTS is one of the free radicals that have been used for assessing antioxidant activity (**Santos *et al.*, 2003**) the extracts were able to mop up free radical generated by loss of electrons and produced a colored nitrogen centered cation by reacting with potassium persulfate for 12–14 h.

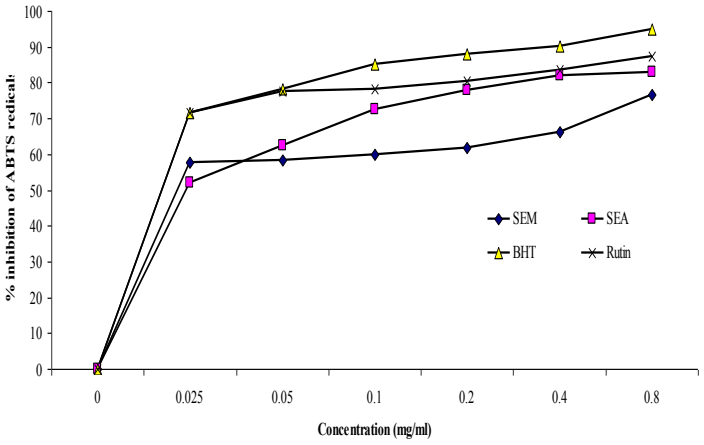

Figure 1 ABTS radical scavenging activity of extracts of *S. aethiopica* leaf

The antioxidant effect of the acetone and methanolic extracts of *S. aethiopica* was analyzed for the scavenging activity on free radical DPPH in a concentration-dependent manner. The maximum scavenging effect exerted by the extract to that exerted by the standards (BTH and Rutin) controls. The activity of the extracts is dose-dependent. The extracts of *S. aethiopica* had a good DPPH scavenging property compared with the control (Figure 2). Therefore, the data obtained from this study justified the ethnomedicinal use of this plant in the treatment of ear infections initiated by oxidative stress (**Sharma *et al.*, 2012**). At all the concentrations tested methanolic extract performed better than the acetone extract. The antioxidant ability of the extracts to donate hydrogen to DPPH radical, thus converting it into stable molecules is due to the presence of the Phytochemicals like phenolic compounds, such as flavonoid, polyphenol, tannins and terpenes as earlier reported (Zheng and Wang, 2001; **Guittat *et al.*, 2003; Sawer *et al.*, 2005**). The redox properties by the Phytochemicals play an important role in adsorbing and neutralizing free radicals **(Badami** *et al.,* 2003). Antioxidant potentials of the extract determined based on the measurement of free radical scavenging potency (hydrogen-donating ability). The radical scavengers donate hydrogen to free radicals. The use of DPPH radical provides an easy, rapid and convenient method to evaluate the antioxidants and radical scavengers (**Olorunnisola *et al.*, 2011**).

Extracts of *S. aethiopica* showed high level of scavenging ability against hydrogen peroxide at 0.8 mg/ml were 62.92, 66.78, 62.81 and 82.05% for methanolic extract, acetone extract, BTH and rutin respectively (Figure 3). Except at the final concentration the hydrogen peroxide scavenging activity of the acetone extract was higher than BTH but lower than rutin. Methanolic extract has a lower scavenging activity than the acetone extract. These plant extract will be able to protect cells against the damaging effect of free radicals generated by hydrogen peroxides (**Ilias and Carlos, 2001; Oyedemi *et al.*, 2010**). Hydrogen peroxide is a highly important reactive oxygen species it has ability to penetrate biological membranes. It may also have a detrimental effect on the cell if it is converted to hydroxyl radical which is toxic to the cell (**Larson, 1988; Fukumoto and Mazza**, **2000; Gulcin *et al.*, 2003**). The acetone extract of *S. aethiopica* was capable of scavenging hydrogen peroxide in a concentration dependent manner.

The ferric reducing/antioxidant power (FRAP) is a non-inhibition and direct test of total antioxidant power method of determining antioxidant potency of biological substances (**Benzie and Strain, 1999**). FRAP assay is simple, speedy and robust assay (**Prior *et al.*, 2005**). It is regarded as a direct test of total antioxidant power. It treats both chemically defined reductant and biologically defined antioxidants as basically equal. In the present study, the highest antioxidant potential was observed in Vitamin C followed by the two the other standard chemicals: Vitamin E and BHT (in decreasing order) (Figure 4). The extracts performance was relatively poor. Acetone extract was better than the methanolic extract at all the concentrations tested. The reducing power of the extract showed to be concentration dependent. The FRAP assay involves neither a pro-oxidant nor an oxidizable substrate. It depends upon the reduction of a ferric tripyridyltriazine complex to the ferrous tripyridyltriazine by a reductant at low pH (**Benzie and Strain, 1999**).

Brine shrimp is a low cost bench bioassay indicative of toxicity to different biological and chemical agents. The plant extracts had lethal effects on the brine shrimp nauplii with LD_{50} of 3.17 and 3.71 ppm for acetone and methanolic extracts respectively as shown in Figure 5. The extracts were classified as toxic to *Artemia salina* at the observed LC_{50}s. The LC_{50}s of the extracts were higher than standard limit (1ppm) (**Prior *et al.*, 2005**). The extracts could be termed considerably toxic.

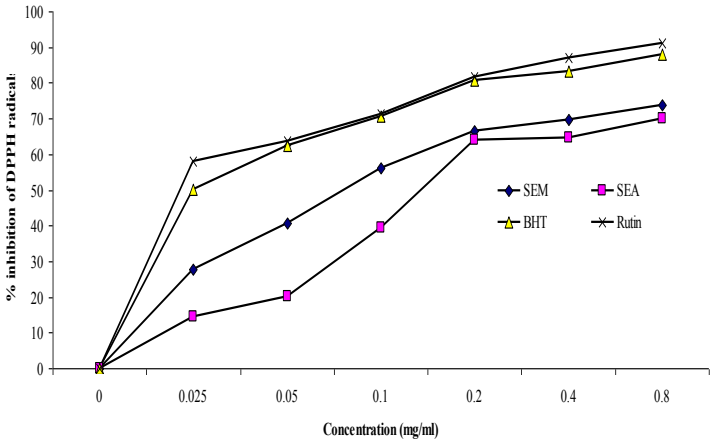

Figure 2 DPPH radical scavenging activity of acetone extract of *S. aethiopica* leaf

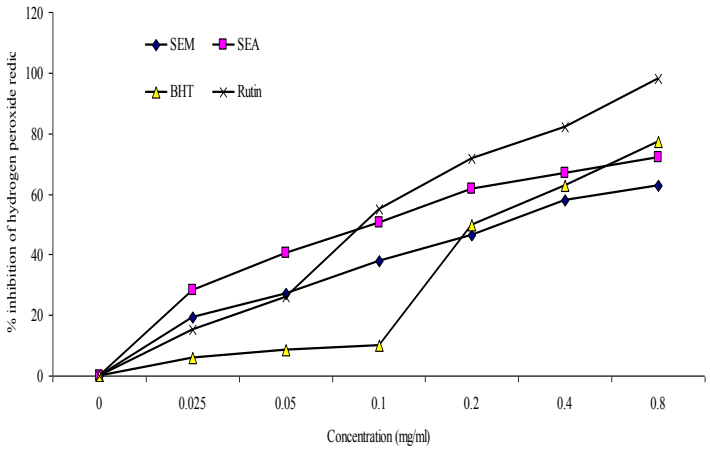

Figure 3 Hydrogen peroxide radical scavenging activity of extracts of *S. aethiopica* leaf

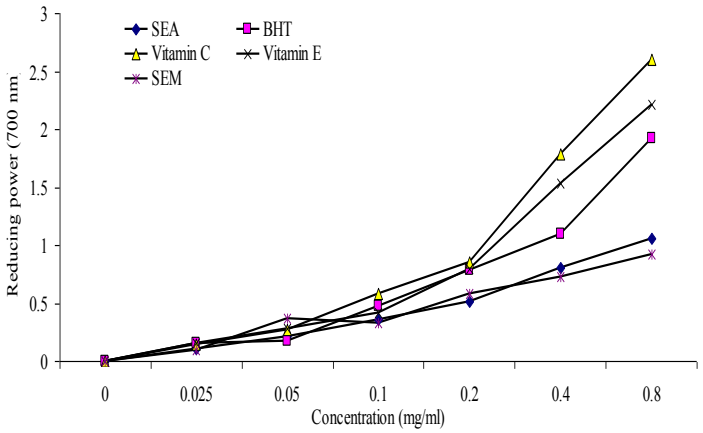

Fig. 5: **Figure 4** Reducing power activities of extracts of *S. aethiopica* leaf

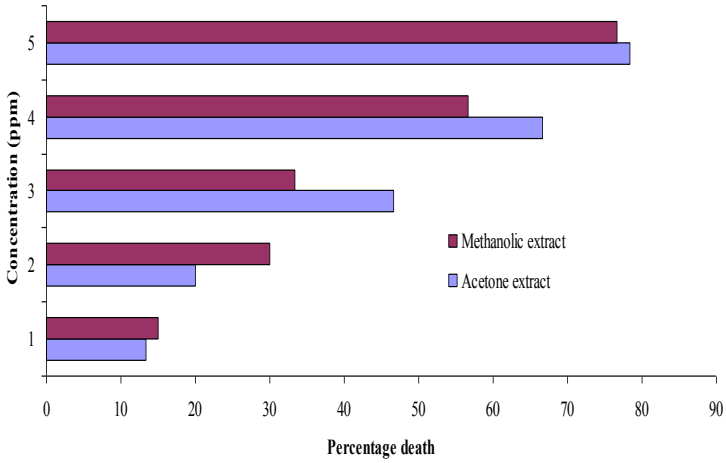

Figure 5 Brine shrimp cytotoxicity of extracts of *S. aethiopica* leaf

The result of the antibacterial assay showed that the extracts had good antibacterial activity against both Gram-positive and Gram-negative bacteria. The MIC and MBC values varied among the tested otitis bacterial pathogens. The susceptibility of all these bacteria to the extracts showed that Gram negative bacteria were relatively more susceptible to the extracts than Gram positive bacteria. This report contradicts the report of Sharma and colleagues (**Sharma *et al.*, 2012**) that reported Gram negative bacteria pathogens associated with ear infections to be more resistant to plant extracts than Gram positives. Since different plants possess varying phytochemicals and these phytochemicals have different mechanisms of actions against pathogens (**Guittat *et al.*, 2003**). Antimicrobial actions of the extract may not be targeted against the cell wall that makes a gram negative pathogen to be more resistant to most antimicrobials as earlier reported. It may be as a result of DNA intercalator and an inhibitor of DNA synthesis through topoisomerase inhibition (**Lisgarten *et al.*, 2002; Guittat *et al.*, 2003**). For the Gram negative bacteria the highest MIC was recorded against *P. aeruginosa* ATCC 19582: 3.125 and 1.5625 mg/ml for acetone and methanolic extracts respectively. The resistance of this pathogen may be as a result of their chemical composition and their ability to form spores. The MIC index however, and expectedly, suggested that the plant extract was bacteriostatic at lower concentration and bactericidal at higher concentration.

Table 2 Antibacterial activity of extracts of *S. aethiopica* leaf against bacteria associated with otitis

Pathogens	Gram Reaction	Extracts					
		Acetone			Methanol		
		MIC	MBC	MBC/MIC	MIC	MBC	MBC/MIC
P. aeruginosa ATCC 19582	-ve	3.125	6.25	2.0	1.5625	12.5	8.0
K. pneumoniae ATCC 10031	-ve	0.3906	3.125	8.0	0.7812	3.125	4.0
Ent. cloaca ATCC 13047	-ve	0.7812	0.7812	1.0	0.7812	0.7812	1.0
E. coli ATCC 25922	-ve	0.7812	3.125	4.0	1.5625	1.5625	1.0
B. cereus ATCC 10702	+ve	1.5625	3.125	2.0	3.125	6.25	2.0
S. aureus ATCC 6538	+ve	3.125	3.125	10.0	1.5625	3.125	2.0
S. aureus OK1	+ve	1.5625	3.125	2.0	0.7812	6.25	8.0
B. pumilus ATCC 14884	+ve	3.125	6.25	2.0	1.5625	3.125	2.0

CONCLUSION

The extracts of *S. aethiopica* (Thunb.) compared with the standard chemicals is very rich in phytochemicals and antioxidant properties. It also showed good antibacterial activity on both Gram negative and Gram positive bacteria although, the effects was more pronounced on Gram negative. This work serves as basic scientific validation of the folkloric uses of the plant for the treatment of ear infection in South Africa. The extract will certainly contain bioactive compounds that could be good candidates for the treatment of drug-resistant ear bacterial pathogens.

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INVOLVEMENT OF EXTRACELLULAR FUNGAL ENZYMES IN BIOREMEDIATION OF TEXTILE EFFLUENT

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ABSTRACT

The plentiful use and reckless discharge of textile effluent to the nature witnessed the rising of water and soil pollution. Biological remediation of these compounds is the most desirable technique to overcome the elevated environmental pollution. Present study evaluates the efficiency of a wild strain of *Irpex lacteus* in decolourisation and degradation of Reactive yellow FG and Reactive orange 2R. Media supplemented with different carbon/nitrogen sources and inoculum size play important role in enhancing the ability in which dextrose and aspargine boosted the process while inoculum size one-three (10 mm diameter) were more significant with solid and liquid decolourisation respectively. The ligninolytic enzyme production under Solid State Fermentation (SSF) was carried out using different lignocellulosic substrates. Among different substrates wheat straw produced highest amount (560.6 IU/ml) of manganese peroxidase. Optimization of particle size and time of incubation were also assorted to define the efficient enzyme activity; where one mm particle size and 6th day of incubation period were the most felicitous. The influence of physico-chemical factors like pH, temperature, reaction time and metal ions were assessed with respect to enzyme activity. The partial purification of crude enzymes was achieved at different percent saturations, where 40% saturated fraction yielded maximum (560.6 IU/ml) MnP activity. Molecular weight of the partially purified enzyme was 58.3 kDa. The degradation of dyes was confirmed with shift of the dominant peaks found on the FTIR graphs.

Keywords: *Irpex lacteus,* dye decolourisation, dye degradation, solid state fermentation, ligninolytic enzymes

INTRODUCTION

The huge manufacturing of the dyes due to their massive applications in textile industries has abundantly enhanced the effluent disposal into the environment. As a technological and scientific development in dye technology, they are synthesized as chemically and photolytically more stable and therefore persist in natural environment (**Rieger *et al.*, 2002**). Consequently leads to worsening of the environment which is inevitably linked with overall quality of life. To mitigate these xenobiotic pollutants, their complete mineralization or transformation into the degradable forms is the only imperative solution on it. Although, many physicochemical techniques are available for efficient mineralization of these dyes, they are very expensive and commercially unattractive. However, biological treatment or biodegradation is an environment friendly and cost-effective alternative to these technologies (**Gueu *et al.*, 2007**).
The Basidiomycetes fungi have the ability of metabolizing lignin. To meet the challenge of lignin degradation, white rot fungi produce one or more of the three principal extracellular enzymes i.e. lignin peroxidises (LiP, EC 1.11.1.14), manganese peroxidase (MnP, EC 1.11.1.13) and laccases (EC 1.10.3.2) (**Hatakka, 1994; Asgher *et al.*, 2004**). Identification of these enzymes generated one of the credible evolutions for the degradation of aromatic xenobiotics and/or environmental pollutants. Perusal of literature demonstrates the potential of white rot fungi to degrade pollutants by producing extracellular ligninolytic enzymes (**Valentin *et al.*, 2007; Wen *et al.*, 2010**) and most of them have been focused on dye decolourisation and degradation (**Enayatzamira *et al.*, 2009; Champagne *et al.*, 2010**). The initial recognition of the white rot fungi for their decolourizing competency lays the foundation for their application in dye degradation. Therefore, the present study paved the way from dye decolourisation to degradation using the potential of white rot fungus *Irpex lacteus*.
From extremely diverse range of the textile dyes, most unanimously used reactive dyes (Reactive yellow FG and Reactive orange 2R) have been opted in the present study. The key role of ligninolytic enzymes yielded by *Irpex lacteus* under solid state fermentation has been emphasized for their ability to degrade the dyes. As the complete purification of enzyme is costly, crude and partially purified enzyme was preferred for their application in enzyme assay and degradation. Influence of different physico-chemical parameters such as inoculum size, effect of pH, temperature, incubation and reaction time, supplementation of carbon/nitrogen sources, and metal ions on enzyme productivity was also investigated. The intermediates formed during degradation of the dyes were analysed by FTIR (Fourier Transform Infrared Spectroscopy).
The main objectives of the present study were: i) to evaluate the potential of *Irpex lacteus* in decolourisation of textile dyes i.e. Reactive yellow FG and Reactive orange 2R; ii) whether the dyes are decolourising or undergoing structural alterations due to enzyme action? and iii) rectification of reactive dyes degradation using ligninolytic enzymes through FTIR.

MATERIALS AND METHODS

Isolation and screening of the fungi

Thirty five strains of wood rot fungi collected from the different forests of Gujarat State (India) were isolated and plated on optimised malt extract agar (MEA) medium. The purified cultures were subjected to Bavendamm's test (**Bavendam, 1928**) for the screening of white rot fungi. Among six screened white rot fungi, KSR-70 was considered for the present studies. For molecular identification fungal DNA was extracted as per **Möller *et al.*, (1992)** and extracted DNA was subjected to Polymerase chain reaction followed by sequencing, BLAST and its submission to the NCBI with Accession No. KJ670229.

Chemicals and dyes

DMAB (3-dimethyl amino benzoic acid), MBTH (3-methyl-2-benzothioazolinone hydrazone hydro chloride), H_2O_2 and Manganese Sulphate ($MnSO_4$) were procured from National chemicals Ltd., (Vadodara, India). The textile dyes used in the present study were kindly provided by dying, printing and processing houses (Gujarat, India). Chemicals required for the biochemical studies were purchased from Qualigens Fine chemicals (Mumbai, India). All the other chemicals used were commercially available products of analytical grade.

Solid plate decolourization

On plate decolourisation of Reactive yellow FG and Reactive orange 2R was performed on the Malt Extract Agar (MEA)-dye plate containing 2% malt and 2.5% agar in the presence of individual dye. The media supplemented with dye concentrations (10, 50, 100, 250 and 500 mg L^{-1}) were prepared with 25 mL medium/plate. These petri plates were inoculated centrally with 10 mm diameter agar disc removed from the actively growing fungi on MEA medium. The decolourisation efficiency was assessed by visual disappearance of dye colours on the plates from 3rd to 15th day of inoculation. Zone of growth and decolourisation were measured at every two days of interval.

Decolourisation in liquid media

Dye decolourisation experiments were performed in 150 mL Erlenmeyer flasks containing 25 mL of 2% Malt Extract Broth (MEB) supplemented with dyes (10 mg L^{-1}). Three discs (10 mm diameter) of fungal inoculums taken from active cultures and inoculated in each flask containing sterilized media. Dye decolourisation was investigated by harvesting the inoculated flasks after 3, 5, 7, 9, 11 and 13 days of incubation. Dye decolourisation was monitored spectrophotometrically by subjecting the filtrate after removing mycelia at the maximum visible wavelength of absorbance (λmax) for individual dyes. All the experiments were performed in triplicates and the average values were considered in calculations. The decolourisation efficiency was expressed as per the following equation.

$$\% \text{ decolourisation} = \frac{\text{Initial absorbance} - \text{observed absorbance}}{\text{Initial absorbance}} \times 100$$

Effect of carbon/nitrogen sources on decolourization

Different sources of carbon (dextrose, sucrose, fructose, and lactose) and nitrogen (ammonium sulphate, sodium nitrite, asparagine, and urea) at the concentration of 10 g L^{-1} were used as co-substrates to investigate their effects on decolourisation. Influence of these sources on the solid plate decolourisation was checked by inoculating the petridishes (containing growth medium, dyes and carbon/nitrogen sources) with a disc (10mm diameter) of fungal mycelium. The diameters (cm) of the decolourisation and growth zone were determined in two perpendicular directions of the plates at every two days of interval. Un-inoculated plates containing dyes and carbon/nitrogen sources were treated as control.
For liquid decolourisation, three plugs of agar discs containing fungal mycelia (10mm diameter) were inoculated in the flask containing MEB (Malt Extract Broth) supplemented with different dyes and carbon/nitrogen sources (1% concentration). Medium without any of the supplement was used as blank, whereas media with dyes and carbon/nitrogen sources but without inoculums were used as control. Decolourisation efficiency of fungal isolates was measured at the interval of every 3 days using UV-visible spectrophotometer (Perkin-Elmer, USA) and per cent decolourisation was calculated as per above mentioned equation.

Determination of enzymatic activity by solid state fermentation (SSF)

Optimisation of different Solid substrates

Different agro-industrial wastes i.e. wheat straw, rice bran, saw dust, ground nut shells, sugarcane bagasse, and banana pseudo-stems were screened to determine the appropriate substrate for maximum ligninolytic enzyme production using Solid State Fermentation (SSF). Agro-industrial wastes were obtained from local agricultural farms, saw dust was acquired from saw mills situated near Vadodara (Gujarat) while and sugarcane bagasse was procured from the sugarcane industry near Vadodara. Among all individually used agro-industrial waste as a substrate, wheat straw was thriving as the best substrate for enzyme production under SSF. All the substrates were inoculated with pure cultures of the fungal strains and crude extract of these substrates was used for enzyme assay.

Optimisation of particle size and incubation time

As wheat straw was found as the best substrate for the maximum enzyme production, optimum size of substrate particle was determined by using different sizes (<1, 1, 1.5, 2, 2.5, 3, 3.5, 4 and >4 mm) of wheat straw to get the high efficient enzyme activity. Similarly, time required for the fungal growth and enzyme production is also evaluated. The optimisation of the incubation period was carried out by harvesting the flasks containing solid substrate covered with the fungal mycelia ate every three days till 18th day of inoculation..

Enzyme production and harvesting

The enzyme production was performed into 250ml Erlenmeyer flasks containing 5g of agro-industrial wastes moistened with 50 mL distilled water. The sterilized production media was inoculated with five plugs (10mm diameter) of fungal inoculum. Flasks were harvested after every three days of inoculation to assess the enzyme activity. Crude extract of extracellular enzymes was prepared by addition of 50 ml phosphate buffer prior to harvesting the flasks. The contents in the flasks were gently beaten and incubated on the rotary shaker for 30 minutes. Liquor obtained was filtered by using Whatman filter paper No. 1 and the filtrate was used as a source of crude enzyme.

