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EXTRACELLULAR PECTINASE ACTIVITY FROM *Bacillus Cereus GC Subgroup A*: ISOLATION, PRODUCTION, OPTIMIZATION AND PARTIAL CHARACTERISATION

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ABSTRACT

A pectin degrading bacterium was isolated from a soil and identified as *Bacillus cereus GC subgroup A* based on its morphological, physiochemical and FAME-GC analysis. The Solid state fermentation at 37 °C for 72h, strain produced pectinase in different substrates; among the substrates orange peel powder and wheat bran powder were good for strain but mixture of orange peel powder and wheat bran powder enhanced enzyme production upto 804 IU/ml. The maximum pectinase production was studied at 37^0 C and pH 7 after 96h of incubation by *Bacillus cereus*. Regarding carbon sources, galactose (0.02%) was the best source for strain; while malt extract (0.1%) was found as the best nitrogen source for *Bacillus cereus.* The enzyme was partially purified by ammonium sulphate precipitation and dialysis was carried out to remove the excess salt. A Lineweaver-Burk analysis showed a *Km* value of 8.3 mg/ml and *Vmax* of 1428.5 IU/ml. The partially purified enzyme exhibited maximal activity at a temperature of 40°C and pH 6.0. The enzyme showed stability towards salts at different concentrations 1mM, 10mM, 50mM and 100mM. In addition to that $CaCl_2$ has increased activity of enzyme at higher concentration too. The amino acid composition of pectinase was also determined by BIOEDIT software. The molecular weight of pectinase was 38304.27 Da (348 amino acids) and was rich in glycine and valine.

Keywords: Pectinase, *Bacillus Cereus,* Fame-GC analysis, Amino acid composition

INTRODUCTION

Pectin or pectic substances are heterogeneous group of high molecular weight, complex, acidic structural polysaccharides with a backbone of galacturonic acid residues linked by α-(1-4) linkages **(Alphons *et al.* 2009; Biswapriya *et al.* 2011)**. They constitute major components of the middle lamella, a thin layer of adhesive extracellular material found between the primary cell walls of adjacent young plant cells **(Kapoor *et.al.* 2001; Hoondal *et.al.* 2000)**. Pectin is known to contain neutral sugars like xylose, Galactose and arabinose which are present in side chains. Pectinases are produced by many organisms such as bacteria **(Horikoshi 1972; Karbassi and Vaughan 1980),** fungi **(Aguillar and Huitron 1990)** and yeasts **(Gainvors and Belarbi. 1995)**. Pectic enzymes have two classes namely, pectin esterases and pectin depolymerases. Pectin esterase has the ability to de-esterifies pectin by the removal of methoxy residues. Pectin depolymerases readily breakdown the main chain of carbohydrate and it was further classified as polygalacturonase (PG) and pectin lyases (PL) Thus, pectinases are group of hydrolytic enzymes, which hydrolyze the pectin molecules and are readily soluble in water. Microbial pectinases account for 10-25% of the global food and industrial enzyme sales **(Singh *et al.* 1999a; Jayani *et al.* 2005; Murad and Azzaz 2011)** and their market is increasing day by day. These are used extensively for fruit juice clarification, juice extraction, manufacture of pectin free starch, refinement of vegetable fibres, degumming of natural fibres, wastewater treatment, curing of coffee, cocoa and tobacco and as an analytical tool in the assessment of plant products **(Alkorta *et al.* 1998)**. Optimization of media is very important to maximize the yield and productivity, and minimize the product cost **(Singh *et al.* 1999b)**. The aim of this study was to isolate the most prominent pectinolytic bacteria from soil samples with unique properties and optimize their fermentation conditions for maximum pectinase production. Owing to the expensive medium ingredients, solid state fermentation (SSF) was tried using wheat bran, orange peel as a carbon source separately and in combinations in order to find out cheap and suitable natural source for production of this industrially important pectinase enzyme.