Enzyme assay

Crude enzyme obtained by SSF was used for the estimation of extracellular MnP (Manganese Peroxidase), MIP (Manganese Independent Peroxidase) and Laccase activities. These activities were determined by spectro-photometric measurement of DMAB (3-dimethyl amino benzoic acid) and MBTH (3-methyl-2-benzothioazolinone hydrazone hydro chloride) as substrates (Vyas *et al.,* 1994). The activities of manganese peroxidises was assayed in 2 mL of reaction mixture containing 100 µL MBTH (1mM), 200µL DMAB (25 mM), 10 µL $MnSO_4$ (20 mM), 10 µL H_2O_2 (10 mM), 1000 µL buffer (0.1M), and 100 µL enzyme. In case of MIP, the same reaction mixture was used as MnP, except the addition of $MnSO_4$. Conversely, in the reaction mixture of laccase, addition of $MnSO_4$ and H_2O_2 were excluded. Oxidation of DMAB and MBTH as chromogen was measured at 590 nm on Shimadzu UV visible spectrophotometer, where reference blanks contained all components except the assayed enzyme.
The enzyme activity was calculated using the molecular extinction coefficient of MnP, MIP and laccase, and expressed in µmol/min^{-1}. One unit (U) of MnP/MIP or laccase was defined as the amount of enzyme necessary to produce one µmol of product per min upon DMAB-MBTH oxidation (590 nm) of the substrate in the reaction mixture under the assay conditions.

Partial purification and Molecular weight determination of Enzyme

Crude enzyme was partially purified by ammonium sulphate precipitation method of **Dawson *et al*., (1969)** at four different per cent saturations i.e. 20, 40, 60 and 80. From all saturated fractions, maximum activity producing fractions were dialysed with membrane filter having 12000-14000 Da cut off value and collected/stored for further characterization.
The molecular weight of the separated proteins was evaluated by 10% SDS-PAGE (Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis) along with standard protein marker (NEB # P7708). Gel electrophoresis was performed by using. Electrophoresis of partially purified enzyme as described by **Laemmli (1970)**, using Medox electrophoresis unit (Bioneeds Instruments, India).

Effect of physico-chemical factors on enzyme activity

Optimisation of enzyme activity with respect to its temperature (10 to 40ºC), pH (2.5 to 6.5), incubation time (5 to 45 minutes) and effect of metal ions (1mM, 0.1mL Mn^{2+}/ Zn^{2+}/ Cu^{2+}/ Ca^{2+}/Mg^{2+}) on enzyme activity was also studied.

Biodegradation analysis by FTIR (Fourier Transform Infrared Spectroscopy)

Degradation of dyes was confirmed by FTIR analysis of treated dyes; where the samples containing 10 mL dye (10 mg L^{-1}concentrations) treated with 500µl of partially purified enzyme and then dried at room temperature and processed for FTIR (Shimadzu 8400) analysis by KBr pellet method at 10^{-4} resolution and 30 scan.

RESULTS AND DISCUSSION

About thirty five strains of wood rot fungi were collected from different forests of Gujarat state. The sterilized fruiting bodies and infected wood samples were processed for the isolation, purification and adaptation of the particular growth media. From all thirty five strains, six were found to be positive with Bavendamm's reaction, showing the complete browning of malt agar medium enriched with 1% tannic acid (**Bavendam, 1928**). Among which, *Irpex lacteus* generating white, cotton-like-fluffy mycelia and radical pattern while growing on malt agar, was selected for the present study.
Irpex lacteus is well characterized for its capacity to decolourise diverse synthetic dyestuffs of main chemical dye groups (**Novontny 2004; Choi 2013**). On the solid media, it totally decolourised Reactive yellow FG and Reactive orange 2R, with 10 mg L^{-1} concentration after 11 and 13 days of inoculation respectively (Fig. 1). All the other four dye concentrations tested in the present investigation were also been decolourised easily by the strain (Fig. 2). However, the dye concentration in the growth media plays important role to determine the time required for complete dye decolourisation from the media (**Koyani *et al*., 2013**). It is directly proportional to increase in concentration of dye with the decrease in rate of decolourisation. However, the time required for the decolourisation increased gradually with the concentrations from 10, 50, 100, 250 to 500mg L^{-1}. Available literature indicates that up to certain concentration of dyes fungal growth occurs but exceeding particular concentration it acts as an inhibitor for growth (**Kim *et al.,* 1995; Eichlerova *et al*., 2006**). In the present study, the

concentration of the dyes up to 100 mg L^{-1} does not affect the fungal growth, but escalating concentration led to the lessening in growth and decolourisation rate both.

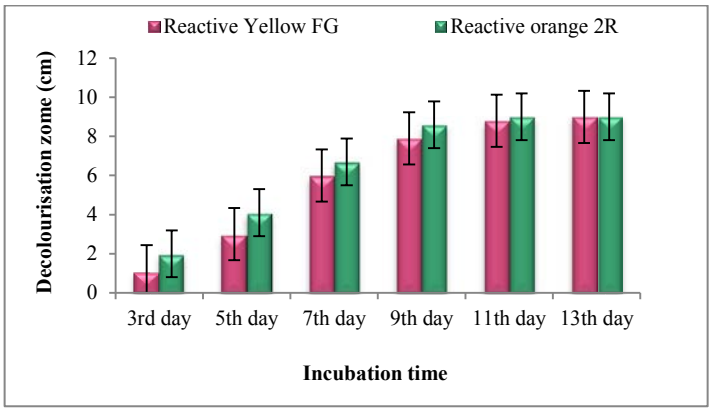

Figure 1 Solid plate decolourisation of two reactive textile dyes i.e. Reactive Yellow FG; Reactive Orange 2R, measured as decolourisation zone (cm) against different incubation time interval (days) using *Irpex lacteus*.

Figure 2 On plate decolourisation of two reactive textile dyes i.e. Reactive Yellow FG (A: Inoculated with *Irpex lacteus*; B: Blank); Reactive Orange 2R (C: Inoculated with *Irpex lacteus* ; D: Control) at five different concentrations (10, 50, 100, 250 and 500 mg L^{-1}- from right to left)

The decolourisation of two reactive dyes with different chemical structures by *Irpex lacteus* was measured as a decrease of visible light absorbance at the wavelength of maximum absorbance ($\lambda_{max,}$ nm) of respective dyes. It bestowed 100% decolourisation of Reactive yellow FG and Reactive orange 2R (10mg L^{-1}) on 11th and 13th day respectively (Fig. 3). *I. lacteus* decolourise different dyes to various extents depending on their complexity of chemical structure (**Novotný *et al.*, 2000; Máximo *et al.*, 2003**). **Novotný *et al.*, (2000)** has also selected *I. lacteus* for its ability to decolourise all the tested dyes with an efficiency of 56–100% within 14 days. However, azo dyes are recalcitrant for decolourisation and could be decolourised to a limited extent (**Revankar and Lele, 2007**). In the present study it was found efficient degraders for both the dyes with concentrations ranging from 10 to 500 mg L^{-1}.

Fungal growth and enzyme production depend upon the growth conditions and the nutrition provided to them (**Sanghvi *et al.*, 2010**). Any of the additional sources to the medium directly influence decolourisation ability of the fungi. Five different carbon sources (Dextrose, Sucrose, Lactose, Maltose and Fructose) and five nitrogen sources (Ammonium sulphate, Urea, Asparagine, Sodium nitrate and Sodium nitrite) were tested for their effectiveness on solid and in liquid medium to enhance the rate of decolourisation. Among them, dextrose enhanced the rate decolourisation while other carbon sources were relatively less proficient. Glucose can serve as a carbon and energy source, and it could support the dye decolourisation (**Sanghvi *et al.*, 2010**). However, there is no unanimity about the role of carbon sources in dye decolourisation**. Carliell *et al.*, (1995)** and **Kapdan *et al.*, (2002)** also recorded that glucose increases rate of decolourisation while others found no effect of it (**Özsoy *et al.*, 2001; Chen *et al.*, 2003**). Supplementation of nitrogen in growth media not only influence the ligninolytic enzyme production by several white rot fungi, but also play an important role in the process of dye decolourisation (**Moldes *et al.*, 2004**). In the present study also, asparagine is reported as an excellent nitrogen source for inducing dye decolourisation while sodium nitrite inhibited the fungal growth.

Different agro-industrial wastes such as saw dust, wheat straw, ground nut shells, rice bran, sugarcane bagasse, and banana pseudo-stems were used as a sole source of carbon without any mineral supplementation in order to determine the most suitable substrate for the production of ligninolytic enzymes. Among all the substrates examined, wheat straw was proved to be unsurpassed lignocellulosic substrate (Fig. 4), for the production of all three ligninolytic enzymes *viz*. MnP, MIP and Laccase. Wheat straw is one of the best substrate known for the production of enzymes. It is the most widely used substrates among all the other substrates that are employed for this purpose (**Valaskova and Baldrian, 2006**). Maximum production of ligninolytic enzymes under wheat straw degradation by fungi has already been reported earlier (**Zhang *et al.*, 2008; Shrivastav *et al.*, 2011**). *I. lacteus* produced 480.36, 440.12 and 195.19 IU mL^{-1} as the highest activity for MnP, MIP and laccase, respectively. According to **Conesa *et al.*, (2002)**, a variation in enzyme production by the fungus is a result of its adaptation to different cultural conditions and substrates on which it. In the present study, MnP is the major protein produced on wheat straw. **Vyas et al., (1994)** and **Hoftrichter *et al.*, (1997)** also reported that some white rot fungi show variation in specific enzyme production when grown on wheat straw.

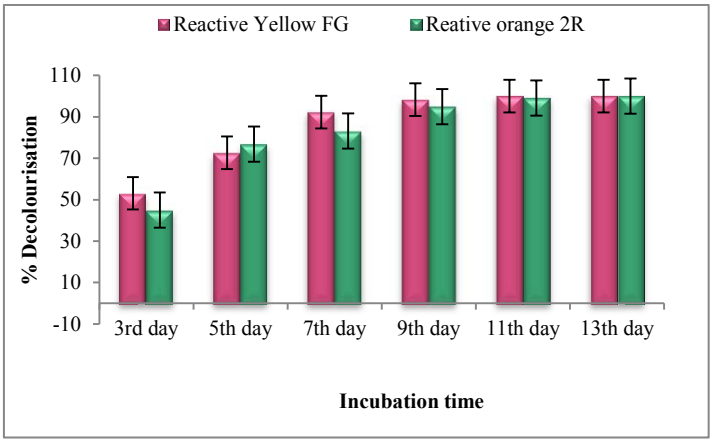

Figure 3 Liquid decolourisation of Reactive Yellow FG and Reactive Orange 2R calculated as %decolourisation obtained at different time interval (days) using *Irpex lacteus*.

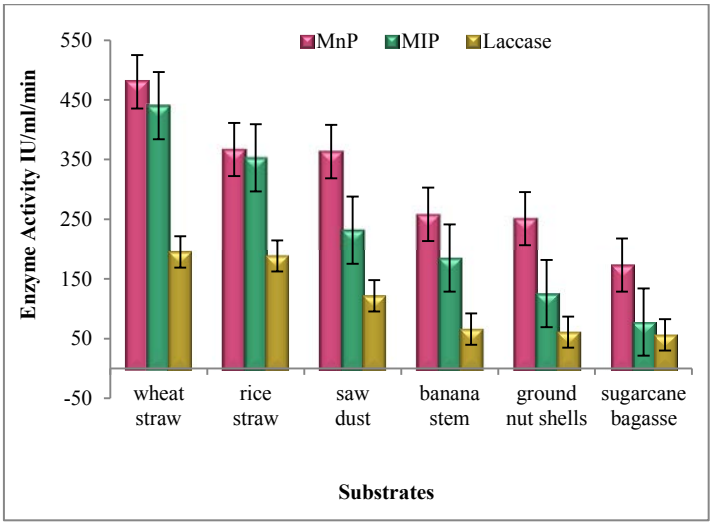

Figure 4 Optimization of different solid substrates for the maximum ligninolytic enzyme production by *Irpex lacteus*

Use of wheat straw as a substrate becomes more exponential when the appropriate particle size of the substrate is used (**Sanghvi *et al.*, 2010**). *I. lacteus* when grown on nine different particle sizes (<1, 1, 1.5, 2, 2.5, 3, 3.5, 4 and >4mm) of substrates, it produced highest MnP (480.36 IUmL^{-1}), MIP (440.12 IU mL^{-1}) and laccase (195.19 IU mL^{-1}) with 1mm sieve size particles. Production of these enzymes was relatively less at particle size smaller than 1mm sieve size. Since the rate of oxygen transfer into the void space affects growth, the substrate should contain particles of suitable sizes to enhance mass transfer (**Pandey, 1992**). Increase in substrate concentration lead to a decrease in enzyme activity (**Gupta *et al.*, 2009**). Therefore, declined enzyme production may be associated with increase in viscosity and decrease in porosity of substrate, which consequently affect the available surface area of substrate and oxygen transfer (**Gupta *et al.*, 2009; Sanghvi *et al.*, 2010, 2011**).

The production of MnP and laccase by different white rot fungi are more common and predominant (**Vyas *et al.*, 1994; Hofrichter *et al.*, 1999**). In the present investigation with *Irpex lacteus*, the optimum ligninolytic enzyme activities of dialyzed partially purified enzyme fraction were 560.6 IU mL^{-1}

(MnP), 534.4 IU mL^{-1} (MIP) and 263.22 IU mL^{-1} (Laccase). The production profile of MnP was elevated that of MIP and laccase activity. The most common ligninolytic peroxidases produced by majority of white rot basidiomycetes and litter-decomposing fungi are MnP (**Wesenberg *et al*., 2003**). **Gupte *et al*., (2007)** and **Kasinath**, (**2002**) have reported absence of MnP activity in *I. lacteus* whereas **Novotný *et al*., (2001)** documented very low level of MnP production by it. We disagree with the earlier reports, since production of manganese peroxidase is a dominant enzyme in *Irpex lacteus*.

About 90% of proteins in the crude extract were filtered out in 80% saturation by ammonium sulphate. The enzyme activities determined from these all the saturated fractions are mentioned in Figure 5. The ammonium sulphate fractions (20-80% saturation) containing about 90% of manganese peroxidase was subjected to molecular weight determination. From all 20, 40, 60 and 80% saturated fractions of crude enzyme; maximum activity (560.6 IU mL^{-1}) of MnP was recorded with 40% saturated fraction (Fig. 4). The enhanced enzyme activity and improved purification fold was noticed when compared with the crude extract (Table 1).These dialysed fractions (12000 to 14000 Da) were subjected to gel electrophoresis where the enzymes appeared as distinct band of 58.3 kDa on SDS-PAGE (Fig. 6). Generally, MnPs of white rot fungi usually have a MW of 45 kDa (**Hofrichter, 2002**). **Shin *et al*., (2005)** isolated MnP from *Irpex lacteus* with the molecular mass of 53.2 kDa, while **Baborová *et al*., (2006)** exhibited the 37.5 kDa MnP from *Irpex lacteus,* which is quite less compared to our results.

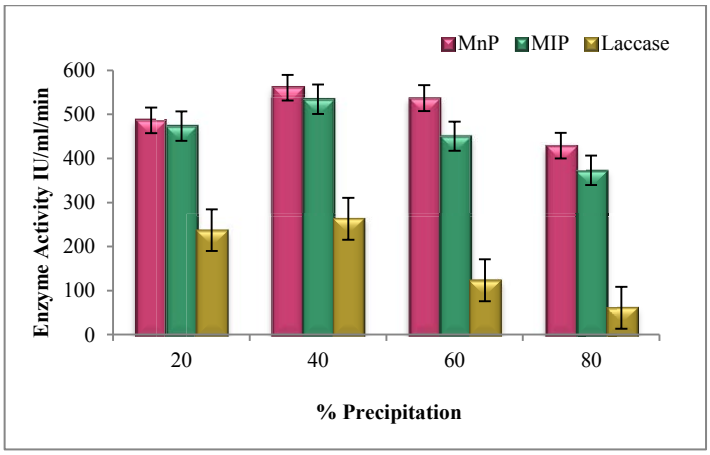

Figure 5 Production profiles of ligninolytic enzymes produced by *Irpex lacteus* at different ammonium sulphate precipitation saturated fractions (20%, 40%, 60%, 80%).

Table 1 Partial purification of Manganese peroxidase

	Enzyme activity U/ml	Specific activity U/mg	Purification fold
Crude extract	480.36	3.90	1.0
$(NH_4)_2SO_4$ (40%)	560.6	22.23	5.7

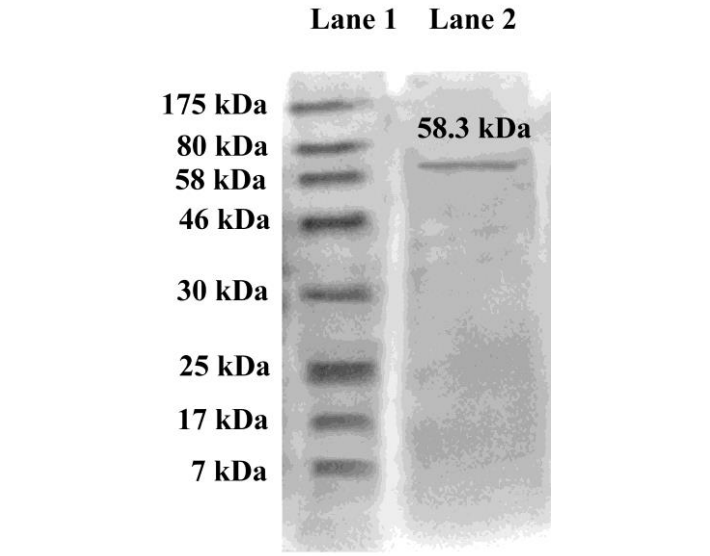

Figure 6 SDS PAGE analysis of partially purified enzyme

Enzymes are highly sensitive to pH change and it is an important factor that controls the activity of peroxidases (**Silva *et al.*, 2008**). The range of pH (2.5 to 6.5) ascribed to the enzyme reaction exhibited pH 5 as the most supportive in utmost enzyme production (Fig. 7). This may be attributed to the fact that change in the pH may alter the three-dimensional structure of the enzymes (**Shulter, 2000**). Temperature is another crucial factor for determining the fungal growth, which is directly related with the fungal biomass, enzyme production and its activity. This is because during the fermentation there is general increase in the temperature of the fermenting mass due to respiration (**Niladevi et al., 2007**). Optimisation of the temperature for attaining the excellent enzyme activity was performed within the range of 5 to 45°C. Available literature suggests that MnP has an optimal activity between 23 and 40°C (**Shin *et al*., 2005**), while **Baborová *et al*., (2006)** isolated highest titre of MnP isoenzymes at 50 to 60ºC. Recently, **Sklenar *et al*., (2010)** has also shown the reasonable temperature range up to 50°C for the production of MnP by *Irpex lacteus*. However, very far from all these reports, 35°C was the flawless match to the optimum enzyme production in the present investigation (Fig. 8).

Baldrian *et al*., (2005) reported that metal ions play important role in the production of enzyme and concluded that some microelements conferred a significant impact on enzyme activity. Here, five different metal ions i.e. Mn^{2+}, Zn^{2+}, Cu^{2+}, Ca^{2+}, and Mg^{2+} were scanned for their ability to enhance the enzyme activity. It is reported that supplementing cultures with manganese and aromatic compounds can stimulate the MnP activity by acting as inducers, enhancers or mediators (**Hoftrichter *et al*., 1997**). According to **Bonnarme and Jeffries, (1990)** and **Scheel *et al*., (2000)**, most of the white rot fungi require manganese in the culture medium to increase MnP activity. In contrast, our results exhibited neutral outcome with any of the inducer. The major ligninolytic enzyme produced by *I. lacteus*, which is not much influenced even after supplementing the inducer such as Mn, Cu, and Pb (**Baldrian *et al*., (2005)**).

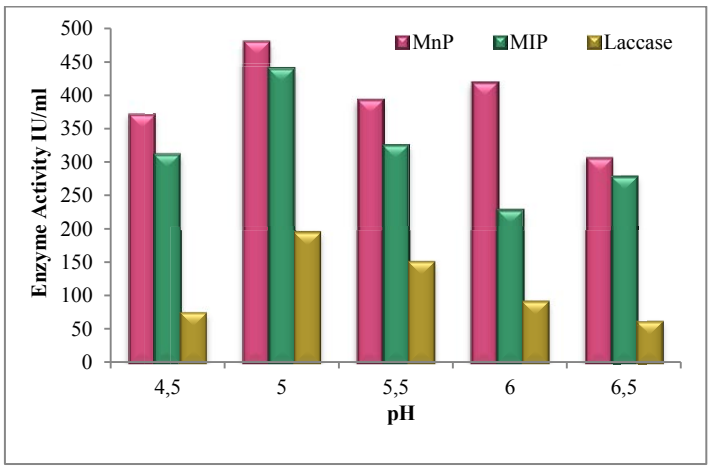

Figure 7 Influence of pH on ligninolytic enzyme activity produced by *Irpex lacteus*.

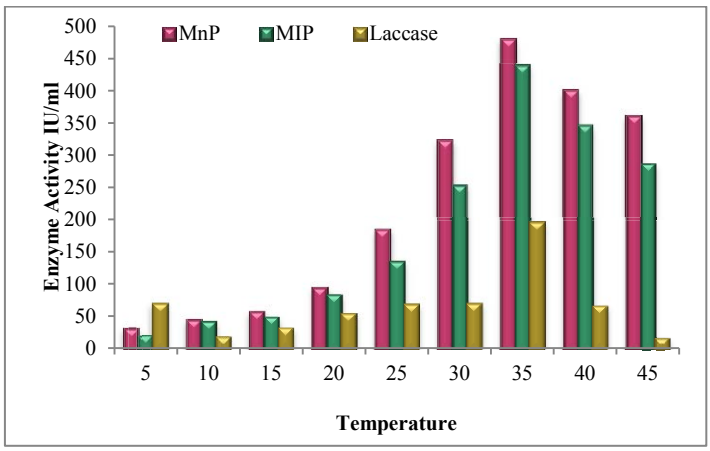

Figure 8 Influence of temperature on ligninolytic enzyme activity produced by *Irpex lacteus.*

Degradation of various xenobiotic compounds including dyes directly entails the ligninolytic system of white rot fungi. The ability of the white rot fungi to degrade a wide range of recalcitrant dyes has generally been associated with the non-specific nature of their lignin modifying enzymes (**Field *et al*., 1993;**

Goszczynski *et al*., 1994). For the confirmation of biodegradation of these compounds by ligninolytic enzymes, FTIR analysis was carried out by earlier workers (**Kalme *et al*., 2007; Dhanve *et al*., 2009; Koyani and Rajput 2014**). In the present investigation, the FTIR spectra of both control (untreated) and treated dyes showed stretching of specific peaks in the region 4000 to 500 cm^{-1}. The stretching vibration at 3456 cm-1 represented the ─N─H stretching which indicates the nature of aromatic amine group present in the parent dye compounds. Azo dyes are aromatic compounds with one or more azo bonds (─N=N─) and presence of ─C─H and ─N=N─ stretching confirmed the azo groups present in the dye, while ─SO_2 stretch represented the presence of sulpha group in the dye structure. All these stretching confirm the dye structures and when the spectrum of control and treated dyes were compared, changes in the positions of these peaks were observed. Shifting of the peaks to another position from their original location indicates degradation of original dye structure. Disappearance of peaks with the vibrations 1545,1345,1230 cm^{-1} and shifting of the peaks 1697 and 1140 cm^{-1} representing the C- C stretching can be noticed in case of treated Reactive yellow FG while peaks 3456 and 1385 cm^{-1} cannot be seen in the treated sample of Reactive orange 2R.Similar changes in the peak of different dyes have already been reported by earlier workers **(Field *et al*., 1993; Goszczynski *et al*., 1994; Kalme *et al*., 2007; Dhanve *et al*., 2009**). The FTIR spectral comparison between control dye and samples treated with ligninolytic enzymes of *Irpex lacteus* showed degradation of both tested dyes (Fig. 9).

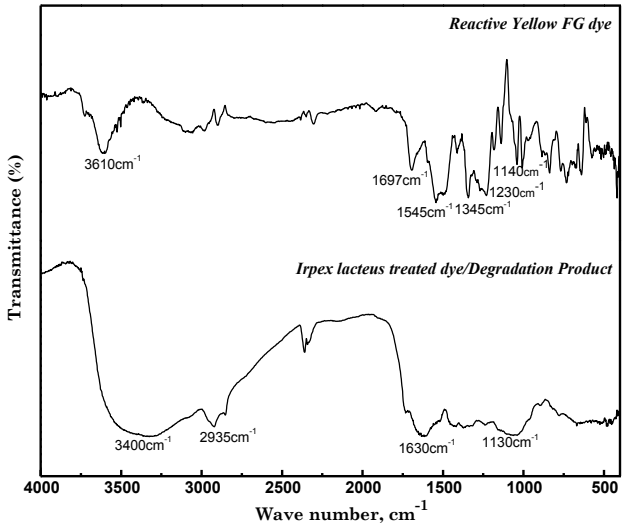

Figure 9A FTIR Spectra of control (upper) and treated dye(lowers)

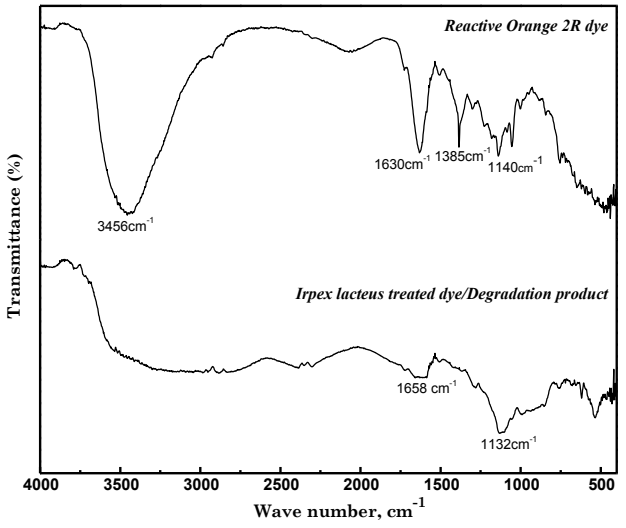

Figure 9B FTIR Spectra of control (upper) and treated dye (lowers)

CONCLUSION

The white rot fungus *Irpex lacteus* manifested its decolourization efficiency for reactive textile dyes which are responsible for significant water pollution. The 100% of decolourization of both reactive dyes is perceived within 13 days. SSF system including the use of agro-industrial wastes is very promising for ligninolytic enzyme production. Production of MnP was optimal when compared with MIP and laccase. *I. lacteus* is a potential fungus for the production of ligninolytic enzymes which offer plausible advantage by their use in biodegradation of reactive textile dyes. Therefore, the output of the present study reflects the possibilities of developing the biodegradation technology for textile dyes in an economic way.

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GENETIC DIVESITY AND EXTRACELULLAR ENZYMATIC ACTIVITY OF *BACILLUS LICHENIFORMIS* STRAINS FROM MILK POWDER

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ABSTRACT

A total of 379 *B. licheniformis* strains isolated from commercial milk powder were characterized genotypically and phenotypically. RAPD analysis yielded three different profiles, which include all isolates in this study, which could be assigned to strain F (n=375) or strain G (n=4), strain F also could be divided into two groups (group 1, n = 117; group 2, n = 258). Clustering by pairwise sequence similarity and phylogenetic relationships between isolates based on comparisons of the 16S rRNA gene sequence, showed two well defined groups. Group I contains all isolates tested belonging to genotype F, and Group II consists of three G genotype isolates. A total of 32 isolates, respecting the representation of each genotype, were randomly selected for extracellular enzymatic activity plate assays. Most isolates (25 out of 32) showed extracellular proteinase, lipase and amylase activity. Hydrolytic activities tested in this study are strain-dependent and none enzymatic activity could be linked to a defined group at genetic level. Preliminar characterization of proteolytic crude enzyme extract suggests the presence of a metal-activated serine protease active at an optimun temperature of 60 ºC. The exoenzymes production and its variation against different factors such as temperature, is isolate dependent so these results indicate that not all *B. licheniformis* strains may mean the same risk to process or product quality**.**

Keywords: *Bacillus licheniformis*, milk powder, RAPD, protease activity

INTRODUCTION

Spore-forming bacilli are common contaminants in dairy products and have been detected throughout the dairy processing, including dairy farm environments, storage and transportation tanks, and dairy processing plants (**Crielly *et al*., 1994; Postollec *et al.,* 2012**). Spore-forming microorganisms enter processing plants from farm environments via milk, and in some cases multiplying within processing stages where conditions are suitable for bacterial growth. Thermal processes based on high temperatures are used in food industries to guarantee stability and safety of the products. However, these thermal treatments are not always sufficient to inactivate all spore-forming bacteria, especially those that are highly heat-resistant. In most cases spore-forming bacteria do not present safety concerns, however they can impact on spoilage and product specification requirements, as in the case of thermophilic bacilli contaminants in milk powders (**Reginensi *et al*., 2011; Ronimus *et al.*, 2003; Rueckert *et al*., 2004; Scott *et al*., 2007**). The presence of the thermophilic bacilli in dairy products is indicator of poor hygiene and high counts are unacceptable, since they can lead to product defects caused by the production of heat-stable enzymes, such as proteinases and lipases, and acids capable to spoil the final product (**Chopra and Mathur, 1984; Cosentino *et al*., 1997; Chen *et al*., 2004**).

In a survey of milk powders from numerous countries, *Bacillus licheniformis, Anoxybacillus flavithermus* and *Geobacillus* spp. constituted 92% of spore-forming species isolated (**Ronimus *et al*., 2003**). *B. licheniformis* is a bacterium commonly found in nature, is the thermophilic bacilli most commonly isolated from raw milk (**Crielly *et al.*, 1994**) and has been described as one of the two predominant species in Uruguayan dairy powders (**Reginensi *et al*., 2011**). It is a Gram positive rod-shaped spore-forming bacteria, mobile and facultative anaerobic. Belonging to *B. subtilis* group of the genus *Bacillus*, further comprising *B. subtilis, B. amyloliquefaciens, B. pumilus, B. atrophaeus, B. mojavensis, B. sonorensis, B. vallismortis, B. firmus, B. lentus* and *B. sporothermodurans* (**Fritze, 2004**). This organism is of great interest not only because of its predominance in the production line of milk powder but also for the broad technological potential of the bacterium itself and its extracellular products (**Schallmey *et al.*, 2004**). Proteases are used in detergent and leather industry, while amylases are used in textile and paper manufacture (**Priest, 1977**). However, these enzymes can be a primary source of spoilage of milk and the manufactured dairy products. Intensive heat treatments (i.e. UHT) at dairy industry are able to lower the load of these microorganism, but exoenzymes may be thermostable (i.e. proteases and lipases) and only partially inactivated with further action on the product (**Chen *et al*., 2003**). Several authors confirmed that bacterial proteinases and lipases found in milk survived all the heat treatments applied during the manufacture of milk powder (e.g., pasteurisation and spray-drying) (**Celestino *et al.* 1997a,b; Chen *et al.,* 2004**), and these enzymes remained active in reconstituted milk even after a further 6 months storage at 25 ºC (**Celestino *et al.*, 1997b**).

Identification of the microorganisms that may be contributing to milk powder spoilage can help in implementing preventive and corrective actions, with an approach directed to find critical control points through the dairy chain. Molecular methods able to rapidly detect and identify thermophilic contaminants are essential to improve the industrial response. *B. licheniformis* can be isolated from commercial milk powder under 'mesophile' and 'thermophile' growth conditions (**Reginensi *et al.*, 2011**). Various methods, including multi-locus enzyme electrophoresis and phenotypic analysis (**Duncan *et al*., 1994**), random amplification of polymorphic DNA (RAPD) (**Ronimus *et al.*, 2003**), *rpoB* and *gyrA* sequencing (**De Clerck and De Vos, 2004**), and bacitracin synthetase gene sequences (**Ishihara *et al*., 2002**) are currently used for genotyping *B. licheniformis*. Two or three different subgroups have been discriminated by this methods. Recently, a multi-locus variable number tandem repeat analysis (MLVA) method and combined with high resolution melt analysis (MLV-HRMA) have been developed for genotyping *B. licheniformis* (**Dhakal *et al*., 2013**). Nineteen genotypes could be identified using this methodology, many of which are correlated with previously defined by RAPD-PCR (**Ronimus *et al.*, 2003**).

In this study, we evaluated genotypic and phenotypic biodiversity of *B. licheniformis* strains isolated from Uruguayan commercial milk powder, with emphasis on the production of extracellular enzymes. A better undestanding of the diversity will allow the validation of industrial process optimization to monitor and track *B. licheniformis* wild strains as an aid to minimize quality and safety problems for food processors.

MATERIAL AND METHODS

Bacterial isolates and culture conditions

Three hundred and seventy nine bacteria identified as *B. licheniformis* isolated from commercial milk powder manufactured in Uruguay belonging to our laboratory collection were used in this study. Isolates were prepared from frozen stocks after growth and transfer twice in fresh Triptic Soy Broth (TSB, Oxoid Ltd., UK) at 37 °C for 24 h prior to growth at different temperatures on Plate Count Agar (PCA, Oxoid Ltd., UK). A 100-fold dilution of each overnight culture was plated in duplicate on PCA and incubated at 37 °C or 55 °C for 24-48 h.

Isolation of total DNA

Bacterial cell cultures were grown overnight in Tryptic Soy Broth (TSB, Oxoid Ltd., UK) and cells were harvested at 10,000 rpm for 5 min in a Spectrafuge 7M tabletop centrifuge (Labnet International Inc., USA). Cell pellets were suspended in 200 µL TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0). DNA was purified using a Genomic DNA purification kit (Fermentas International Inc., USA) following the manufacturer's instructions. Purified DNA was suspended in 40 µL TE buffer and used as template in amplification reactions. DNA concentration was determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific Incorporation, Wilmington, DE, USA).

RAPD-PCR analysis

Random amplification of polymorphic DNA (RAPD) analysis was carried out in 25 µL reaction mixtures containing 1× Thermo buffer (Fermentas, USA), 2.5 mM $MgCl_2$, 200 µM of each dNTP (Fermentas, USA), 1U Taq polymerase (Fermentas, USA), 1 µM primer OPR13 (5′-GGACGACAAG-3′) and 20 ng template DNA. PCR amplifications were done in a Corbett CG1-96 thermal cycler with a palmtop computer interface (Corbett Research Ltd., Cambridge, UK). PCR cycling parameters included a denaturation step at 94 °C for 3 min and 45 s; 35 cycles each consisting of 94 °C for 15 s, 36 °C for 15 s and 72 °C for 2 min; and a final extension step at 72 °C for 4 min. (**Ronimus *et al*., 1997**). Control reaction mixtures lacking template DNA were included with each analysis. RAPD-PCR reactions were electrophoresed on 1.8 % agarose-gels using 0.5× TBE buffer (45 mM Tris, 45 mM Boric acid, 1 mM EDTA, pH=8.0) as running buffer at 10 V/cm for 1 h, stained with GoodView Nucleic Acid Stain 5%(v/v) (SBS Genetech Co. Ltd, China) and visualized and photographed on a UV transilluminator.

Isolate identification by 16S rDNA sequence analysis

Several isolates having distinct OPR13 RAPD profiles were identified by 16S rDNA sequence analysis. Twenty-five microliter reaction mixtures contained 1× Thermo buffer (Fermentas, USA), 2.5 mM $MgCl_2$, 200 µM of each dNTP (Fermentas, USA), 1U Taq polymerase (Fermentas, USA), 0.2 mM of each PCR primer (fD1 and rD1) and 20 ng template DNA. Primers fD1 5′-AGAGTTTGATCCTGGCTCAG-3′ and rD1 5′-AAGGAGGTGATCCAGCC-3′ were used to amplify a 1540 bp genome fragment with 16S rRNA gene sequences (**Weisburg *et al.*, 1991**). PCRs were performed using an initial denaturation step at 94 °C for 7 min, then 35 cycles of 94 °C for 1 min, 56 °C for 1 min and 72 °C for 1 min and a final extension at 72 °C for 10 min, and experiments included negative controls with no added DNA template. Amplified fragments were purified and sequenced by Macrogen Sequencing Service, Korea, using an ABI PRISM 3730XL capillary sequencer (Applied Biosystems, CA, USA). DNA sequences were compared with those of the NCBI BLAST database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify type strains with highest similarity, and were aligned against each other using Clustal W package (**Thompson *et al.*, 1994**). Phylogenetic trees were constructed using neighbourjoining method (**Saitou and Nei, 1987)** by MEGA4 software (**Tamura et al., 2007)**.

Enzymatic activity

From a total of 379 isolates of *B. licheniformis* genotypically characterized, a total of 32 isolates, respecting the representation of each genotype, were randomly selected for extracellular enzymatic activity. The proteolytic activity was tested by culturing the strains on 50% skim milk agar and incubating the plates at 37°C and 55ºC for 24 – 48 h. Presence of clear zone of hydrolysis around the colonies was taken as positive for proteolysis. The lipolytic activity was tested by culturing the strains in Spirit Blue agar supplemented with 30% of lipase reagent (Difco, Becton Dickinson and Company, Nevada, USA) and incubating the plates at 37°C for 3 days and screening the plates for the presence of clear zone of hydrolysis as described by **Starr and Burkholder (1942)**. The amylolytic activity was tested by culturing the strains on PCA plates supplemented with 1% soluble starch (w/v, Difco) and incubating the plates at 37°C for 24 – 48 h. Amylase activity was detected by flooding the plates with Gram's iodine solution (0.203 g of I_2 and 5.2g of KI in 100 ml of aqueous solution), active isolates were detected as bright clear haloes around the colonies.

Preparation of crude enzyme

All proteolytic isolates were cultivated in 250 ml nutrient broth with vigorous shaking (150 rpm) at 37 °C for 30 h. The culture was then centrifuged at 15,000 rpm for 30 min at 4 ºC. The cell free culture supernatant was filtered using 0.22 µm membranes (Millipore, Bedford, MA, USA) and maintained at -40 ºC for subsequent experiments.

Quantification of proteolytic activity

Determination of the enzyme activity was performed using the azocasein method (**Andrews and Asenjo, 1986**). 250 µl of the crude enzymes were incubated at 37 °C in 500 µl mixture containing 1% azocasein and 0.5 M tris-(hydroxymethyl)-aminomethane (TRIS) buffer pH 7.5 for 1 h. To terminate the reaction, 0.5 ml of 1.5M trichloroacetic acid (TCA) was added. All samples were allowed to stand for 15 min and the supernatant was collected after centrifugation (10,000 rpm; 15min). Protelytic activity was determined by measuring the absorbance at 340 nm. One unit of enzyme activity (U) was defined as the amount of cell-free supernatant required to increase one unit of absorbance at 340 nm in the assay conditions.

Effect of temperature on proteolytic activity and influence of protease inhibitors

The effect of temperature on enzyme activity was determined by carrying out the enzyme assay in a temperature range between 30-70 °C at pH 7.5. Enzyme activity at each temperature was measured as described above. The effects of active site inhibitors on protease activity were studied using phenylmethylsulfonylfluoride (PMSF), iodoacetimidate and ethylene diamine tetra acetic acid (EDTA). To determine whether the activity of proteases could be affected, each inhibitor was added to the crude enzymes and incubated at 35 ºC for 20 min. Protease activity was then measured using the azocasein method.

RESULTS AND DISCUSSION

RAPD genotyping of *Bacillus licheniformis* strains.

Three hundred and seventy nine *B. licheniformis* isolates were characterized genotypically. RAPD analysis of isolates yielded three different RAPD profiles, which include all isolates in this study (Fig. 1). All three profiles matched those described previously (**Ronimus *et al.*, 2003; Rüeckert *et al*., 2004; Reginensi *et al*., 2011**), corresponding to strain F (n = 375), and strain G (n = 4), although strain F could be divided into two groups (group 1, n =117 ; group 2, n = 258). A more detailed analysis of the RAPD patterns showed that almost all isolates identified as *B. licheniformis* belonged to these two groups. Both profiles corresponded to strain F, but differed from each other by the presence of an additional band of approximately 650 bp in group 2 (Fig. 1, Lanes 3, 6, 7, 9,10). A third group within this species had a different RAPD profile corresponding to strain G (Fig. 1, Lanes 12 to 14), but this group had a small contribution to *B. licheniformis* contamination in the powdered milk samples (4 out of 379 isolates). Distribution of *B. licheniformis* isolates agreed with other reports in which isolates of strain F (groups 1 and 2) were the dominant isolates in milk powder from New Zealand and Australia (**Ronimus *et al*., 2003, Dhakal *et al*., 2013**) as well as in milk powders manufactured in other countries (**Rüeckert *et al*., 2004**). The regular occurrence of *B. licheniformis* in powdered milk samples is likely due to the widespread distribution as a frequent contaminant in the dairy environment, like feed concentrate (**Scheldeman *et al.*, 2005; Vaerewijck *et al*., 2001**), feces (**Scheldeman *et al*., 2005**), soiling of the udder and teats (**Waes, 1976**) and raw milk (**Scheldeman *et al*., 2005**).

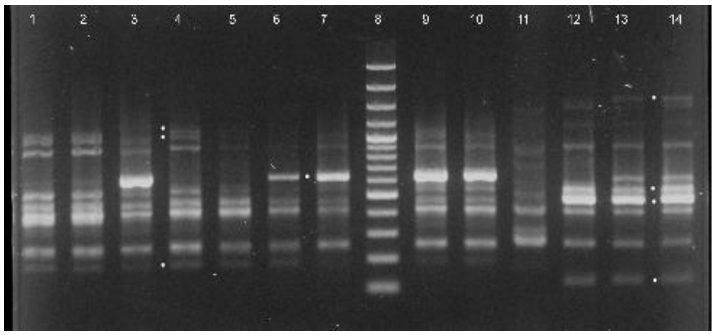

Figure 1 RAPD fingerprint profile of *B. licheniformis* isolates . Lanes 1, 2, 4, 5 and 11 *B. licheniformis* F isolates subgroup 1; lanes 3, 6, 7, 9,10, *B. licheniformis* F isolates subgroup 2 with distinctive band at 650 bp; lanes 12–14 *B. lichenifomis* G isolates; lane 8 DNA molecular mass standard ((Generuler 100bp

DNA Ladder Plus, Fermentas, bandas: 3000pb, 2000pb, 1500pb, 1200pb, 1000pb, 900pb, 800pb, 700pb, 600pb, 500pb, 400pb, 300pb, 200pb, 100pb). * Characteristics bands defined for each genotype are indicated.

Pairwise sequence similarity and phylogenetic relationships between isolates were inferred from comparisons of the 16S rRNA gene sequence. Clustering into two groups well defined was observed (Fig. 2). Group I contains all isolates tested belonging to genotype F, and Group II consists of three G genotype isolates. **Ronimus *et al.* (2003)** reference sequences of F (AY751766.1) and G (AY672764.1) isolates were also included and they appear clustered in group I and II, respectively. Our results corresponded well with previous findings of two different lineages within *B. licheniformis* that seem to have evolved differently (**De Clerck and De Vos, 2004, Madslien *et al*., 2012**). Several techniques were used to study the genotypic diversity among isolates of *B. licheniformis* from different sources. On the basis of different DNA fingerprinting methods two major groups were determined (**De Clerck and De Vos, 2004, De Jonghe *et al*., 2008, Banyko and Vyletelova, 2009**), as well as by *gyrA, rpoB* and *bac* sequence analysis (**De Clerck and De Vos, 2004, Ishihara *et al.*, 2002**). A recent analysis of diversity within the species *B. licheniformis* typing was performed by multilocus isolates sequences food contaminants. The evolutionary relationship inferred by the analysis of six "house keeping " genes, showed the presence of two groups or lineages (major one from the other) that appear to have evolved separately (**Madslien *et al.*, 2012**).

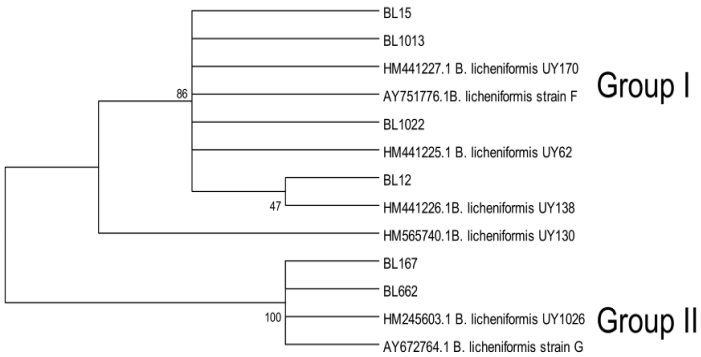

Figure 2 Phylogenetic tree obtained by Neighbor-joining analysis based on 16S rRNA gene sequences showing the phylogenetic position of isolates and type strains. Bootstrap values expressed as percentages of 1000 replications are shown at the branch points.

Extracellular enzymatic activity

A total of 32 isolates, respecting the representation of each genotype, were randomly selected for extracellular enzymatic activity plate assays. *Bacillus* species generally synthesise several extracellular enzymes, the maximum synthesis of which normally occurs in the late exponential and early stationary phases of growth, before sporulation (**Priest, 1977**). The results showed the ability of *B. licheniformis* isolates to produce exoenzymes with different hydrolytic activities (Table 1). Most isolates (25 out of 32) were screened as capable of degrading casein, starch and lipids. All the 32 tested isolates showed lipolytic activity, 26 isolates were able to hydrolyze starch, while the ability to hydrolyze casein was found in 30 and 31 isolates when incubation temperature were 37 and 55 °C, respectively (Table 1). None hydrolytic activity could be linked to a defined genetic group. Hydrolytic activities tested in this study were strain-dependent and proteolytic activity could eventualy (1 out of 31 isolates) be temperature dependent. This is an important fact to consider in the context of milk processing, and not all *B. licheniformis* strains represent the same risk to process or product quality.