MATERIALS AND METHODS:

Isolation, Selection and Maintenance of culture

In view of getting efficient pectin degrading cultures, soils rich in pectic waste and fruit waste samples were scrutinized from fruit processing area, sewage of juice centres of different locations. The 1g of all sample were inoculated in 100 ml of pectin broth (0.1% w/v yeast extract, 0.2% w/v $NaNO_3$, 0.1% KH_2PO_4, 0.05% KCl , 0.05% $MgSO_4.7H_2O$, pH 6.0) supplemented with 0.2% w/v pectin and incubated on rotary shaker 120 rpm at 37 °C for 7 days. 0.1ml of previously enriched culture was inoculated in 100 ml of freshly prepared pectin broth supplemented with 0.5% w/v of pectin for next 7 days of enrichment. After several serial transfers in pectin broth, each time with increasing pectin concentration up to 2.0%, the soil sample was finally subjected for isolation of pectinase producers. After fifth week of enrichment, 0.1 ml of broth was spread on the surface of pectin containing agar medium plate and incubated at 37 °C for 24h. Different bacterial colonies were picked from each plate and streaked on the same medium. The pure cultures of bacteria used in the present investigation were transferred on to the slants of same composition and incubated at the same optimized conditions. Periodic transfers were made at regular intervals and every time.

Preliminary screening of pectinase producing bacterial isolates by well plate method

Purified bacterial isolates were preliminary screened for pectinase activity by well plate method **(Hannan *et al.* 2009)**. Supernatant from overnight incubated bacterial cultures (1.0 optical density at 600 nm) were used in all experiments to determine the pectinase activity. Modified medium containing 0.3% KH_2PO_4, 0.6% Na_2HPO_4, 0.2% NH_4Cl, 0.5% NaCl, 0.01% $MgSO_4.7H_2O$, 1.5% agar supplemented with 0.2 % pectin were prepared. After solidification of the medium, well of 5 mm in diameter were made in the agar with the help of cork-borer and filled with 50 µl of cell supernatant. After incubation for 24 h at 37°C, plates were flooded with iodine solution containing 0.25% iodine, 0.5% potassium iodide and 30 ml of 20% ethanol **(Cappuccino and Sherman 2002)**.

The pectinase activity was observed by a clear zone around the well. The result was observed by measuring the diameter of the clear zone.

Morphological and biochemical characterization of pectinase producing bacterial isolates

Biochemically, isolated strain of the bacterium was characterized by using carbohydrate utilization tests. Tests were also carried out to determine reaction with casein, starch, gelatine hydrolysis, catalase and determination of nitrate reduction **(Aneja 2007; Peter *et al.* 1986)**.

FAME-GC Analysis

The cultures were obtained from soil sample and pure cultured onto Trypticase Soy Broth Agar (TSBA) media. The fatty acids were extracted from loop full of culture with 65% chloroform and 35% methanol. 0.7% NaCl solution was added to the crude extract to remove methanol and water soluble components of extract. The aqueous layer was siphoned off and the organic phase was dried by anhydrous sodium sulphate. The pooled dried extract was concentrated under a stream of nitrogen. The recovered lipid was reconstituted in 0.5 M methanolic KOH and hydrolyzed. Hexane and methyl tert-butyl ether were used to quench the reaction. The recovered organic phase was pooled and analyzed by GC. As the bacteria are killed in the saponification step of the extraction, there is little infectivity concern with handling of the sample once this step is concluded. FAMEs are more volatile than their respective fatty acids and therefore more suitable to GC analysis. The Sherlock software automates all analytical operations and uses a sophisticated pattern recognition algorithm to match the unknown FAME profile to the stored library entries for identification **(Anju Rajan *et al.* 2011)**.

Optimization of pectinase production

The optimization study of the following parameters was done for better growth and production for enzyme.

Effect of pH on pectinase production

50 ml of production medium was prepared and pH of the medium was adjusted to 2, 3, 4, 5, 6, 7, 8 and 9.The sterilized production medium were inoculated and incubated for 3 days under the shaking condition at 37°C. The enzyme activity was studied. Sterile uninoculated production medium was used as blank for pectinase assay.

Effect of carbon and nitrogen source on pectinase production

Different carbon sources (0.2 g/l; Sorbitol, Lactose, Starch, Glucose, Sucrose, Fructose, maltose and Galactose) and nitrogen sources (1 g/l; Urea, Ammonium nitrate and ammonium sulphate, Malt extract, Beef extract, peptone and tryptone) were chosen .The effect of these different carbon and nitrogen sources on pectinase production under optimal pH 7.0 and temperature 37°C were examined.