Table 1 Qualitative assessment of extracellular enzymatic activity of *B. licheniformis* isolates.

Isolates	Proteolytic isolates		Amylolytic isolates	Lipolytic isolates
	37 ºC	55 ºC	37 ºC	37 ºC
Strain F group 1	14/14	14/14	9/14	14/14
Strain F group 2	13/14	14/14	14/14	14/14
Strain G	3/4	3/4	3/4	4/4

All *B. licheniformis* strains that exhibited a clear zone around their colonies on skim milk agar were grown on nutrient broth for 30 hours and tested for proteolytic activity by the azocasein assay. As well as other *Bacillus* species, production of protease by this *B. licheniformis* is dependent on cell growth, reaching the maximum yield at early stationary phase (**Olajuyigbe and Ajele, 2008)**. In the crude enzyme caseinolytic protease activity assay, no significant difference (P>0.05) among genotypic groups F and G was observed, but different levels of proteolytic activity were expressed by particular bacterial isolates (Fig. 3). BL1026 and BL27 showed highest proteolytic activity amongst all *B. licheniformis* isolates tested (Fig. 3). Consequently, these isolates were selected for further studies.

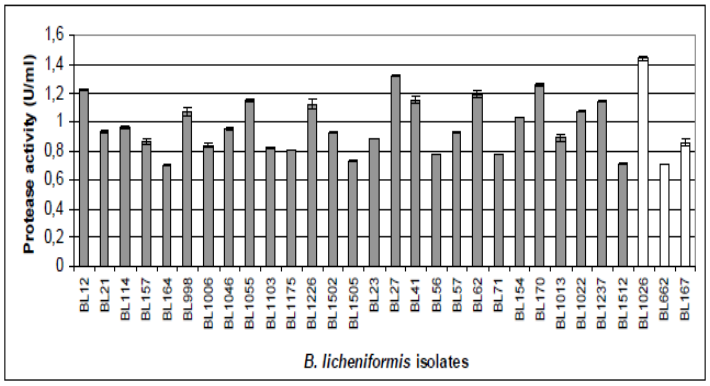

Figure 3 Proteolytic activity of *B. licheniformis* strain F (■) and strain G (□) isolates performed using azocasein method (**Andrews and Asenjo**, 1986). Experiments were performed in triplicate, and error bars indicate ±SD.

Effect of temperature and inhibitors on protease activity

Proteases produced by mesophilic and thermophilic bacteria have been reported to be heat stable. The effect of temperature on protease activity was determined for isolates BL1026 and BL27 at temperatures between 30 and 70 °C. Temperature profiles on enzyme activity are shown in Figure 4. Optimum temperature for the enzyme was found to be 60 ºC for both isolates. Similar results were reported by other researchers where optimum temperature of 60 ºC was recorded for proteases from other *B. licheniformis* strains (**Öztürk *et al.*, 2009; Bezawada *et al.*, 2011; El Hadj-Ali *et al*., 2007**).

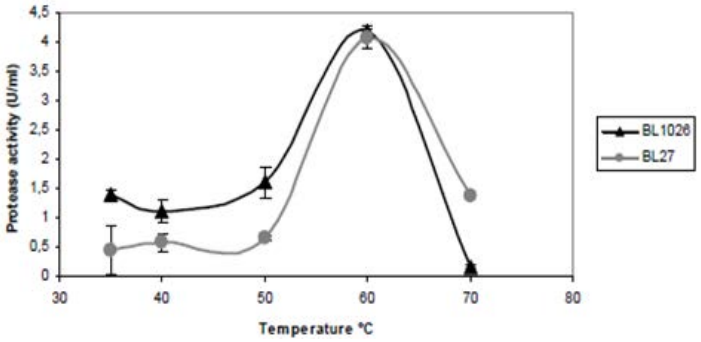

Figure 4 Effect of temperature on protease activity produced from *B. licheniformis* strains BL1026 and BL27. Experiments were performed in triplicate, and error bars indicate ±SD.

Inhibition studies primarily give an insight of the nature of an enzyme. The effect of a variety of enzyme inhibitors, such as chelating agent and group-specific reagent, on the activity was investigated (Table 2). The enzymes were strongly inhibited by the serine protease inhibitor (PMSF), indicating that both isolates produced serine proteases. Furthermore, the enzymes were equally affected when preincubated with EDTA, a metalloprotease inhibitor. Partial inhibition of many serine porteases by chelating agents has been reported (**El Hadj-Ali *et al*., 2007, Fakhfakh *et al*., 2009**) indicating the probable interaction of ions on the stabilization or activation of conformational structure of enzymes. These data indicate that the preparations contains a metal-activated enzyme with a serine residue at the active site.

Table 2 Effect of inhibitors on activity of protease crude enzyme extract obtained from *B. licheniformis* strains BL1026 and BL27.

Strain	Relative activity in the presence of [a]			
	No inhibitors	1 mM PMSF	1 mM EDTA	0,1 mM Iodoacetamide
BL1026	100	25 ± 3,4	24 ± 6,9	98 ± 0,4
BL27	100	19 ± 3,7	17 ± 1,7	99 ± 6,0

[a]The relative activity was calculated using the A340nm value of the control reaction (no inhibitors) as 100% enzyme activity. Experiments were performed in triplicate and SD is indicated.

Many commercial proteases (Protemex®, Neutrase®), mainly neutral or alkaline are produced by microorganisms belonging to the genus *Bacillus*. Most of these proteases have been cloned and characterized. These enzymes were characterized as serine proteases, cysteine proteases or metalloproteases. Only a protease of this genus was characterized as atypical aspartic protease described in *B. licheniformis* (**Carroll and Setlow, 2005**). Generally, *Bacillus* species have higher extracellular and intracellular proteolytic activity than other bacteria and they commonly produce subtilases, members of the superfamily of subtilisin-like serine proteinases (**Siezen and Leunissen, 1997**). These enzymes are active at neutral or alkaline pH. Many peptidases of this family are thermostable and the typical substrate for these enzymes is casein. Indications are that proteases produced by *B. licheniformis* isolates belong to this family but needed more comprehensive characterization.

CONCLUSION

These results confirm the diversity at genetic and phenotypic level of *B. licheniformis* isolated from milk powder. Up to now, it seems difficult to link a phenotypic characteristics to a defined group at genetic level. The study highligths the existence of groups with similar behaviors or characteritics but other strains with higher differences. The results of this work show that the exoenzymes production and its behaviour at different temperature tends to be isolate dependent. Further studies must be developed to determine the impact of enzyme activity and the number of microorganisms in the quality of dairy products.

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CHARACTERIZATION OF TYROSINASE ENZYME FROM NATIVE *BACILLUS MEGATERIUM* SP. STRAIN M36

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ABSTRACT

Tyrosinase is a type 3 copper-containing enzyme that catalyzes the conversion of l-tyrosine to L-DOPA and finally to melanin. In this study tyrosinase enzyme from native *Bacillus megaterium* sp. strain M36, was produces, characterized and used to produce L-DOPA. The M36 tyrosinase enzyme showed optimum monophenolase and diphenolase activity at pH 7.5 and conserved its maximum activity over than 95 % at pH ranging from 6.5 to 8.0. The M36 tyrosinase enzyme showed optimum monophenolase and diphenolase activity at 40 °C also, the enzyme conserved 100% of its original activity at 4-45 °C. The M36 tyrosinase enzyme was inhibited strongly by β-mercaptoethanol and about 90% by 5mmol of EDTA (a chelating agent). Although the enzyme was activated at the presence of 1mM SDS, it was strongly inhibited at high concentration of SDS (above 15mM). In TLC analysis, the transformation of L-tyrosine to L-DOPA was conspicuously detected.

Keywords: Melanin, monophenolase, diphenolase, TLC

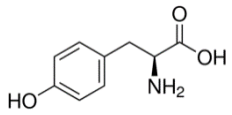
INTRODUCTION

Tyrosinase is a type 3 copper-containing enzyme that has been found widely distributed in microorganisms, plants and animals **(Claus and Decker, 2006).** Tyrosinase catalyses the hydroxylation of monophenol to o-diphenol (monophenolase or cresolase activity) and the oxidation of diphenol to o-quinones (diphenolase or catecholase activity). O-quinones are converted in to melanin by using nonenzymatic steps and molecular oxigene **(Decker and Tuczek, 2000). Howard *et al*., in 1948**, elucidated the biosynthetic pathway for melanin formation by tyrosinase enzyme. In mammals, tyrosinase catalyzes the biosynthesis of melanin pigments, which contributes to a fundamental part of the skin protection against UV radiation. It is also related to the browning reactions of fruit and vegetables **(Seo *et al.*, 2003)**. Tyrosinases have several biotechnological applications relying on the ability of the enzymes to oxidize both small phenolic molecules and protein-associated phenolic groups, i.e. the side chain of the amino acid tyrosine. Tyrosinase enzyme has very important role in bioremediation **(Marino *et al*., 201128)**, production of L-DOPA, the preferred drug for treatment of Parkinson's disease and other antioxidants (having crucial application in medical field) **(Xu *et al*., 2012)**, food industry **(Allouche *et al*., 2004)**, textile industry **(Franciscon *et al*., 2012)** and production of melanin **(Kumar *et al.*, 2011)**. Recently, because of increasing application of the tyrosinase enzyme, the interest in the isolation of new tyrosinase enzyme has been increased. Up to present, several tyrosinase enzyme from microbial strains such as *Bacillus thuringiensis* **(El-Shora and Metwally, 2008)**, *Pseudomonas putida* F6 **(McMahon *et al.*, 2007)**, *Ralstonia solanacearum* **(Hernandez-Romero, 2005)**, *Rhizobium etli* **(Pinero *et al*., 2007)**, *Streptomyces antibioticus* **(Marino *et al*., 2010)**, *Thermomicrobium roseum* **(Kong *et al*., 2000)**, *Streptomyces* sp. REN-21**(Ito and Inouye, 2005)**, *Verrucomicrobium spinosum* **(Fairhead and Thony-Meyer, 2010)** have been isolated and characterized. Most of the strains have multicatalytic functions such as peroxidase and laccases in addition to tyrosinase activity, these characteristics make more restrictions for the strains to be used in industrial and pharmaceutical applications **(Dastager *et al*., 2006)**, any way some strains which produce only tyrosinase enzyme has been isolated from soil samples **(Freddi *et al*., 2006)**. These strains are appropriate for industrial applications.

The commercial production of tyrosinase enzyme is mostly reported from the common mushroom *Agaricus bisporus*. Extensive research regarding this enzyme has been carried out using this mushroom tyrosinase. The mushroom's tyrosinase enzyme exhibits relatively low pH and temperature stability and its purification is relatively hard, as compared to bacterial tyrosinases **(Seo *et al*., 2003).** To date, this is the first time that isolation and characterization of a native tyrosinase enzyme from *Bacillus megaterium* strain was carried out.

MATERIAL AND METHODS

In this research all material for making medium were bought from sigma and merck. Also the substrate (l-tyrosine) was bought from sigma. According to its information wrote in sigma, L-tyrosine has the following properties; form: fine crystals and fragments, colour: white, molecular weight: 181.19 g/mol, water solubility: 0.479 g/l at 25 °c, formula: C9H11NO3



Production and partial purification of the m36 tyrosinase enzyme

Culture condition for tyrosinase enzyme production by the *Bacillus megaterium* sp. strain M36had been optimized previously and it was as follow: temperature (36 °C), pH (7.0), incubation time (16 hour), agitation (170rpm) , l-tyrosine (0.4mg/ml), yeast extract (0.05%), tryptone (0.423%), NaCl (3.4%) and CuSo4 (148.4µM). The native *Bacillus* sp.M36 was cultured at optimized culture condition and in order to enzyme extraction, to start with, the cell free extract was prepared then the extract was subjected to ammonium sulfate precipitation and dialysis.

For cell free extract preparation, the medium culture was centrifuged at 6000g for 10 min at 4°C when OD_{530} of medium culture was 1.3, Then the obtained supernatant was stored at +4°C and the pellets were washed twice in ice-cold 50mM potassium phosphate buffer, pH 7.0. After that the pellets were resuspended in 0.1M sodium phosphate pH 7.0 containing an inhibitory bacterial proteases cocktail (1: 4, µl: mg cell mass) and disrupted by sonication. The homogenate was centrifuged at 14000g for 15min. The supernatant achieved both by the previous centrifuge at 6000g and by centrifuge at 14000g were used as a cell free extract **(Lopez-Serrano *et al*., 2002; McMahon *et al*., 2007; Michalik *et al*., 1976)**. The cell free extract was subjected to precipitation with ammonium sulfate (40, 50, 60, 70, 75, 80, 85 and 90% saturation) for 1 hour with gentle stirring. After fractionation with ammonium sulfate, the precipitated proteins are

recovered by centrifugation at 12000g for 30 minute and are dialyzed against 50mM sodium phosphate buffer, pH 6.8 with 0.02% sodium azide, 0.01mM $CuSO_4$. The fractions were tested to tyrosinase activity and active fractions were stored at –20 ° C without loss of activity **(El-Shora and Metwally, 2008)**. Protein contents of the samples were determined by Bradford method using bovine serum albumin (BSA) as the standard **(Kohashi *et al*., 2004).**

Enzyme assay

Tyrosinase activity is assayed by using L-tyrosine and L-DOPA as substrates. The appropriate concentration of the enzyme was determined before the enzyme activity was assayed and an aliquot of the enzyme solution is added to a 0.1M sodium phosphate buffer (pH 6.8) containing 1mM L-tyrosine and L-DOPA , and the formation of dopachrome is monitored by measuring the absorbance at 475 nm **(Rao *et al*., 2013)**. The initial rate is used for the calculation of tyrosinase activity. One international unit (IU) of tyrosinase activity is defined as the amount of enzyme required to oxidize 1µmol of L-tyrosine to dopachrom per minute under the above conditions, which was calculated using the molar extinction coefficient of dopachrome ($3600M^{-1}\ cm^{-1}$) by the following equation:

$$\mathrm{IU/ml} \sim \mu\mathrm{mol/\ min\ /ml}) = \frac{\mathrm{absorption/\ min} \cdot \mathrm{assay\ volume\ (ml)} \cdot \mathrm{dilution\ factor} \cdot 10\,000}{\varepsilon_{\mathrm{nm}}(\mathrm{l} \cdot \mathrm{mol}^{-1}\ \mathrm{cm}^{-1}) \cdot 1\ \mathrm{cm} \cdot \mathrm{enzyme\ volume\ (ml)}}$$

Effect of pH and temperature on enzyme activity and stability

For this purpose, 200µl of enzyme solution (protein content, 0.05 mg/ml) was added to 1800µl buffer containing 1mM of L-dopa for diphenolase and 1mm of l-tyrosinae for monophenolase activity and incubated for 45min. The effect of pH on monophenolase activity was investigated by analyzing the activity at different pH values (pH 4, 5, 6, 7, 8, 9, 10, 11 and 12) and for diphenolase activity pH (4-7.5) were tested because L-DOPA spontaneously converted to dopachrome at pH values above 7.5. pH value in which the enzyme showed maximum relative activity was determined as optimum pH for the enzyme activity **(Burhan *et al*., 2003; McMahon *et al*., 2007)**.
Also, the enzyme activity was analyzed at a range of temperatures from 10 to 70°C (10, 20, 30, 40, 50, 60 and 70) and the temperature showing maximum relative activity was determined as an optimum temperature for the enzyme activity. In order to ascertain of the temperature stability, the enzyme solutions in different tubes are incubated at various temperatures in the range from 0°C to 70°C for 2 hour then residual activity is assayed in enzyme assay condition **(Liu *et al*., 2004)**.

Effect of detergents on enzyme activity

To examine the effects of sodium dodecyl sulphate (SDS), ethylene diamine tetraacetic acid (EDTA), Urea, Tween-80, TritonX-100, β-Mercaptoethanol and PMSF are analyzed by incubating enzyme in the presence of these detergents and substrate **(Aygan *et al*., 2009; Caf *et al*., 2012)**.

Kinetic study of M36 tyrosinase enzyme

The initial rate of enzyme reaction for l-tyrosine and L-DOPA was determined at various concentrations. The resulting data was analyzed and the K_m and V_{max} values are calculated by Michaelis–Menten and Hill equation $v_i = \frac{V_{max}[S]}{k_m+[s]}$ and Lineweaver-burk equation $\frac{1}{v_i} = \frac{K_m}{V_{max}}\frac{1}{[S]} + \frac{1}{V_{max}}$ **(Mc Mahon *et al*., 2007; 45 Zanjani *et al*., 2009)**. After addition of 200µl of enzyme solution (protein content, 0.05mg/ml) to potassium phosphat (50mM, pH, 7) containing various concentrations ranging from 0.02 to 0.8 for tyrosine and 0.06 to 2.0 for L-DOPA, the reaction medium with L-DOPA and with L-tyrosine was incubated at room temperature for 30min and 45 min, respectively. After that the reaction medium with L-tyrosine was diluted 5 times and reaction medium with L-DOPA was diluted 10 time and both of them was subjected to study of OD_{475} by spectrophotometer. The obtained data was used to calculation of velocity.

Electrophoretic study

The enzyme solution was loaded in several well of Non-denaturing PAGE (8% w/v) and after separating protein bands, a single lane of the gel was sliced out of the gel using a clean scalpel. The tyrosinase enzyme related band was stained by placing the gel slice in substrate solution (l-tyrosine (0.1mg/ml) and $CuSO_4$ (50µM) in phosphate buffer (0.1M, pH 7)) for 60 min. The formation of a dark-brown band indicated the position of the tyrosinase enzyme. The remaining lanes of the gel were placed in 50mM phosphate buffer, pH 7.0. Using the activity stained lane as a guide to the location of tyrosinase, the corresponding band was sliced out of the unstained lanes. The gel slice was homogenized and resuspended in a 50mM phosphate buffer and left overnight at 4 ◦C. The gel suspension was centrifuged at 12000 g for 10 min to remove remaining gel fragments and the obtained supernatant was subjected to SDS-PAGE (12%) analysis for determination of the tyrosinase enzyme molecular weight **(Arikan, 2008)**.

Thin layer chromatography analysis of the reaction mixture

The conversion of L-tyrosine to L-DOPA by M36 tyrosinase enzyme was analyzed by thin layer chromatography. For this purpose, phenol-water system (75:25) (w/v) was used as a mobile phase and 3% ninhydrin in n-butanol as spray and staining reagent. Besides of TLC analysis, **(Rani *et al*., 2007; Raval *et al*., 2012)**.

Statistical analysis

All experiments were conducted in three replicates; data generated were subjected to statistical analysis using Microsoft Excel and presented as mean_SE.

RESULT AND DISCUSSION

Preparation of the M36 tyrosinase enzyme

The enzyme was precipitated by ammonium Sulfate 85% and centrifugation at 13000g and dialyzed against 50mM sodium phosphate buffer (pH 6.8 containing 0.02% sodium azide and 0.01mM $CuSO_4$).

Effect of pH and temperature on enzyme activity and stability

The result of this research showed that the M36 tyrosinase enzyme had maximum monophenolase and diphenolase activity at pH, 7.5 (Figure 1). This result was in accordance with tyrosinase enzyme originated from *Streptomyces sp*. REN-21 (pH 7.0) **(Ito and Inouye, 2005)**, *Rhizobium etli* CFN42 (pH 7.5) **(Pinero *et al*. 2007)** and *Pseudomonas putida* F6 (pH 7.0) **(McMahon *et al*., 2007)** notwithstanding, the tyrosinase enzyme from *B. thuringiensis* **(Liu *et al*., 2004)** and *T. roseum* **(Kong *et al*., 2000)** have shown to have maximum activity at 9.0 and 9.5, respectively. The M36 tyrosinase enzyme could conserve its maximum activity over than 95 % at pH (6.5-8.0). Before pH (6.5) and above pH (8.0) the activity and stability of the enzyme was dropped. These findings are similar to the finding of Shuster and Fishman **(2009)**.
The tyrosinase enzymes have two cupper in its active site and each of the two metal atoms; Cu_A and Cu_B, of the active site are coordinated by three conserved histidines which are located in a 'four α-helix bundle' **(Claus and Decker, 2006)**. The α-helix is structured by hydrogen bonds. Generally changing of pH value (extremely basic or acidic) causes changes in the charge of H-bond donor and acceptor groups, it can rearrange the H-bonds and change the conformation/folding of the protein.

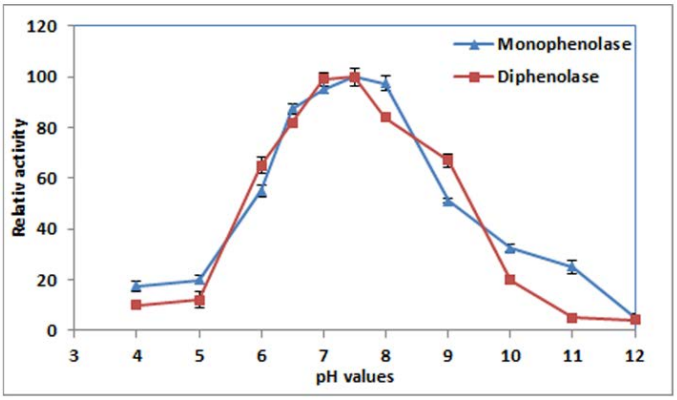


Figure 1 Effect of pH on activity and stability of the *Bacillus megaterium* M36 tyrosinase enzyme (monophenolase). The enzyme showed maximum activity (0.52 IU) at pH=7.5 and 97.5% of its maximum activity at pH=8.0. At pH lower than 6.5 and higher than 8.0 the activity of the enzyme was steeply decreased.

The M36 tyrosinase enzyme showed optimum monophenolase and diphenolase activity at 40 °C also, the enzyme conserved 100% of its original activity at 4-45 °C (Figure 2). The monophenolase and diphenolase activity of the enzyme was deeply decreased at temperature below 30 °C and above 55 °C, probably this result was related to that, the tyrosinase enzyme has mostly composed from α-helix, on the other hand α-helix is more flexible than the others structures. This result was more or less closed to other investigations.
The M36 tyrosinase enzyme showed up to 95% activity at temperature ranges from 35 °C-45°C, in contrast to this, the activity of tyrosinase enzyme from *P. putida* F6 **(McMahon *et al*., 2007)** has been decreased dramatically at temperature above 30°C and the enzyme of *Streptomyces michiganensis* DSM **(Philipp *et al*., 1991)** has showed optimum activity at 33°C. Moreover there is some reported tyrosinase enzymes whit higher optimum temperature. Also the

M36 tyrosinase enzyme was different with the tyrosinase from *Rhizobium etli* CFN42 (50°C) **(Pinero *et al*., 2007)**, *Bacillus (HR03)* (55°C) **(Dalfard *et al*., 2006)**, *Bacillus thuringiensis* (75°C) **(El-Shora, Metwally, 2008)** and *Thermomicrobium roseum* (70°C) **(Kong *et al*., 2000)**. The M36 tyrosinase enzyme conserved its original activity at 45°C, contrary to this *Trichoderma reesei* **(Cura *et al*., 2010)** tyrosinase started to lose its activity relatively quickly at temperature above 30°C.

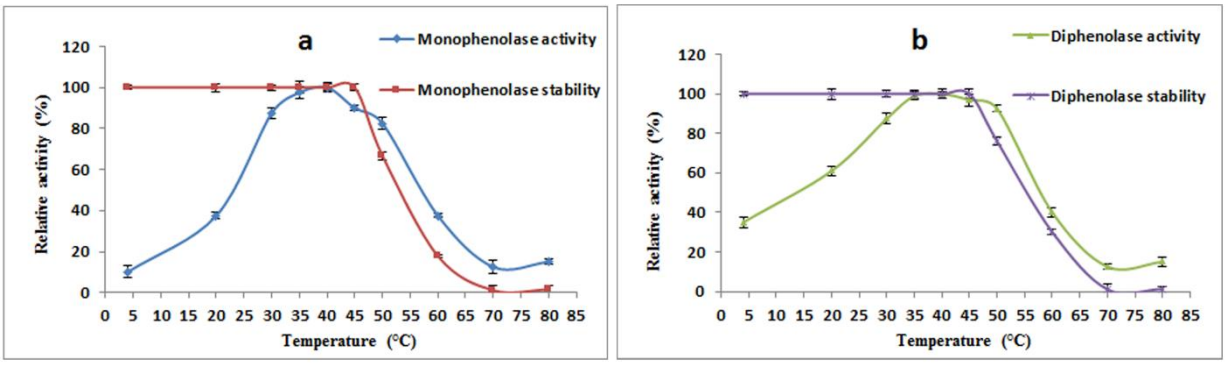


Figure 2 Effect of temperature on a) monophenolase (L-tyrosine as a substrate) and b) diphenolase activity (L-DOPA as a substrate) of the *Bacillus megaterium* M36 tyrosinase enzyme. The enzyme showed maximum monophenolase (0.56IU) and maximum diphenolase activity (0.62IU) at 40°C. Both of the monophenolase and diphenolase activity were conserved 100% at temperature 0-45°C, after that the enzyme loosed its activity.

Effect of detergents on enzyme activity

The M36 tyrosinase enzyme was studied in presence of various inhibitors (Figure 3a). The enzyme was inhibited strongly by β-mercaptoethanol. β-mercaptoethanol is a reducing agent which inhibit dopachrom and melanin synthesis by reducing qinones (an intermediate) to L-DOPA. Similar results were obtained for *Bacillus megaterium* tyrosinase **(Shuster and Fishman, 2009)**, and *Thermomicrobium roseum* tyrosinase **(Kong *et al*., 2000)** that was completely inhibited by β-mercaptoethanol (1mmol). The M36 tyrosinase was inhibited about 90% by 5mM EDTA (a chelating agent). The agent can inhibit the enzyme by chelating of Cu from its active site. Similarly *Bacills megaterium* tyrosinase was inhibited up to 27% by 1mM EDTA **(Shuster and Fishman, 2009)** and *Bacillus (HR03*) tyrosinase enzyme was partially inhibited by 1mM EDTA **(Dalfard *et al*., 2006)**. In contrast to the result of this research tyrosinase enzyme from *Bacillus thuringiensis* **(El-Shora and Metwally, 2008)** was activated at high concentration EDTA from 200 to 400mM. Effect of different concentration of SDS (0.2- 30mM) on the M36 tyrosinase enzyme was studied. Although the enzyme was activated at the presence of 1mM SDS, it was strongly inhibited at high concentration of (above 15mM) SDS (Figure 5b). Previously, activating effect of SDS on tyrosinase enzyme from *Xenopus laevis* **(Wittenberg and Triplett, 1985)**, *A. bisporus* **(Espin and Wichers, 1999)**, *Bacillus sp.* **(Dalfard *et al*., 2006)** and *Bacillus megaterium* **(Shuster and Fishman, 2009)** has been reported which was in agreement with our result. According to the paper published by Gandia-Herrero, although, active site of enzyme is not affected by SDS; a stepwise conformational change affected the enzyme activity by increasing accessibility of its active site to the substrate **(Gandía-Herrero *et al*., 2005)**.

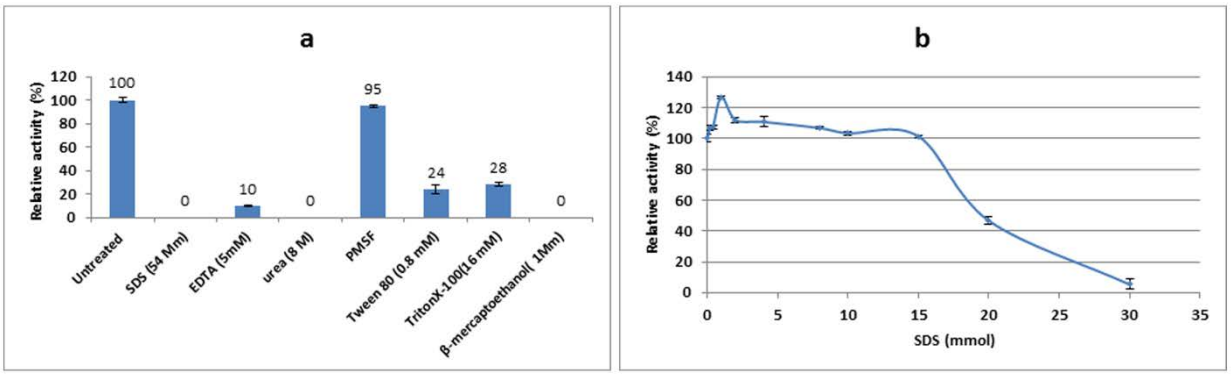


Figure 3 a) Effect of inhibitors on *Bacillus megaterium* M36 tyrosinase activity. The activity of the sample containing the enzyme without any of the additives was considered as control (100%). **b)** Effect of SDS concentration (w/v) on the *Bacillus megaterium* M36 tyrosinase activity. The enzyme showed maximum activity (126.6%) at the presence of 1mM of SDS and its activity was gradually decreased at the SDS concentration more than 1mM, so that it reached to 5.3% at concentration of 30mM. Sample having no SDS in reaction mixture was considered as a control (100%)

Production of L-DOPA from L-tyrosine

In TLC analysis, the transformation of L-tyrosine to L-DOPA was conspicuously detected. Ascorbic acid, used to prevent further oxidation of L-DOPA, did not give interfering spots (Figure 4).

Kinetic study of M36 tyrosinase enzyme

The M36 tyrosinase enzyme was shown to obey Michaelis-Menten kinetics when L-tyrosine and L-DOPA was used as a substrate. The K_m value of M36 tyrosinase for l-tyrosine (0.15mM) was lower than L-DOPA (0.58mM). The obtained V_m was 1.7μM.min^{-1}.ml^{-1}for l-tyrosine and 6.2μM.min^{-1}.ml^{-1} for L-DOPA. K_m value of M36 tyrosinase enzyme was similar to the previously reported K_m values with the l-tyrosine, for example ; 0.2mM for *Agaricus bisporus* **(Selinheimo *et al.* 2009)** and 0.19mM for *Rhizobium etli* CFN42 **(Cabrera-Valladares *et al*., 2006)**, also it is higher than the value (0.075mM) reported for *Bacillus megaterium* **(Shuster and Fishman, 2009)** and it is less than the values 0.563mM, 1mM, 0.421mm reported for *Bacillus huringiensis* **(El-Shora and Metwally, 2008)** , *Streptomyces* sp. REN-21**(Ito and Inouye, 2005)** and *Verrucomicrobium spinosum* **(Fairhead and Thony-Meyer, 2010)**, respectively. The K_m value of M36 tyrosinase enzyme for L-DOPA was higher than K_m value of tyrosinase from *Agaricus bisporus* (0.17mM) **(Selinheimo *et al*., 2009)**, *P. putida* F6 (0.33) **(McMahon *et al.*, 2007)** and *Bacillus megaterium* (0.35mM) **(Shuster and Fishman, 2009)** for the same substrate, but it was lower than K_m values of tyrosinase from *Trichoderma reesei* (7.5mM) (Selinheimo *et al*., 2009), *Rhizobium etli* CFN42 (2.44mM) **(Cabrera-Valladares *et al*., 2006)**, *Streptomyces antibioticus* (8.9mM) **(Marino *et al*., 2011)**, *Streptomyces castaneoglobisporus* (8mM) **(Kohashi *et al.*, 2004)** and *Verrucomicrobium spinosum* (7mM) **(Fairhead and Thony-Meyer, 2010)**.

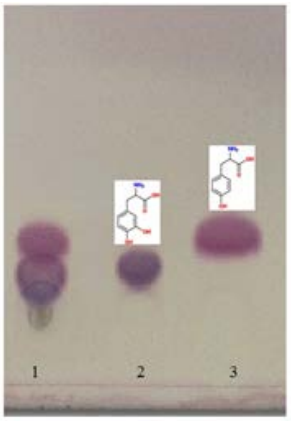


Figure 4 TLC analysis of L-tyrosine conversion to L-DOPA by *Bacillus megaterium* M36 tyrosinase enzyme

Electrophoresis and enzymatic activities in gel

After dialysis, tyrosinase M36 was electrophoresed by using native polyacrylamide gel (8%), after specific staining, a distinct band was detected. by extracting of the tyrosinase enzyme from native gel using the method mentioned in material methods, and the enzyme was subjected to SDS-PAGE (12%) analysis. This analysis showed almost 34kDa bond of the enzyme (Figure 5). This result was similar to the result of Shuster and Fishman **(2009)** who have demonstrated the tyrosinase from *Bacillus megaterium* to be almost 35kDa.

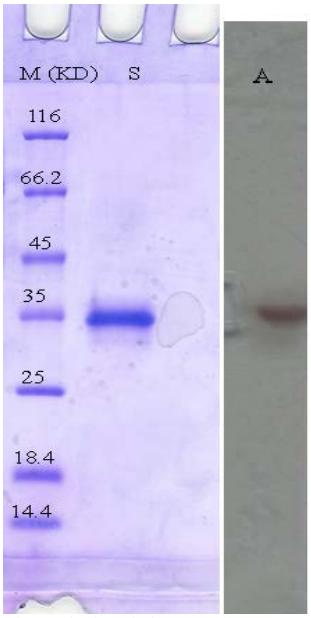


Figure 5 Electrophoresis analysis of the *Bacillus megaterium* M36 tyrosinase Enzyme. Lane (A) shows tyrosinase activity, lane (T) shows the enzyme molecular weight almost 34KDa, almost 15µg of protein was loaded, lane (M) shows protein marker.

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CHARACTERIZATION OF DIARRHEAGENIC *ESCHERICHIA COLI* ISOLATED FROM RAW BEEF, MUTTON, AND INTESTINES SOLD IN OUAGADOUGOU, BURKINA FASO

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ABSTRACT

Diarrheagenic Escherichia coli are zoonotic bacteria commonly present in animal gut. The aim of our study was to determine the prevalence of diarrheagenic *E. coli* isolated from raw meats and intestines in open markets of Ouagadougou, Burkina Faso. A total of 450 samples were collected from beef, mutton, beef intestine and sheep intestine, in respective number 175, 175, 50 and 50. Diarrheagenic *E. coli* were isolated by using standard microbiological methods and then Multiplex Polymerase Chain Reaction was used for characterization. Among the pathotypes, enteropathogenic *E. coli* was identified by serotypage (slide agglutination). A 30% (135/450) were *E. coli*. 30% (40/135) of *E. coli* strains provided the virulence genes. 14% of Shiga toxin producing *Escherichia coli*, 13% of shiga toxin producing *Escherichia coli*-enterotoxinogenic *Escherichia coli*, 1% of enteroaggregative *Escherichia coli*, 2% of enteroinvasive *Escherichia coli* and 1% of enterotoxinogenic *Escherichia coli*. 41% (55/135) were enteropathogenic Escherichia coli belong to serotypes: (5%), O119 (3%), O127 (16%), O125 (9%), O126 (18%), O128 (5%), O114 (5%), O124 (5%), O142 (7%). This study show contamination of slaughter animal with diarrheagenic *E. coli* pathotypes in Burkina Faso. Precaution can take of mutation breeding level.

Keywords: Beef, Mutton, Raw intestines, Diarrheagenic *E. coli*, Burkina Faso

INTRODUCTION

Meat and meat products, second food outbreak are associated several cases of collective foodborne diseases worldwide (**EFSA, 2012; OMS, 2015**). Meat, being it a nutrient-rich substrate, can support the growth of a wide range of microorganisms, which also include *Escherichia coli*. The latter has received much attention as a potential public health threat due to the morbidity and mortality rates associated with outbreak and sporadic cases of human illness (**Paton and Paton, 1998**). However, people who have died from diarrheal diseases were estimated to 2 million cases (**OMS, 2015**), including a large proportion cases from the consumption of contaminated food. In Burkina Faso, many studies showed that diarrheal diseases are caused by *E. coli* (**Bonkoungou *et al.*, 2012; Timbiné *et al*., 2013; Dembélé *et al*., 2015**). Various diseases have been reported due to ingestion of food contaminated with pathogenic *Escherichia coli* (**Fadi *et al*., 2012; Kagambèga *et al*., 2012; Croxen *et al*., 2013**). There are several pathovars of *Escherichia coli* described as clinical generate indication. Pathogenic strains are divided into intraintestinal *E. coli* pathogens causing diarrhea and extraintestinal *E. coli* causing a variety of infection in both humans and animals (**Jafari *et al*., 2012**). Diarrheagenic *E. coli* possess virulence factors which are responsible for their pathogenicity. They include enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enterohaemorrhagic *E. coli* (EHEC), and diffusely adherent *E. coli* (DAEC) (**Nataro and Kaper, 1998; Igbeneghu and Lamikanra, 2014; Saeed *et al*., 2015**). Among them enteropathogenic *Escherichia coli* (EPEC) is a major cause of infantile diarrhea among children under five years in developing countries (**Jafari *et al*., 2012; Kotloff *et al*., 2013; Croxen *et al*., 2013; Dembélé *et al*., 2015**).

In Burkina Faso there are a few data concerning the prevalence of diarrheagenic *E. coli* in food stuffs. Therefore, the objective of our study was to determine diarrheagenic *E. coli* pathotypes from meat and intestines samples sold at some open markets in Ouagadougou and to identify the prevalent serogroups of EPEC.

MATERIALS AND METHODS

Study design and sampling

Sampling of raw meats and intestines of beef and sheep were carried out in twenty-five (25) open markets of Ouagadougou. Overall 450 samples: 175 samples of beef, 175 samples of mutton, 50 samples of beef intestine and 50 samples of sheep intestine were collected from each animal after slaughtering. Approximatively 400g were collected aseptically from October 2011 to October 2012. The samples were placed in sterile plastic bags and transported to the laboratory and kept at 4°C until microbial examination within 2h.

Isolation and identification of *E. coli*

Samples processing

The **ISO 4832, 1991** lightly modified were used for the isolation of *E. coli*. Twenty five (25) g of each sample was transferred in to 225 ml of buffered peptone water (Liofilchem, Italy) and was homogenized in a stomacher LAB BLENDER 400 (Sewar, England). The suspension of meat was incubated at 37°C.

Isolation of *E. coli*

After 24 h of incubation two (2) loopful of pre-enrichment broth were streaked onto Eosin Methylene Blue (EMB) agar (Liofilchem, Italy) and Violet Red Bile Lactose (VRBL) agar (Liofilchem, Italy). The plates were incubated at 44 °C for 18-24 hours. Suspect *E. coli* colonies were appeared metallic green on EMB agar and as small colonies purple with a purple cloud on VRBL agar.

Identification of *E. coli*

The suspect colonies were selected and streaked onto Mueller Hinton agar (Liofilchem, Italy). Confirmation was carried out by biochemical microbiology method based on negative urease (Bio-Rad, French), negative citrate (Liofilchem, Italy), positive indole (Bio-Rad, France), positive lactose (Lioflchem, Italy) and positive orthonitrophenyl-β-D-galactopyranoside (ONPG) (BioMerieux, France). The *E. coli* strains isolated were confirmed by API 20E (BioMérieux, France) system and API 20E interpretation was done by API 20E catalogue.

Multiplex polymerase chain reaction (16 plex PCR)

The 16-plex PCR was used to detect simultaneously 16 genes of 5 main pathogroups of *E. coli* (STEC, STEC-ETEC, EAEC, EIEC and ETEC) as describe by **Antikainen *et al*., (2009)**. The genes investigated and primers used are described in (Tab 1). DNA extraction was performed using heating method (**Moyo *et al*., 2007**). A loopful of bacterial growth of Mueller Hinton agar plate was suspended in 1.5 ml of distilled sterile water. The mixture was boiled for 10 min and centrifuged for 10 min at 11337 rpm. The supernatant was collected and used in the PCR reactions. One (1) µl of supernatant was added to 19µl reaction mixture containing 1U of Taq DNA polymerase (AccuPower, korea), deoxyribonucleic triphosphate (250 mM), Tris HCl (pH 9,0) (10 mM), KCl (30 mM), $MgCl_2$ (1.5 mM), and PCR primers (*escV, bfpB, stx1, stx2, LT, STIa, STI, invE, a*stA, *aggR, pic, uidA*, *hly, , eaeA, ipaH ent*) (100 µM) (STEC, STEC-ETEC, EAEC, EIEC, ETEC). Thermocycling conditions were as follows: 30s at 98°C, followed by 35 amplification cycles of 98°C for 30s, 62.5°C for 60s and 72°C for 90s with a final extension of 72°C for 10 min on a thermal cycler (Perkins Helmer Cetus, USA). Following PCR, the reaction products were separated to electrophoresis in (2% weight/volume) agarose gel, stained with ethidium bromide solution (Prolabo, France) and visualized under UV light (Applex, France). Reference strains (FE 102301 (*stx2, eae, escV, ent* EHEC-*hly*), FE 95562 (*stx1,* EHEC-*hly, estla, astA, uidA*), IHE 56822 (*aggR, pic, astA, uidA*), RHE 6647 (*invE, ipaH, uidA*), FE 94725 (*elt, astA*) and IHE 50246 (*uidA*)) were used in each PCR run

Table 1 Oligonucleotides primers used for Multiplex PCR reaction

Pathotype	Target gene	Primer Sequence (5'to3')	PCR product size (bp)	Concentration (µM)	Reference
STEC	*Stx2*	MP3-stx2A F:GTTTTGACCATCTTCGTCTGATTATTGAG	324	0.4	Müller *et al*., 2007
		MP3-stx2A-R:AGCGTAAGGCTTCTGCTGTGAC		0.4	
	eaeA	eae-F:TCAATGCAGTTCCGTTATCAGTT	482	0.1	Müller *et al*., 2007
		eae-R:GTAAAGTCCGTTACCCCAACCTG		0.1	
	escV	MP3-escV-F:ATTCTGGCTCTCTTCTTCTTTATGGCTG	544	0.4	Müller *et al*., 2007
		MP3-escV-R:CGTCCCCTTTTACAAACTTCATCGC		0.4	
	ent	ent-F:TGGGCTAAAAGAAGACACACTG	629	0.4	Müller *et al*., 2007
		ent-R:CAAGCATCCTGATTATCTCACC		0.4	
	Stx1	MP4-stx1A-F:CGATGTTACGGTTTGTTACTGTGACAGC	244	0.2	Müller *et al*., 2007
		MP4-stx1A-R:AATGCCACGCTTCCCAGAATTG		0.2	
EAEC	*aggR*	MP2-aggR-F:ACGCAGAGTTGCCTGATAAAG	400	0.2	Müller *et al*., 2007
		MP2-aggR-R:AATACAGAATCGTCAGCATCAGC		0.2	
	pic	MP2-pic-F:AGCCGTTTCCGCAGAAGCC	1,111	0.2	Müller *et al*., 2007
		MP2-pic-R:AAATGTCAGTGAACCGACGATTGG		0.2	
EIEC	*invE*	MP2-invE-F:CGATAGATGGCGAGAAATTATATCCCG	766	0.2	Müller *et al*., 2007
		MP2-invE-R:CGATCAAGAATCCCTAACAGAAGAATCAC		0.2	
	ipaH	ipaH-F: GAAAACCCTCCTGGTCCATCAGG	437	0.1	Vidal *et al*., 2005
		ipaH-R:GCCGGTCAGCCACCCTCTGAGAGTAC		0.1	
ETEC	*elt*	MP2-LT-F:GAACAGGAGGTTTCTGCGTTAGGTG	655	0.1	Müller *et al*., 2007
		MP2-LT-R:CTTTCAATGGCTTTTTTTTGGGAGTC		0.1	
	astA	MP2-astA-F:TGCCATCAACACAGTATATCCG	102	0.4	Müller *et al*., 2007
		MP2-astA-R:ACGGCTTTGTAGTCCTTCCAT		0.4	
E.coli	*uidA*	MP2-uidA-F:ATGCCAGTCCAGCGTTTTTGC	1,487	0.2	Vidal *et al*., 2005
		MP2-uidA-R:AAAGTGTGGGTCAATAATCAGGAAGTG		0.2	

Legend. STEC = *E. coli* producing shiga toxine, ETEC = *E. coli* enterotoxinogenic, EAEC = *E. coli* enteroaggregative, EIEC = *E. coli* enteroinvasive, PCR=polymerase chain reaction, µM=micromolaire, pb=paire de base.

Serotyping

EPEC serogroups were identified by slide agglutination test using nonavalent, trivalent and monovalent antisera (Bio-Rad, France) according to the method described by **Neter *et al*., (1955)** lightly modified. The first test witness was carried out with physiological solution to check if the strain was not autoagglutinable. If not, trivalent I (O111 + O55 + O26), II (O86 + O119 + O127), III (O125 + O126 + O128) and trivalent IV (O114 + O124 + O142) antisera were used. Finally, monovalent antisera were used according to the manufacturers. Only strong agglutination occurring within 1 min was considered to be positive reaction.

RESULTS AND DISCUSSION

Total prevalence of *E. coli*

Foodborne diseases are of utmost concern for public health due to their direct impact on consumer. Our study showed that 30% (135/450) among 450 samples analyzed were isolated *E. coli* (tab 2). The highest prevalence was isolated from beef with 33% (57/175), followed by mutton 26% (46/175). The same prevalence 32% (16/50) of *E. coli* was isolated from beef and sheep intestines. This high prevalence is sometimes responsible of the non-conformity of meat processing environment such as reported by many authors (**Barro *et al*., 2007; Ilboudo *et al*., 2010; Kagambèga *et al*., 2012**).

Table 2 Prevalence of *E. coli* strains on meats and intestines samples

Samples (n=450)	Number of isolates
Beef (n=175)	57 (33 %)
Beef intestine (n=50)	16 (32 %)
Mutton (n=175)	46 (26 %)
Sheep intestine (n=50)	16 (32 %)
Total prevalence	135 (30 %)

Legend: n = number of sample, % = percentage.

Prevalence of diarrheagenic *E. coli* pathotypes

From 135 *E. coli* strains, 59% (80/135) were identified by 16 plex PCR among others 30% (40/135) *E. coli* strains provided the virulence genes. Five (5) *E. coli* pathotypes were identified: STEC 14% (18/135), STEC-ETEC 13% (17/135), EAEC 1% (1/135), EIEC 2% (3/135) and ETEC 1% (1/135) in the meat samples. The higher prevalence was observed in STEC and STEC-ETEC. The genes *uidA* were identified only in 30% (40/135) *E. coli* strains; however, they were considered like non diarrheagenic *E. coli* (tab 3).

Table 3 Prevalence of *E. coli* carrying different virulence genes

Virulence genes																
Pathotypes	*stx*$_1$	*stx*$_2$	*eae*	*escv*	*ent*	EHEC-*hly*	*bfpb*	*aggr*	*pic*	*inve*	*ipah*	*elt*	*estla*	*estb*	*asta*	*uida*
Beef (n=33)																
STEC	-	2	3	4	1	-	-	-	-	-	-	-	1	-	-	-
STEC-ETEC	7	-	1	-	-	1	-	-	-	-	-	-	6	-	6	7
EAEC	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-
EIEC										1	1	-	-	-	-	1
ETEC												1	-	-	1	1
Beef intestine (n=7)																
STEC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
STEC-ETEC	2	-	-	-	-	-	-	-	-	-	-	-	1	-	-	2
EAEC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
EIEC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ETEC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mutton (n=29)																
STEC		4	-	-	-	-	-	-	-	-	-	-	4	-	-	-
STEC-ETEC	5	-	-	-	-	1	-	-	-	-	-	-	-	-	4	5
EAEC	-	-	-	-	-	-	-	1	1	-	-	-	1	-	-	1
EIEC	-	-	-	-	-	1	2	-	-	2	1	-	1	-	-	2
ETEC	-	-	-	-	-	-	-	-	-	-	-	1	-	-	1	1
Sheep intestine (n=11)																
STEC	-	2	1	1	-	-	-	-	-	-	-	-	-	-	-	-
STEC-ETEC	2	-	-	-	-	-	-	-	-	-	-	1	-	-	1	2
EAEC	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-
EIEC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ETEC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Legend: STEC = *E. coli* producing shiga toxine, ETEC = *E. coli* enterotoxinogen, EAEC = *E. coli* enteroaggregative, EIEC = *E. coli* enteroinvasive, - = no prevalence, n = number of gene.

Otherwise, five (5) mainly pathotypes of *E. coli* were detected by 16 plex PCR. The *stx* gene is the most detected with a high prevalence of STEC. In fact, STEC is responsible for diseases in humans and animals whose clinical spectrum includes hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP) (**Nataro and Kaper, 1998**). Globally, the prevalence of STEC is higher in meat than other foods in Burkina Faso and other countries (**Kagambèga *et al*., 2012; Rahimi and Nayebpour, 2012; Isibor *et al*., 2013; Mori *et al*., 2014**). However, the low prevalence of ETEC, EAEC and EIEC pathotypes were recorded respectively in mutton and beef only. These results are similar to studies conducted in Senegal, Burkina Faso, Morocco and Iran (**Gassama-Sow *et al*., 2004; Kagambèga *et al*., 2012; Fadi *et al*., 2012; Jafari *et al*., 2012; Sarker *et al*., 2013**). The most important mode of transmission diarrheagenic *E. coli* is the contaminated meat. Person to person transmission is possible, but not frequent because there is the high infectious dose considered as a major cause of infantile diarrheas in developing countries.

Prevalence of enteropathogenic *E. coli* serogroups

41% (55/135) were identified EPEC. These EPEC belong to the 12 serogroups (O111, O26, O55, O86, O119, O127, O125, O126, O128, O114, O124, and O142) with different proportions (tab 4). In all the strain isolated, the 12 serogroups were observed in beef. The most representative EPEC was the serogroup-O126 18% and the less representative was EPEC-O119 4%. 44% (24/55), 16% (9/55), 31% (17/55), 9% (5/55) of beef, mutton, beef intestine and sheep intestine respectively were contaminated by EPEC.

Table 4 Prevalence of enteropathogenic *E. coli* in the beef, sheep and intestines raw meats

Samples	Trivalent I			Trivalent II			Trivalent III			Trivalent IV			Total EPEC
	O111	O26	O55	O86	O119	O127	O125	O126	O128	O114	O124	O142	
Beef	1(2%)	1(2%)	3(5%)	1(2%)	1(2%)	4(7%)	2(4%)	5(9%)	2(4%)	1(2%)	1(2%)	2(4%)	24(44%)
Beef intestine	1(2%)	-	-	-	-	-	1(2%)	2(4%)	-	2(4%)	2(4%)	1(2%)	9(16%)
Mutton	1(2%)	2(4%)	2(4%)	2(4%)	1(2%)	5(9%)	2(4%)	2(4%)	-	-	-	-	17(31%)
Sheep intestine	-	1(2%)	1(2%)	-	-	-	-	1(2%)	1(2%)	-	-	1(2%)	5(9%)
Total EPEC	3(5%)	4(7%)	6(11%)	3(5%)	2(4%)	9(16%)	5(9%)	10(18%)	3(5%)	3(5%)	3(5%)	4(7%)	55(100%)

Legend: -= none, EPEC = enteropatogenic *E. coli*, % = percentage

EPEC identified in raw meats and intestines from the open market shown different proportions in this study. This pathogroup was isolated in several similar studies (**Kagambèga *et al*., 2012; Fadi *et al*., 2012; Mori *et al*., 2014**) from raw meats. The prevalence of EPEC is highest and are most isolated from human than foods and animal, but the transmission of this pathogen to human occurs through various mechanism: consumption of contaminated ground meat and drinking unpasteurized milk (**Kagambèga *et al*., 2012; Isibor *et al*., 2013; Bagré *et al*., 2014**); consumption contaminated water, vegetables and juice (**Isibor *et al*., 2013; Bsadjo Tchamba *et al*., 2014**). The slaughtering and the transformation are the step of production generally considered as trunks of risk in frame of the prevention of EPEC that contaminated the meat (**Cohen and Karib, 2006**). According to several authors, EPEC constitutes the first cause of diarrhea in infant and child less than five years old in Burkina Faso (**Bonkoungou *et al*., 2012; Dembélé *et al*., 2015**), and other authors **Croxen *et al*., (2013); Igbeneghu and Lamikanra, (2014); Saeed *et al*., (2015)**.

A high proportion of STEC, STEC-ETEC and EPEC strains were obtained in this study from beef and mutton raw meats and intestines considering these animals as potential zoonotic reservoir of STEC, STEC-ETEC and EPEC. Prevent cross contamination in food preparation areas by thoroughly washing hands, counters, cutting boards, and utensils after they touch raw meat. Knowledge of transmission routes and vehicles allows consumers to be educated on reducing risky behavior that can decrease their risk for infection.

CONCLUSION

The current study confirms the presence of DEC strains in beef and mutton. Also cross contamination by contact with areas of selling previously contaminated with raw meat and contact with the raw meat itself can lead a factor risk for consumers. The preventives measures must be integrated in the slaughterhouse and in the nice practices of hygiene of production. The prevalence of virulent *E. coli* indicated risk exposure of the population.

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ANTIBACTERIAL ACTIVITY OF CARAWAY ESSENTIAL OIL AGAINST *STAPHYLOCOCCUS AUREUS* ISOLATED FROM PATIENTS WITH FURUNCULOSIS

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ABSTRACT

Furunculosis is a common skin disease caused by *Staphylococcus aureus*. Infection is characterized by a deep inflammation of the hair follicle. It leads to abscess formation with accumulation of pus and necrotic tissue. Treatment with antibiotics is often ineffective and patients often suffer from recurrent episodes. The aim of the study was to determine the antibacterial activity of caraway essential oil (CEO) against *S. aureus* isolated from patients with furunculosis. *S. aureus* strains were characterized by a different virulence factors and resistance to antibiotics. Effect of CEO was evaluated against 15 strains of *S. aureus* isolated from patients and against 3 control strains. The susceptibility to antibiotics was determined using Kirby-Bauer disk diffusion method. The presence of genes encoding virulence factors was determined with PCR method. The presence of slime was examined with Congo red method. The composition of the CEO was evaluated by gas chromatography method with mass selective detector. The minimum inhibitory concentration (MIC) of CEO was determined by serial dilutions in tryptic soy broth containing 10% NaCl. Analysis of the oil composition showed that the predominant component was cuminaldehyde (46.7%). CEO showed inhibition activity against control strains and patients strains. The MIC values of essential oil ranged from 10 to 12 µL/mL and from 7 to 67 µL/mL, respectively. Lack of correlation between the antibiotic resistance of the bacterial strains, presence of genes, presence of slime and their sensitivity to essential oil was found. CEO can be used as alternative antibacterial agent in supportive treatment patients with furunculosis.

Keywords: *Staphylococcus aureus*, *Carum carvi*, caraway oil, essential oil, furunculosis

INTRODUCTION

Furunculosis (boils) is a common skin disease caused by *Staphylococcus aureus*. This infection is characterized by painful and deep infections of the hair follicle. Even mild lesions are very painful and often leave a scars. Many furunculosis strains acquired resistance to different antibiotics. Therefore, antibiotic treatment is frequently not effective, especially in patients who suffer from recurrent episodes **(El-Gilany & Fathy, 2009; Holtfreter *et al.*, 2011)**. Recurrence of boils is an occurrence of many lesions over a period of months or years, in the same patient. Boils are often located on the limbs and neck, and leave a scar after recovery **(Demos *et al.*, 2012)**. If several adjacent lesions are coalesced form carbuncles as a result **(Ibler & Kromann, 2014)**.
It has been observed that furunculosis most often spreads among family members. Direct contact with an infected person is a major risk factor for the development of furuncles. Other independent factors include diabetes, infection with HIV, alcoholism, anemia, previous antibiotic therapy, skin diseases (especially atopic dermatitis), previous hospitalization, the multiplicity of lesions, poor personal hygiene, deficiency of mannose binding lectin and impaired function of neutrophils **(El-Gilany & Fathy, 2009; Stevens *et al.*, 2010; Demos *et al.*, 2012; Ibler & Kromann, 2014)**. The most important risk factor of recurrent furunculosis is the exposure to the source of infection - most likely nasal carriage of *S. aureus*. Many patients after removal of furuncles have relapses of the disease **(Guzik *et al.*, 2005)**.
The genus *Carum* is an important genus of the *Umbelliferae* (known as *Apiaceae*) family **(Laribi *et al.*, 2013)**. Among *Carum* genus the most important medicinal plant is *Carum carvi* L. (Caraway). This crop has been cultivated for long time in different parts of the world i.e. Europe, Egypt, Australia, China and Iran **(Keshavarz *et al.*, 2013)**. High amount of the oil is found in the caraway seeds, which are most frequently used in the food and cosmetics industry **(Darougheh *et al.*, 2014)**. Caraway essential oil (CEO) extracted from caraway fruits is rich in essential oils i.e.: carvone, limonene, germacrene D and transdihydrocarvone. Also, caraway seeds contain trace amounts of other compounds including acetaldehyde, furfural, carveole, pinene, thujone, camphene and phellandrene **(Darougheh *et al.*, 2014; Moubarz *et al.*, 2014)**. Caraway is used in medicine as a remedy for indigestion, persistent ailments of the digestive system and the status of systolic. Moreover, it is used as a laxative, carminative and an appetite stimulant. CEO increases lactation in pregnant women and alleviates menstrual pain **(Villarini *et al.*, 2011; Keshavarz *et al.*, 2013)**. CEO is used in phytomedicine as an antibacterial, antioxidant, antiproliferative and antitumor agent **(Laribi *et al.*, 2009b; Sadiq *et al.*, 2010)**. Essential oil from *C. carvi* has also fungicidal, insecticidal and diuretic properties **(Laribi *et al.*, 2009a)**.
S. aureus is a Gram-positive, extracellular bacterium colonizing i.a. human and animal skin. In predisposing circumstances, it is responsible for the high number of skin infections, i.e. impetigo, cellulitis, folliculitis, furunculosis, subcutaneous abscesses, infected abrasions, ulcers and wounds **(Krishna & Miller, 2012)**. About 30% of the healthy individuals are persistent nasal *S. aureus* carriers. This bacterium permanently colonizes the anterior nares in 10-20% of the population and transiently – in 30-50% **(Ryu *et al.*, 2014)**. Colonization also occurs at other body sites. Bacterium can be isolated from warm and moist skin folds (for example in the groin and under pendulous breasts) **(Ibler & Kromann, 2014)**. *S. aureus* nasal carriage plays an important role in chronic or recurrent furunculosis. According to **Masiuk *et al.* (2010)** patients suffering from chronic furunculosis are concomitantly the carriers of *S. aureus* localized in nose.
The aim of the study was to determine the antibacterial activity of CEO against *S. aureus* strains isolated from patients with furunculosis. Investigated strains demonstrated the presence of different virulence factors and resistance to antibiotics.

MATERIAL AND METHODS

Bacterial strains

15 *S. aureus* strains isolated in 2003-2008 from patients with furunculosis were analyzed in the Department of Microbiology and Diagnostic Immunology, Pomeranian Medical University in Szczecin. The material was inoculated on the following sets of solid media: Chapman agar and Columbia agar with addition of 5% sheep blood (bioMérieux. Poland). Agar plates were incubated at 35±1°C in

aerobic atmosphere for 24 h. *S. aureus* strains were identified on the basis of colonies morphology, positive catalase test, positive Staph-Kit test (bioMérieux, Poland) and positive coagulase test (Institute of Biotechnology, Sera and Vaccines, Biomed, Poland).
S. aureus ATCC 25923, *S. aureus* ATCC 29213 and *S. aureus* ATCC 43300 were used as a control strains, the property of Department of Microbiology and Diagnostic Immunology, Pomeranian Medical University in Szczecin.

Detection of slime production by light microscopy

Slime production of *S. aureus* isolates was evaluated by Congo red method with modification (crystal violet as a positive dye), according to the protocol of **Korres *et al.* (2013)**. Cultures *S. aureus* were incubated in trypticase soy broth (TSB) (Difco, USA) at 35±1°C for 24 h. After incubation, 2 drops of the bacterial suspension were mixed with 2 drops of crystal violet solution. Next, the mixture was smeared on a glass slide, washed with distilled water and air-dried. Slides were stained with Congo red solution (1 min) and air-dried. Afterwards, slides were examined with optical microscope (Olympus, Japan) and photographed. As positive result was indicated by appears a colourless halo around the bacterial cells against a pink background.

DNA isolation

Total DNA of *S. aureus* was isolated with a DNeasy Blood and Tissue Kit (Qiagen, Germany), according to the manufacturer's instructions. DNA was stored at 4°C.

Detection of *S. aureus* virulence factors by PCR

Set of 5 Multiplex PCR was established to detect the genes as follows: a) *sea, seh, sec* and *tst*, b) *see, seb, sem, sel* and *seo*, c) *sed, etd, eta* and *sek*, d) *sei, ser, seu* and *sep* and e) *sen, seg, seq* and *sej*, as reported previously **(Holtfreter *et al.*, 2011)**. Single PCR was performed for the detection PVL (*luk-PV*), methicillin-resistant *S. aureus* (MRSA) (*mecA*) and exfoliative toxin B (*etb*).
Single and Multiplex PCRs were performed with the GoTaq Flexi DNA Polymerase System (Promega, USA), as described previously **(Holtfreter *et al.*, 2011)**. Amplification was performed in a Thermocycler Perkin Elmer Gene Amp System 9600 (Applied Biosystems, USA). DNA was amplified with the following thermal settings: the initial denaturation (2 min, 94°C); 35 cycles of annealing at 55°C (94°C, 15 s; 55°C, 20 s; 72°C, 40 s), final extension (74°C, 10 min). The amplified DNA was purified with QIAquick purification kit (Qiagen, Germany). All PCR products were resolved by electrophoresis in 1.5% agarose gels (Sigma Aldrich, Germany) in 1xTris-borate-EDTA buffer (BioRad, France), stained with ethidium bromide (Sigma Aldrich, Germany), and visualized under UV light. 100-1500 bp DNA Ladder (Promega, USA) was used for precise sizing of PCR products. As positive controls used reference strains **(Baba *et al.*, 2002; Wu *et al.*, 2010; Holtfreter *et al.*, 2011)**.

Essential oil analysis

CEO used in this study was obtained from Vera-Nord Company, Poland (commercial producer of plant essential oils and aromatic substances). The oil exhibited a strong and characteristic odor. It was intended to the production of cosmetics and household chemistry products.
The analysis of CEO composition was performed by gas chromatography method with mass selective detector (GC-MS) using an Agilent 6890N gas chromatograph with a 5973N mass selective detector. The resolution of analytes was achieved using a HP-5MSI column (5% phenyl/95% dimethylpolysiloxane), 30 m x 0.25 mm I.D. and 0.25 µm film thickness. The column temperature was programmed as follows: initial temperature 60°C, ramp rate 8°C/min, final temperature 300°C (hold 5 min). Helium was used as carrier gas at a flow rate of 1.2 mL/min. The injector temperature was set at 250°C, MS quad: 150ºC; MS source: 230ºC. Mass spectra were obtained using electron impact ionization at 70 eV in full scan mode (mass range: 20–500 m/z).
Before the analysis 100 µL of tested essential oil was dissolved in 1 mL of acetone (p.a.). The identification of the CEO components was based on the comparison of their mass spectra with the reference spectra from NIST 02 library. The relative contents of the particular compounds in essential oil were their peak area percentages in a total ion chromatogram.

Screening susceptibility of bacteria to antibiotics

The antimicrobial susceptibility of *S. aureus* isolates was performed in accordance with the European Committee on Antimicrobial Susceptibility Testing recommendations **(EUCAST, 2013)**. Susceptibility to: ciprofloxacin - CIP (5 µg.disk^{-1}), gentamycin - GE (10 µg.disk^{-1}), trimethoprim-sulfamethoxazole - SXT (1,25/23,75 µg.disk^{-1}), mupirocin - MUP (10 µg.disk^{-1}) and cefoxitin - FOX (30 µg.disk^{-1}) (Becton Dickinson, USA) was evaluated with disk diffusion method performed with Mueller-Hinton agar (MHA) (bioMérieux, Poland) inoculated with a suspension (1.5x10^8 CFU.ml^{-1}) of the *S. aureus* isolates. The plates were incubated at 35±1°C for 18±2 h and inhibition zones were measured. Strains resistant to FOX were considered as MRSA.
The D-test was performed with clindamycin - CC (2 µg.disk^{-1}) and erythromycin - E (15 µg.disk^{-1}) (Becton Dickinson, USA). These disks were placed 20 mm apart on the MHA plate seeded with the test strain. *S. aureus* strains resistant to CC and E were considered to have constitutive macrolides, lincosamides and streptogramins B resistance (cMLS$_B$) phenotype. Moreover, strains with flattening of the susceptible zone of inhibition to CC adjacent to the E disk (D-shape) were considered to exhibit resistance inducible phenotype to macrolides, lincosamides and streptogramins B (iMLS$_B$). Strains with circular zone around CC were considered to exhibit MS$_B$ phenotype (macrolides and streptogramins B resistance) **(Saderi *et al.*, 2011)**.

Broth microdilution method - determination of the minimum inhibitory concentration (MIC)

The microdilution test was conducted in 96-well plates according to **Urbaniak *et al.* (2014)** with some modification. A dilution series of the CEO was obtained using 1% Tween 80 (Difco, USA) solution as the solvent. The final concentrations were 100-0.5 µL/mL. Each well received 100 µL of the specific concentrations of the CEO and TSB with addition 10% NaCl (Chempur, Poland) inoculated with 10 µL bacterial suspension (1.5x10^8 CFU/mL). The positive solvent control was completed with 100 µL of 1% Tween 80 solution. The final volume in each well was 110 µL. The microplates were covered with parafilm and incubated for 24 h at 35±1°C. Inhibition of bacterial growth was confirmed by cultivation preincubated plates on Columbia agar with addition 5% sheep blood. The MIC values were defined by the lowest concentration of the CEO that inhibits the growth of the microorganism. The control wells solvent does not affect the growth of all tested bacterial strains. Each MIC test was conducted with three replicates.

RESULTS AND DISCUSSION

Chemical composition of the CEO

The results of qualitative and quantitative analysis of CEO purchased from Vera-Nord Company are shown in Table 1. The main constituent of essential oil was cuminaldehyde (46.7%) followed by β-pinene (10.3%), durene (9.7%), γ-terpinene (8.9%) and limonene (4.7%). A total of 17 components were identified in the oil, accounting for 84.2% of the total oil (Table 1).
It has been proven that the main components of essential oil depends on many environmental and genetic factors and oil extraction method **(Aćimović *et al.*, 2014)**. A number of studies examining the essential oil content and composition of CEO have been performed. For example, research conducted by **Dawidar *et al.* (2010)** showed that Egyptian commercial essential oil from *C. carvi* L. contained D-carvone (42.61%) and D-limonene (33.53%) as its two main components. D-carvone and limonene were also found to be the main components of the commercial CEOs provided by three independent companies (Etol, Dragoco and Pollena-Aroma) **(Simic *et al.*, 2008, Dimić *et al.*, 2012; Gniewosz *et al.*, 2013)**. Our studies demonstrated different results than these experiments in which essential oils did not contain cuminaldehyde – the main component identified in commercial CEO provided by Vera-Nord Company (Table 1). However, research conducted by **Razzaghi-Abyaneh *et al.* (2009)** showed that cuminaldehyde was present in essential oil extracted from *C. carvi* seeds. The caraway oil from Iran contained cuminaldehyde (22.08%) and γ-terpinene (17.86%) as its two main components, followed by *p*-cymene (7.99%).

Presence of slime

18 *S. aureus* strains were examined with Congo red method. The slime production of *S. aureus* was recorded in 12 (66.7%) isolates (Table 2). On the microscopic slides, a spherical, Gram-positive cocci were surrounded by a well-defined halo on a pink background (Figure 1).
Biofilm formation is important ability of bacteria and plays essential role in increased resistance to antimicrobial agents **(Podbielska *et al.*, 2010)**. In addition, slime interferes with phagocytosis and enhances adhesion to host tissue and inanimate objects. According to **Gündoğan *et al.* (2006)** there is lack of correlation between antibiotic resistance and slime production among *S. aureus* observed. The results showed that not all of resistance strains produced an extracellular substances. In addition, we noticed that 6 susceptible strains also produced slime (Table 2).
In our study *S. aureus* extracellular matrix did not affect on the MIC values. Both high and low concentration CEO did not impact on the presence of slime. It may indicate that slime did not play significant role in CEO attack on the bacteria surface.

Table 1 Chemical composition of the commercial essential oil of *C. carvi* L.

Compound	CAS #	Retention time (min)	Relative content (%)
α-Thujene	2867-05-2	3.64	0.3
α-Pinene	80-56-8	3.75	0.8
β-Pinene	127-91-3	4.39	10.3
β-Myrcene	123-35-3	4.48	0.3
α-Phellandrene	99-83-2	4.73	0.4
Durene	95-93-2	5.09	9.7
Limonene	138-86-3	5.14	4.7
γ-Terpinene	99-85-4	5.61	8.9
p-Cymene	99-87-6	6.13	0.3
Terpinen-4-ol	562-74-3	7.61	0.2
Cuminaldehyde	122-03-2	8.92	46.7
Phellandral	21391-98-0	9.32	0.3
Carvacrol	499-75-2	9.85	0.3
β-Caryophyllene	87-44-5	11.62	0.2
α-Bergamotene	17699-05-7	11.80	0.2
β-Farnesene	18794-84-8	12.09	0.3
β-Bisabolene	495-61-4	12.92	0.3
Total			**84.2**

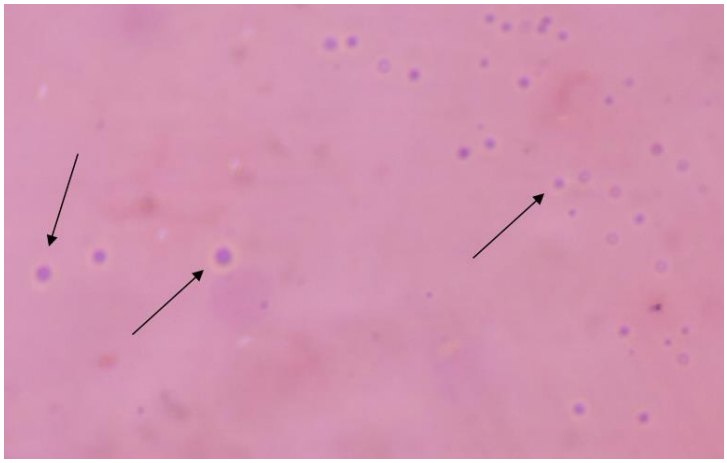

Figure 1 Microscopic views of slime formation of strain number 7 (x1,000 magnification, light microscopy) isolated from patient with furunculosis. Slimes (pointed with arrows) were visualized by double staining with crystal violet and Congo red.

Distribution of genes

In total, 80% (12/15) of the furunculosis strains were *luk-PV* positive and 13.3% (2/15) were *mecA* positive (Table 2). *S. aureus* produces variety of virulence factors, according to available data only PVL is associated with furuncles formation **(Demos *et al.*, 2012)**. Research performed by **Masiuk *et al.* (2010)** showed that most of methicillin-susceptible *S. aureus* (MSSA) isolates from patients with furunculosis harbored *luk-PV* genes. These same conclusions are reported in other reviews **(Yamasaki *et al.*, 2005; Cupane *et al.*, 2012)**.

The presence of genes of exfoliative toxins *eta, etb, etd* and toxic shock syndrome toxin gene (*tst*) was not confirmed (Table 2). Exfoliative toxins A and B (ETA and ETB) are exotoxins produced by *S. aureus,* which are involved in staphylococcal scalded-skin syndrome (SSSS) and bullous impetigo **(Jursa-Kulesza *et al.*, 2009)**. In our study no ETA and ETB was observed. Study conducted by **Bukowski *et al.* (2010)** shown that ETD-producing *S. aureus* strains are mainly isolated from furuncles or abscesses, but not from SSSS. However, in our research all isolates were *etd* negative.

Among enterotoxins genes (SEs) (*sea* to *seu*) only *seb* and *sel* were detected. Among all tested strains, 33.3% (5/15) were *seb* positive and only 1 strain (6.7%) was *sel* positive (Figure 2, Table 2). These genes are located on a pathogenicity island such as SaPI3 (*seb, sek, seq,* or *seb, sel* and *sek*), SaPIm1/n1 (*tst, sec* and *sel*) and SAPIj50 (*tst, sec* and *sel*) **(Yamamoto *et al.*, 2013)**. Enterotoxin B is mainly responsible for food poisoning outbreaks and can cause toxic shock syndrome **(Pinchuk *et al.*, 2010; Karauzum *et al.*, 2012)**. A recent study performed by **Sina *et al.* (2013)** showed that *S. aureus* strains isolated from skin and soft tissue infections (SSTI) such as furuncles, skin abscesses and cellulitis; and bone infections often harbored *seb*. Moreover, research conducted by **Masiuk *et al.* (2010)** indicated that *seb* was present among about 25% strains isolated from furuncles.

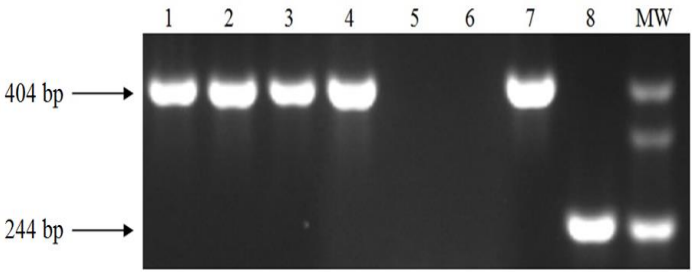

Figure 2 Multiplex PCR amplification for detection of *S. aureus* genes. Lines 1-4, 7: *seb* (404 bp); lines 5-6: no template DNA; line 8: *sel* (244 bp); MW - molecular weight standard.

Susceptibility testing

2 MRSA (resistant to FOX) and 13 MSSA were isolated from furuncles. Of the all isolates, 46.7% (7/15) were resistant to at least one of the antibiotics tested. 46.7% (7/15) showed resistance to CC and E in disk diffusion testing. In D-test, 33.3% isolates exhibited $cMLS_B$ resistance and 13.3% had $iMLS_B$ resistance phenotype. MS_B resistance phenotype as not detected. Details of the prevalence of antimicrobial resistance in tested isolates are shown in Table 2.

Significant increases in prevalence of resistance to antibiotics have been observed over the past years. The misuse and overuse of antibiotics causes that antibiotics are no longer that effective in therapy. According to **Lewis & Jorgensen (2005)** many community-acquired MRSA (CA-MRSA) responsible for SSTI often show MLS_B mechanism. All MRSA strains tested in present study demonastrated this type of resistance mechanism. Furthermore, we have found that 33.3% MSSA strains were resistance to CC and E (detected $cMLS_B$ and $iMLS_B$ resistance phenotypes). Study performed by **Patel *et al.* (2006)** indicated that $iMLS_B$ was detected in 33% CA-MRSA and 56% hospital-acquired MRSA isolates. Research conducted by **Saderi *et al.* (2011)** showed similar results. In this experiment *S. aureus* strain was isolated from the purulent lesions of the skin. Prevalence of $cMLS_B$ and $iMLS_B$ resistance phenotypes were 92.8% and 6.4%, respectively. This indicates that *S. aureus* strains with $cMLS_B$ phenotype were much more frequent than with $iMLS_B$ phenotype, which is similar to our results.

The activity of CEO against tested bacterial strains

Both control and all clinical strains derived from patients with furunculosis were sensitive to CEO (Table 2). The inhibition of growth for all analyzed strains *S. aureus* after applying of CEO was observed. In the case of MSSA strains, the MIC values of essential oil were 26.7 ± 5.8 - 66.7 ± 11.5 µL/mL, which was larger than that produced by the MIC values of control strains number 1 and 2 (both 10 ± 8.7 µL/mL). In the case of MRSA strains, the MIC values of CEO were 6.67 ± 2.9 - 30 ± 10 µL/mL, which was also larger than that produced by the MIC values of control strain number 16 (11.7 ± 7.6 µL/mL). The largest number of isolates was inhibited by concentrations of 46.7 ± 5.8 µL/mL (4 antibiotic-susceptible strains and 1 $cMLS_B$ strain).

In recent years, interest in essential oils is significantly observed. Essential oils are used in natural medicine to treat a wide range of infections caused by bacteria. Commercial essential oil was tested against *S. aureus* and demonstrated antibacterial activity against these strains **(Gniewosz *et al.*, 2013; Alboofetileh *et al.*, 2014)**. **Seidler-Łożykowska *et al.* (2013)** have studied the activity of CEO obtained from fruit originated from different genotypes against *S. aureus*. The authors demonstrated that MIC analyzed essential oil ranged from 0.2 - 1.6 mg/mL. Moreover, in this study the authors have proved that carvone can be recognized as a one of the active component. Others have reported MIC of CEO against *S. aureus* varied from 0.1 to 3 µL/mL **(Di Pasqua *et al.*, 2005; Mohamed *et al.*, 2013; Simic *et al.*, 2008; Tarek *et al.*, 2014)**. In these studies, GC-MS analysis of essential oil shown that the main components were carvone and limonene in contrast to our study. Moreover, tested by us CEO demonstrated higher MIC against *S. aureus* strains responsible for furunculosis than above-mentioned results.

Table 2 Characteristic of *S. aureus* strains

Strain number	Laboratory number	MIC of CEO (µL/mL)	Susceptibility patterns							Phenotypic resistance	Genes				Slime
			CIP	E	CC	GE	SXT	MUP	FOX		*luk-PV*	*mecA*	*SEs, tst*	*eta, etb, etd*	
1.	ATCC 29213	10 ± 8.7	S	S	S	S	S	S	S	-	-	-	-	-	+
2.	ATCC 25923	10 ± 8.7	S	S	S	S	S	S	S	-	-	-	-	-	+
3.	9418	46.7 ± 5.8	S	S	S	S	S	S	S	-	+	-	-	-	-
4.	3455	46.7 ± 5.8	S	S	S	S	S	S	S	-	+	-	*b*	-	+
5.	6147	33.3 ± 5.8	S	S	S	S	S	S	S	-	+	-	*b*	-	+
6.	1218	46.7 ± 5.8	S	S	S	S	S	S	S	-	+	-	-	-	-
7.	8786	46.7 ± 5.8	S	S	S	S	S	S	S	-	+	-	-	-	+
8.	3442	56.7 ± 5.8	S	S	S	S	S	S	S	-	-	-	*l*	-	-
9.	3121	63.3 ± 5.8	S	S	S	S	S	S	S	-	+	-	-	-	+
10.	9647	26.7 ± 5.8	S	S	S	S	S	S	S	-	+	-	-	-	-
11.	7756	66.7 ± 11.5	S	R	R	S	S	S	S	$cMLS_B$	+	-	*b*	-	+
12.	4661	43.3 ± 5.8	S	R	R	S	S	S	S	$cMLS_B$	+	-	*b*	-	-
13.	9589	46.7 ± 5.8	S	R	R	S	S	S	S	$cMLS_B$	+	-	*b*	-	+
14.	247	56.7 ± 11.5	S	R	R	S	S	S	S	$iMLS_B$	-	-	-	-	+
15.	694	36.7 ± 5.8	S	R	R	S	S	S	S	$iMLS_B$	+	-	-	-	-
16.	ATCC 43300	11.7 ± 7.6	S	S	S	S	S	S	R	MRSA	-	+	-	-	+
17.	2966	6.67 ± 2.9	S	R	R	S	S	S	R	MRSA, $cMLS_B$	+	+	-	-	+
18.	7293	30 ± 10	S	R	R	S	S	S	R	MRSA, $cMLS_B$	-	+	-	-	+

Legend: MIC - minimal inhibitory concentration, CEO - caraway essential oil, R - resistant, S - susceptible, CIP - ciprofloxacin, E - erythromycin, CC - clindamycin, GE - gentamycin, SXT - trimethoprim-sulfamethoxazole, MUP - mupirocin, FOX - cefoxitin, $cMLS_B$ - constitutive MLS_B phenotype, $iMLS_B$ - inducible MLS_B phenotype, MRSA - methicillin-resistant *S. aureus*, *luk-PV* - Panton-Valentine leukocidin gene, *mecA* - methicillin resistance gene, *SEs* - enterotoxins genes, *tst* - toxic shock syndrome toxin gene, *eta, etb, etd* - exfoliative toxin A, B, D genes.
MIC values are expressed as means ± standard deviation.

According to **Peter (2012)** CEO extracted from *C. carvi* L. is generally safe for internal and has no toxic effect. Although it may cause skin irritation if used in high concentration. Moreover, CEO can irritate the eyes. Therefore, CEO should not be used directly on the skin. In addition, study performed by **Morshedi *et al.* (2015)** showed that cuminaldehyde (the main component identified in commercial CEO provided by Vera-Nord Company) is a nontoxic compound. These authors demonstrated no toxic effect on the cells.

CONCLUSION

There were no significant differences in MIC values depending on the susceptible/resistance to antibiotics, virulence genes and presence of slime. Commercial CEO has inhibitory effects on growth of *S. aureus* strains isolated from patients with furunculosis, regardless of the degree of resistance to antibiotics and virulence of the strain. CEO can be used as an alternative antibacterial agent in the treatment of furunculosis (especially in persons suffer from recurrent episodes).

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INFLUENCE OF RECONSTITUTION TEMPERATURE ON SURVIVAL OF *CRONOBACTER SAKAZAKII* IN POWDERED INFANT FORMULA

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ABSTRACT

Cronobacter spp. are emerging opportunistic pathogens commonly associated with the feeding of powdered infant formula (PIF). This study aimed to investigate the ability of *C.sakazakii* (ATCC 29544) to survive heat injury in laboratory growth medium and powdered infant formula (PIF) in comparison to *Escherichia coli* (ATCC 25922) and other selected *Cronobacter* strains. As part of this work the thin agar layer (TAL) method for the recovery of heat-injured foodborne pathogens was evaluated for use with *C.sakazakii* (ATCC 29544) and *E.coli* (ATCC 25922). We report that heat treatment at 55°C for 10 minutes had no significant effect ($P > 0.05$) on *C.sakazakii* (ATCC 29544) viability in peptone or PIF. Significant differences in survival of *Cronobacter* strains after this heat treatment were identified indicating that heat tolerance in this genus may be strain dependent. PIF did not enhance survival of this organism in comparison to other media. Results indicate that a decrease in temperature of 5°C from the recommended 70°C for the reconstitution of PIF was sufficient to produce a significant increase in survival of *C.sakazakii* (ATCC 29544). Reconstituted PIF stored at room temperature was shown to support the survival and proliferation of this pathogen indicating that temperature abuse during PIF preparation and storage may significantly increase the risk of disease caused by these opportunistic pathogens.

Keywords: *Cronobacter,* powdered infant formula, heat-injury, thin agar layer

INTRODUCTION

PIF is consumed by millions of infants throughout the world every day, and is generally considered as safe (**Pagotto & Farber, 2009**), however it is not a sterile product and can harbour pathogens such as *Cronobacter, Salmonella* and *Staphylococcus* (**Wang *et al.*, 2012**). In 2004, the Food and Agriculture Organisation of the United Nations (FAO) and the World Health Organisation (WHO) jointly convened an expert meeting on *Enterobacter sakazakii* (now *Cronobacter* spp.) and other microorganisms in powdered infant formula (PIF) and concluded that intrinsic contamination of PIF with *E.sakazakii* (now *Cronobacter* spp.) and *Salmonella* has been a cause of infection and illnesses in infants. This includes severe disease which can lead to serious developmental sequelae and death (**FAO/WHO, 2004**). The World Health Organisation (WHO) recommends reconstitution of PIF using boiled water that has cooled slightly but not below a temperature of 70°C. It is recommended that this be checked using a sterile thermometer (**FAO/WHO, 2004**). In contrast to this, manufacturers of this product most often recommend using boiled water which has been cooled for 30 minutes with no specific requirements given for exact temperature or recommended methods of checking temperature before reconstitution. A common risk factor in many reported *Cronobacter* (previously *Enterobacter sakazakii*) outbreaks is temperature abuse of reconstituted formula (**Osaili & Forsythe, 2009**). Heating of prepared milk to 80°C - 90°C or mixing with boiling water is likely sufficient to kill most microorganisms but is not recommended due to the potential loss of nutritional value, especially in terms of adverse effects on the vitamin content of PIF (**Agostoni *et al.*, 2004**). It is currently recommended that reconstituted PIF be stored below 4°C and for no more than 4 hours (**FAO/WHO, 2004**) and that leftover product be discarded and never reheated or added to subsequent preparations (**Osaili and Forsythe, 2009**).

In 2007, a year-long study based on samples taken from a PIF processing plant, reported that the prevalence of the organism in specific locations of the plant was as high as 31% while the prevalence in the final product was 2.5%. It was suggested based on these findings, other scientific studies and surveys from FAO/WHO, that PIF may be contaminated at frequencies ranging from 0-33% (**Mullane *et al.*, 2007**). PIF and milk powder have been suggested as the main sources of *Cronobacter* infection and the vehicles responsible for 50-80% of infections caused by these organisms (**Kim *et al.*, 2008**). Microorganisms in food processing or preparation environments are exposed to a wide range of chemical, physical and nutritional stresses (**Osaili and Forsythe, 2009**). The detection of food-borne pathogens should accommodate this by aiming to detect both normal and injured microorganisms. An injured cell has been defined as a cell that survives a stress but loses some of its distinctive qualities (**Busta, 1978**). In practical terms this means many injured cells will be capable of forming colonies on non-selective media, but not on selective media (**Harstell, 1951**) or may display an extended lag-phase in comparison to uninjured cells (**Wu, 2008**). Therefore it is desirable to allow injured cells to recover before isolation or enumeration by customary procedures in order to avoid an underestimation of microbial content. The reader is directed to the review by **Wu (2008)** for a description of the numerous methods available for the recovery of injured bacterial cells. The method of recovery chosen for this study was the thin agar layer (TAL) method developed by **Kang and Fung (2000)**. This one-step method is relatively convenient to prepare and use and has been utilised effectively to recover heat-injured *Salmonella typhimurium* (**Kang and Fung, 2000**). The method has been found to be more effective than selective media alone for the recovery of heat-injured foodborne pathogens due to its incorporation of an initial exposure to non-selective medium (**Wu & Fung, 2001**). This present study includes an evaluation of the TAL method for the recovery of heat-injured *C.sakazakii* (ATCC 29544) and *E.coli* (ATCC 25922) cells.

It is not clear how stringently the guidelines for the safe preparation and storage of PIF are complied with in the home and hospital settings and education of potential users of PIF therefore remains paramount to ensure its safe preparation and the avoidance of outbreaks of infectious disease (**FAO & WHO, 2004**). The inconsistencies existing in the recommendations for the safe preparation of PIF may prove to be extremely important in terms of preventing infection from intrinsic microorganisms, as even low numbers of organisms in this genus are considered to be a risk factor (**FAO & WHO, 2004**). This present study compares the efficacy of currently recommended preparation methods in terms of ability to reduce survival of *C.sakazakii* (ATCC 29544) cells added at the time of preparation. In addition to the inconsistencies existing in the preparation guidelines for PIF, there are also discrepancies in the existing literature related to the *Cronobacter* genus. Differences in growth characteristics and tolerance to environmental stresses such as temperature resistance are reported to be strain dependent in some studies (**Osaili & Forsythe, 2009; Strydom *et al.*, 2012**) and

characteristic of the entire genus in others (**Iversen *et al.*, 2004; Nazarowec-White & Farber, 1997b)**. This issue is further confounded by the relatively recent taxonomic reclassification of this organism from a single species (*Enterobacter sakazakii*) to the novel genus *Cronobacter* (**Iversen et al., 2008**). Comprehensive studies published prior to this reclassification lack species specific data needed to describe the genus, now containing seven individual species (**Joseph *et al.*, 2011**). The disparities in the available information on this group of pathogens emphasise the need for research such as the present study which compares multiple strains and species of *Cronobacter* in order to gain data with practical applications.

MATERIAL AND METHODS

Source of Bacterial Strains and Growth Conditions

Type strains of *Cronobacter sakazakii* (ATCC 29544), *Escherichia coli* (ATCC 25922) and *Cronobacter muytjensii* (ATCC 51329) were obtained from the American Type Culture Collection (ATCC), Virginia, USA. Type strains of *Cronobacter turicensis* (DSM 18703) and *Cronobacter malonaticus* (DSM 18702) were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ), Braunschweig, Germany. Additional strains of *C.sakazakii* (ATCC 29004, ATCC 12868) were also obtained from the ATCC. *C.sakazakii* (NCTC 8155) was obtained from the National Collection of Type Cultures (NCTC), Salisbury, UK. It should be noted that this strain is currently named *Enterobacter sakazakii* in the collection but was reclassified as *C.sakazakii* after the recent taxonomic change (**Iversen *et al.*, 2008**). All strains used in this study were stored in a Microbank™ (Pro-Lab Diagnostics) at -80°C. Fresh cultures were obtained by direct inoculation of Nutrient Agar (Cruinn Diagnostics, Dublin, Ireland) formulated according to ISO 6579:2002, ISO 10273:2003 and ISO 16654:2001 and tested in accordance with ISO/TS 11133-2:2003. Liquid cultures were obtained by direct inoculation of Nutrient broth (Cruinn Diagnostics, Dublin, Ireland). Inoculated media were incubated overnight at 37°C. Pure cultures on solid media were stored at 4°C for no more than 2 weeks. All culture media were obtained from Cruinn Diagnostics unless otherwise stated.

Preparation of TAL plates

The chosen selective media, Harlequin CSA-DFI-*Cronobacter sakazakii* Agar-DFI Formulation and Harlequin *E.coli*/Coliform Medium, were prepared according to the manufacturer's instructions. The TAL plates were prepared according to the method described by **Kang and Fung (2000**) using tryptone soya agar (TSA) as a non-selective medium.

Thin agar layer method evaluation

Overnight cultures of all bacterial strains were prepared in nutrient broth and diluted in sterile peptone water to an optical density of 0.08-0.13 at 625 nm. 5 ml aliquots of sterile peptone water were heated and maintained at 55°C or 70°C in a water bath. 50 µl of *C.sakazakii* or *E.coli* alone or a 1:1 mixture of *C.sakazakii* (ATCC 29544) and *E.coli* (ATCC 25922) were added to the heated peptone water. Inoculated samples were maintained at the specified temperatures for 10 minutes. After this time the samples were cooled quickly by placement in slush ice. 100 µl of each sample was removed before and after the heat treatment and used to prepare serial dilutions for plating on the prepared solid media. The plates were incubated at 37°C for 24 hours after which time the visible colonies were counted.

Establishment of reconstitution temperatures

500 ml of distilled water was heated on a hot plate and allowed to boil for approximately 30 seconds and stood at room temperature. Water temperature was monitored using a thermometer and recorded every 5 minutes for one hour. This was repeated in triplicate and on three independent testing days in order to obtain average temperature values for specific time periods in the cooling process.

Preparation of bacteria and PIF reconstitution

Bacterial cultures were prepared and diluted as described previously. The concentration of PIF used was chosen from the table provided by the manufacturer as the concentration most suitable for feeding neonates (0.75 g / 10 ml water). 100 µl of the diluted bacterial culture was added to the dry PIF immediately prior to the addition of water. The PIF was reconstituted using boiled water that had cooled to 70˚C, 65˚C, 60˚C or 55°C. Immediately after the addition of water at the specified temperatures 100 µl of each sample was removed and used to prepare serial dilutions and spread plates on selective agar. The remaining inoculated PIF samples were incubated at room temperature for 2 hours. The process of preparing serial dilutions and spread plates was repeated each hour. Plates were incubated at 37°C overnight before conducting colony counts and calculating CFU/ml for each testing interval.

Influence of PIF on survival rates

Heat treatment using *C.sakazakii* (ATCC 29544) was performed according to the method previously described. Dilutions were plated on selective agar only and PIF was used alongside peptone for both 55°C and 70°C heat treatments. Six additional strains of *Cronobacter* were included for heat treatment in PIF at 55˚C for 10 minutes in order to establish any differences in heat tolerance between particular strains of this organism.

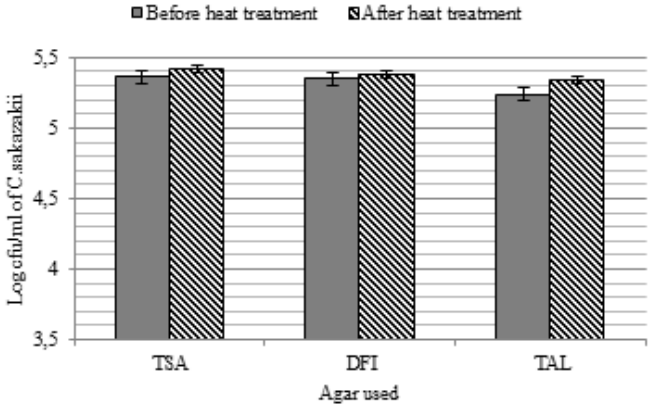
Statistical Analysis

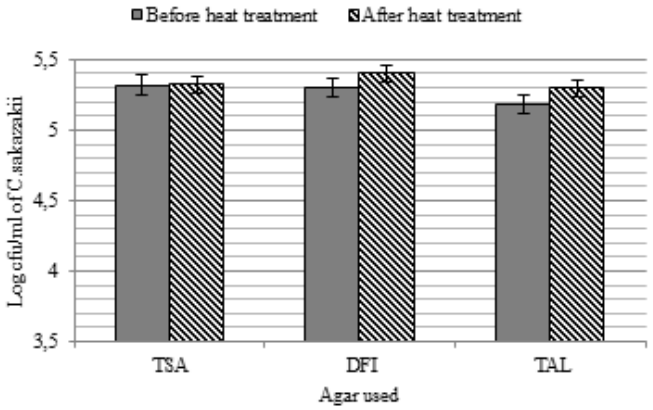
Results for all tests described were recorded and analysed using Microsoft® Excel Software. Methods of presenting the data obtained were chosen based on a literature review of similar studies. Statistical analysis was performed using the paired Student t-test. P values < 0.05 were considered significant, P values < 0.01 as very significant and P values < 0.001 as highly significant. Error bars on graphs represent ± standard error of the mean (SEM).

RESULTS

TAL method evaluation

The thin agar layer (TAL) method was evaluated for the recovery of heat-injured *C.sakazakii* (ATCC 29544) and *E.coli* (ATCC 25922) in both pure and mixed cultures. The mixed culture experiments aimed to assess the TAL method in terms of its ability to retain the selective characteristics of the original selective medium i.e. inhibition of growth of other organisms and/or distinctive colour change in organism of interest. *C.sakazakii* produced characteristic circular cream to yellow colonies on the TSA plates as expected. *E.coli* (ATCC 25922) produces pale cream colonies of roughly the same size on this agar and distinguishing between the two species on the mixed culture plates was therefore difficult. The selective *Cronobacter* medium (DFI) produced colonies of a similar size to those on the TSA for *C.sakazakii.* The distinctive colour change of the colonies to dark green was clearly visible. This colour change did not occur in the *E.coli* colonies and they were also noticeably smaller than on the TSA plates, indicating that some inhibition of growth had occurred. The TAL plates produced the desired colour change but no inhibition of *E.coli* (ATCC 25922) was visible indicated by colonies of both species being of the same size. The quantity of bacteria recovered (represented by log colony forming units/ml) by each medium after heat treatment for 10 minutes at 55˚C is illustrated in figure 1. The same information for pure *C.sakazakii* (ATCC 25944) and mixed culture with *E.coli* (ATCC 25922) after heat treatment at 70˚C for 10 minutes is provided in figure 2.





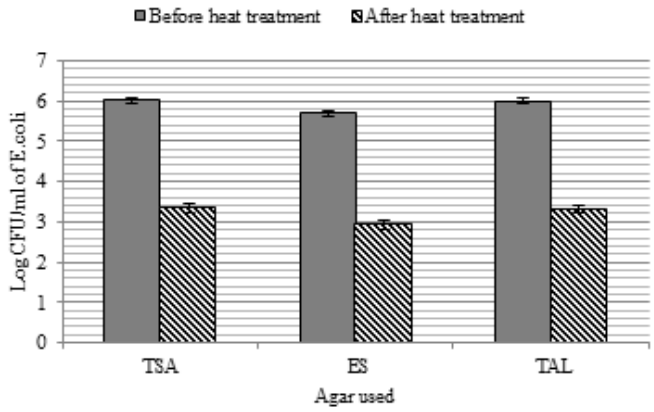


Figure 1 Log CFU/ml of *C.sakazakii* (ATCC 29544) in pure culture (A) and mixed culture (B) and *E.coli* (ATCC 25922) in pure culture (C) before and after heat treatment for 10 minutes at 55°C. Tryptone soya agar (TSA), *C.sakazakii* selective agar (DFI), *E.coli* selective agar (ES) and thin agar layer plates (TAL)

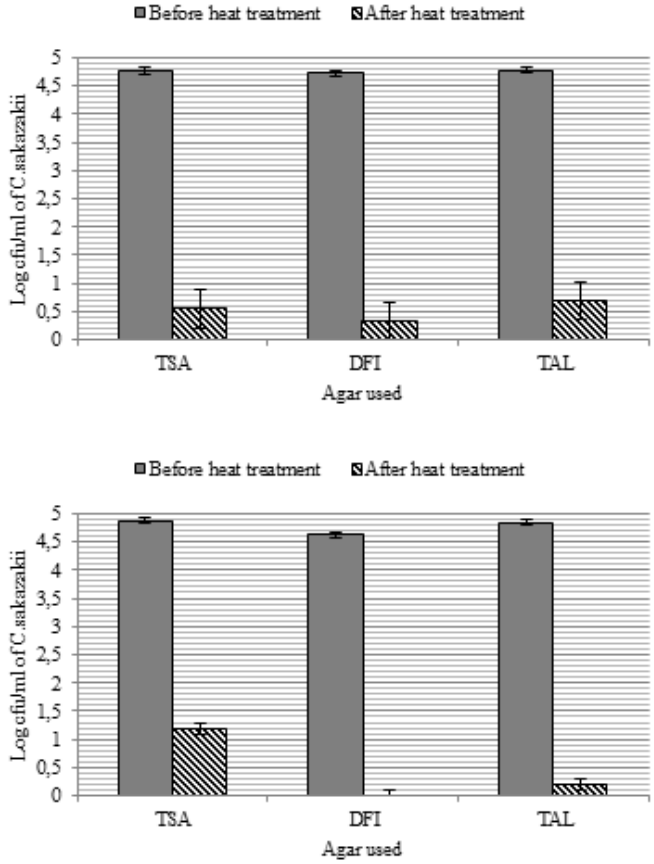


Figure 2 Log CFU/ml of *C.sakazakii* (ATCC 29544) in pure culture (A) and mixed culture (B) before and after heat treatment for 10 minutes at 70°C using tryptone soya agar (TSA), *C.sakazakii* selective agar (DFI) and thin agar layer method (TAL)

Effect of PIF on *C.sakazakii* (ATCC 29544) heat-treatment survival

An investigation into the effect of PIF on bacterial survival of heat treatment for 10 minutes at 55˚C and 70˚C was carried out using *C.sakazakii* (ATCC 29544). The survival of this organism after these treatments was assessed in terms of log CFU/ml of bacteria and is illustrated in figure 3. This investigation was furthered by comparing the survival rates of six additional *Cronobacter* species after heat treatment for 10 minutes at 55˚C in PIF. The results of this investigation are represented in figure 4.

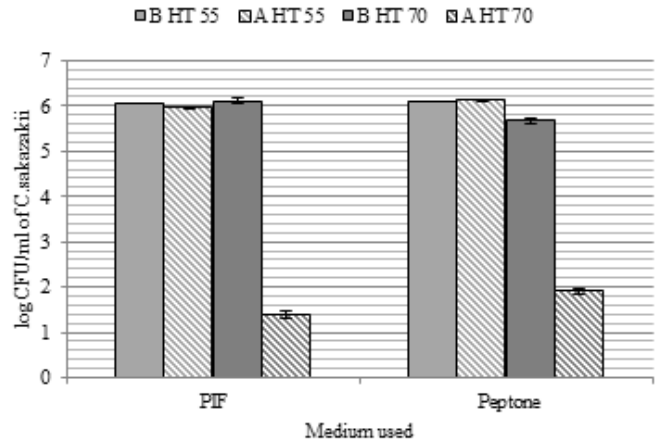


Figure 3 Log CFU/ml of *C.sakazakii* (ATCC 29544) in PIF and peptone before heat treatment at 55°C (B HT 55), after heat treatment at 55°C (A HT 55), before heat treatment at 70°C (B HT 70) and after heat treatment at 70°C (A HT 70)

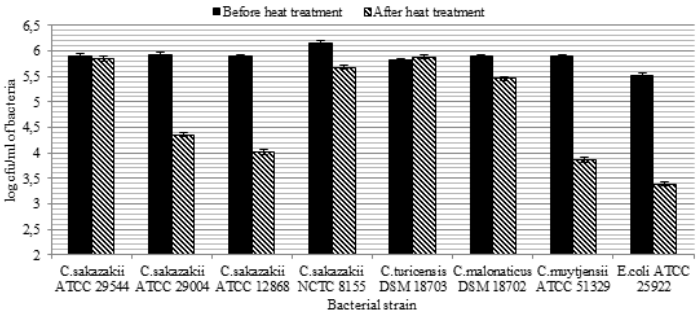


Figure 4 Comparison of selected *Cronobacter* species and *E.coli* (ATCC 25922) survival after 10 minute heat treatment at 55˚C

Effect of PIF reconstitution method on *C.sakazakii* survival

In accordance with the manufacturer's instructions for the preparation of PIF, it is recommended that boiled water be allowed to cool at room temperature for 30 minutes before use. An investigation was carried out in order to establish if this method complied with WHO recommendations which state that water should not be allowed cool below 70˚C for the safe reconstitution of PIF (**FAO/WHO, 2004**). A number of temperatures within this range were then chosen to be used in a further investigation in which PIF was spiked with a known concentration of *C.sakazakii* (ATCC 29544) before reconstitution with water allowed to cool to set temperatures. Bacterial survival was assessed immediately and after one and two hours of standing at room temperature. The collected data are represented in figure 5.

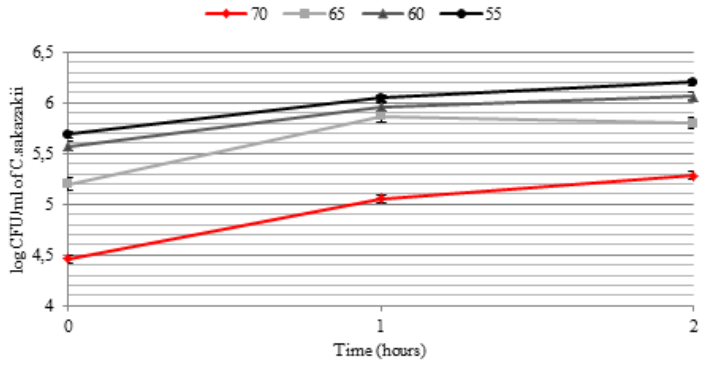


Figure 5 Survival and growth of *C.sakazakii* (ATCC 29544) after PIF reconstitution at a range of temperatures (55°C, 60˚C, 65˚C, 70°C)

DISCUSSION

TAL method evaluation

The TAL method was successful in producing colony colourization associated with the specific selective medium and facilitated superior identification between the bacteria compared to non-selective agar. However, the colonies of both species were much larger than on either the DFI or TSA plates, making counting

considerably more difficult due to colonies merging with surrounding colonies. *E.coli* (ATCC 25922) colonies were similar in size to those of *C.sakazakii* (ATCC 29544) indicating a reduction/lack of inhibition that had been visible when using the selective agar alone. The TAL method has previously been shown to be effective in recovering more cells after heat-injury than selective agar alone in a number of foodborne pathogens. The method has been evaluated for a range of species including *Salmonella typhimurium* (**Kang and Fung, 2000; Wu and Fung, 2001**) *Escherichia coli* O157:H7, *Listeria monocytogenes, Staphylococcus aureus* and *Yersinia enterocolitica* (**Wu and Fung, 2001**) and recovered significantly more cells for each organism than the respective selective agar alone. Interestingly, the findings of this study do not correlate with previous studies in that there was no significant difference between the selective, non-selective and TAL agars in terms of number of bacteria recovered ($P > 0.05$). This was apparent in pure and mixed cultures and both before and after heat treatment for 10 minutes at 55°C (See figures 1 and 2). This experiment was repeated using TAL plates that incorporated agar selective for *E.coli* (Harlequin E.coli/Coliform Medium). As was observed in previous studies (**Wu and Fung, 2000; Kang and Fung, 2001**), a significant reduction in the number of viable *E.coli* cells occurred.

The World Health Organisation (WHO) recommends that water should be no less than 70°C for the reconstitution of powdered infant formula. This experiment was therefore repeated at this higher temperature to observe any effect on the recovery of *C.sakazakii* (ATCC 29544) cells. There was a very significant drop in bacterial numbers after treatment at this temperature for 10 minutes ($P < 0.01$). This finding was expected as it has been shown that exposure of species of *Cronobacter* to temperatures above 68°C for even short periods of time can significantly reduce the number of surviving cells **(Nazarowec-White & Farber, 1997a)**. The TAL method again did not recover significantly more cells than the other agars used ($P > 0.05$). In the mixed culture experiment the TSA produced significantly higher counts than the other agars ($P < 0.05$). The bacterial species used in this study share very similar colony morphology on this non-selective agar, increasing the likelihood of counting errors occurring. This agar is therefore not suitable for use in mixed culture assays and was included for comparison purposes only. It can be concluded that the TAL method for the recovery of heat-injured cells, in this case, is not significantly more effective than the use of selective agar alone. The volume of agar used and the added time for the preparation of plates for this method are therefore not justified. Further experiments utilised selective agar alone on the basis of these findings.

The influence of medium on *C.sakazakii* (ATCC 29544) survival

It has been suggested that the high total solids, fat and sugar content of PIF may have protective effects on bacteria during drying and reconstitution (**Kim *et al.*, 2008; Osaili & Forsythe, 2009**). This claim was investigated by comparing *C.sakazakii* (ATCC 29544) survival after heat treatment at 55°C and 70°C in PIF and peptone. After heat treatment at 55°C for 10 minutes, there was no significant drop in the number of *C.sakazakii* (ATCC 29544) recovered for either medium ($P > 0.05$). This correlates with the data generated previously from the TAL method evaluation. After heat treatment at 70°C for 10 minutes there was a highly significant drop in the number of cells recovered for both media ($P < 0.001$). However there was no significant difference found between survival of *C.sakazakii* (ATCC 29544) in PIF and survival in peptone ($P > 0.05$). This is an indication that PIF does not incur any protective benefits in terms of prolonged survival at higher temperatures in a liquid medium. The composition of PIF may, however, improve the survival of *Cronobacter* spp. during desiccation. The desiccation resistance and persistence of these bacteria in infant formula has been reviewed previously (**Osaili & Forsythe, 2009**) and will therefore not be discussed in detail here.

A notable finding of this study was that there was no significant drop in *C.sakazakii* (ATCC 29544) numbers after the heat treatment at 55°C ($P > 0.05$). The five foodborne pathogens mentioned that have been previously used to evaluate this method, all dropped significantly in terms of CFU/ml after the heat treatment step described (**Wu and Fung, 2001; Kang and Fung, 2000**). It has been shown that *C.sakazakii* has an array of survival mechanisms which include resistance to desiccation and osmotic stresses, in particular the ability to synthesise a capsule under certain conditions (**Osaili and Forsythe, 2009**). This may explain the apparent lack of effect observed for the heat treatment step used here. This was investigated further by repeating the heat treatment at 55˚C for 10 minutes with the inclusion of six additional strains of *Cronobacter* and the type strain, *E.coli* (ATCC 25922). This was done in order to establish any differences between strains in terms of heat tolerance at this temperature in PIF. As shown in figure 4, the tolerance to heat treatment at this temperature was not present in all strains of *Cronobacter*. Four out of the seven strains tested were not affected or showed only a slight decrease in number after the treatment while *C.sakazakii* (ATCC 29004), *C.sakazakii* (ATCC 12868) and *C.muytjensii* (ATCC 51329) numbers were significantly reduced ($P < 0.05$) by the heat treatment. It has previously been reported that thermal resistance in this genus is strain dependant (**Strydom *et al.*, 2012**) but in contrast to these findings, other studies have reported no significant difference between strains (**Iversen *et al.*, 2004**). This inconsistency in findings to date highlights the importance of studies such as the one conducted here in terms of elucidating the survival and growth characteristics of these organisms in PIF.

It has previously been shown that pasteurisation is sufficient to eliminate *Enterobacteriacae* including *Cronobacter* spp. **(Lin & Beuchat, 2007)**. One potential route of entry into the powdered infant formula (PIF) production line and/or the products is the post-pasteurisation addition of plant-derived supplements such as starches and proteins, that are potentially contaminated with *Cronobacter* spp., without the use of an additional heating step (**Schmid *et al.*, 2009**). Some studies have suggested that the high prevalence of Cronobacter spp. in PIF may be, in part, due to an unusually high tolerance to dry conditions. This is thought to be inferred by the organism's ability to produce trehalose. Trehalose is a compatible solute which protects bacteria from dry conditions by stabilising phospholipid membranes and proteins (**Kim et al., 2008**). While the importance of trehalose production has not yet been fully elucidated, it is clear that the organism has an array of survival mechanisms which includes resistance to desiccation and osmotic stresses, in particular the ability to synthesise a capsule under certain conditions (**Osaili and Forsythe, 2009**). It is possible that the findings of this study, showing that some strains possess a tolerance to the heat treatment described, may be due to the presence of such an ability or mechanism. Further research into the cause of the observed tolerance to heat treatment and the reasons for its absence in certain strains of *Cronobacter* may yield useful insights into the control and prevention of infections caused by the presence of these organisms in PIF.

Influence of reconstitution temperature on *C.sakazakii* (ATCC 29544) survival and growth in PIF

As previously stated, the World Health Organisation (WHO) recommends reconstitution of PIF using boiled water that has cooled slightly but not below a temperature of 70°C. It is recommended that this be checked using a sterile thermometer (**FAO & WHO, 2004**). Manufacturers of PIF most often recommend using boiled water which has been cooled for 30 minutes before use for the preparation of PIF. In order to compare these methods in terms of *C.sakazakii* (ATCC 29544) survival in reconstituted PIF an average temperature for water cooled at room temperature for 30 minutes was calculated. On average after 30 minutes of cooling the water had cooled to 68°C ± 2.9˚C. A standard volume (500ml) and controlled conditions were used in this experiment but it can be presumed that in the home/hospital setting the volume of water and the size and type of container used would also have an effect on the rate of cooling and increase the variability of the rate of cooling. A range of temperatures were therefore selected for testing (55, 60, 65 and 70°C).

As expected, the addition of water cooled to 70˚C caused an immediate and highly significant drop in bacterial numbers in comparison to the other temperatures tested ($P < 0.001$). However, bacterial growth at room temperature did occur in the following two hours, represented in figure 5 by a significant increase in log CFU/ml from the time of reconstitution ($P < 0.05$). At each testing interval the difference between the 70˚C reconstituted PIF and the three other temperatures tested remained significant ($P < 0.05$). This indicates that even a small decrease in temperature from the recommended 70˚C to 65˚C would cause a significant increase in the survival rate of any intrinsic *Cronobacter* contamination in the PIF, subsequently increasing the risk of infection in the home/hospital setting. This drop of 5˚C was found to occur after only a 5-10 minutes deviation from the recommended 30 minute cooling period (data not shown). It should be noted that *Cronobacter* contamination in PIF would likely be at much lower levels than are represented here. It is likely therefore, that the log reduction in bacterial numbers caused by the reconstitution at 70˚C would be sufficient to completely eliminate the low levels of viable cells seen in contaminated batches of PIF. These findings emphasise the importance of complying with WHO recommendations for the safe preparation of PIF products and suggests that the current manufacturer instructions may be inadequate to prevent future outbreaks of *Cronobacter* infection. It is suggested that additional instructions regarding the use of a set volume of water and the checking of temperature with a sterile thermometer be adopted by manufacturers of PIF.

CONCLUSION

It can be concluded that that the temperature of water used for reconstitution has a very significant effect on the survival and growth of *C.sakazakii* (ATCC 29544) in PIF. Tolerance to heat treatment at 55˚C for 10 minutes was found in multiple strains but was absent in some indicating that certain traits are strain dependent. Future research should include the effect of reconstitution temperature on biofilm formation and growth at 4˚C, factors which may also be contributing to the risk of infection from these organisms. It is recommended that manufacturers of PIF should review their instructions to consumers for the preparation of this product in order to ensure the lowest possible risk of infection from these opportunistic pathogens.

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