Solid state fermentation

The effect of different substrates (orange peel, wheat bran and orange peel+ wheat bran) was studied. The substrates were obtained by drying the peels of orange in an oven maintained at 50°C till they were completely dried and whereas wheat bran was purchase dried form. The dried peels and the wheat bran were then grinded to powder. Solid state fermentation (SSF) was carried out in 250 ml Erlenmeyer flasks that contained 5 g of orange peel/wheat bran /wheat bran+ orange peel and 5 ml of distilled water (moistening agent). The flasks were sterilized at 121°C for 15 minutes. 1ml of inoculum was added, mixed well and incubated at 37°C for 72h.At the end of incubation period, the flasks were taken out and the content of each flask were extracted with 25 ml of sterile distilled water. Petri plates containing autoclaved modified MS medium supplemented with 0.2 % pectin were prepared. After solidification of the medium, well of 5 mm in diameter were made in the agar with the help of cork-borer and filled with 50 µl of cell supernatant. After incubation for 24 h at 37°C, plates were flooded with iodine solution. The pectinase activity was observed by a clear zone around the well. The activity of pectinase was measured according to standard method (Miller 1959).

Production of pectinase

Fermentations were carried out by inoculating the culture into a liquid medium composed of (0.2% $NaNO_3$, 0.1% KH_2PO_4, 0.05% KCl and 0.05% $MgSO_4$ at pH 7), 0.1% yeast extract and substrate as the main carbon source pectin at 0.5%. After incubation for 96 hours at 37°C under shaking conditions, the media was centrifuged. The supernatant was the source of enzymatic extract and was used for pectinase activity assay.

Pectinase Assay

Pectinase activity was measured according to Miller's method **(Miller 1959)**. Briefly, 0.5ml of cell free supernatant was incubated with 0.5ml of pectin in 0.1M acetate buffer with pH 6.0 and the reaction mixture was incubated at 40°C for 10 minutes in static condition. After adding 1ml of DNS reagent, the mixture was boiled for 5 minutes at 90°C. The reaction was stopped by adding 1ml of Rochelle's salt. Then the mixture was diluted by adding 2ml of de-ionized water. The absorbance was measured spectrophotometrically at 595 nm. A standard graph was generated using standard glucose solution. One unit of Pectinase activity was defined as the amount of enzyme which liberated 1µM glucose per minutes.

Determination of Protein Concentration

Protein of all enzymatic preparations was determined according to Lowry **[Lowry *et al.* 1951]** using bovine serum albumin as the standard. Readings were carried out in a spectrophotometer at 660 nm.

Purification of pectinase enzyme

Ammonium sulphate precipitation

Culture filtrate solution was treated with 70% of saturated ammonium sulphate solution. The crude enzyme was continuously stirred using magnetic stirrer and kept at 4°C for overnight, the enzyme was centrifuged at 10,000 rpm for 15 minutes, then supernatant was discarded and the pellets were dissolved in minimum volume of 0.5M phosphate buffer of pH 7.

Dialysis

About 8cm of the dialysis tube was cut and placed in 100 ml of 2% w/v sodium bicarbonate. 1 mM EDTA was added to chelate any metal ions. It was boiled for 10 minutes and was again washed in boiling distilled water for 10 minutes. The boiling process was repeated with distilled water again. The activated dialysis bag was filled with the enzymes and sealed from the both sides without any air bubbles. The bag was kept in 500ml of 50 mM phosphate buffer (pH 7.0) solution on a magnetic stirrer in ice cold condition for 8 hours. The buffer was changed frequently for every hour to avoid equilibration.

Effect of pH on catalytic activity of pectinase

The activity of pectinase was evaluated at different pH values. The partially purified enzyme was incubated using 0.1 M of buffers, in the range between pH 3 - 10, under assay conditions and the amount of glucose liberated was determined. Buffers used were sodium-acetate (pH 3.0 - 6.0), sodium-phosphate (pH-7), Tris-HCl (pH 8.0 - 10). The enzyme was incubated for 10 minutes at 40°C at different pH in the presence of substrate (10mg/ml) and the enzyme activity was determined by Miller's method.

Effect of Temperature on catalytic activity of pectinase

The pectinase activity was tested at different temperatures viz., 10 to 80°C. Sodium-phosphate buffer (pH-7) to maintain optimum pH of enzyme was used in the system and the enzyme substrate reaction was carried out at different temperatures, after which the velocity of enzyme reaction was measured.

Effect of Different Salts on catalytic activity of pectinase

The effect of salts (i.e. NaCl, $CaCl_2$ and NH_4Cl) on the enzyme activity were tested at different concentrations (1mM, 10mM, 50mM and 100mM) by incubating with the purified enzyme. After the exposure, enzyme activity of each sample was measured by Miller's method.

Determination of K_m and V_{max} of pectinase

The Michaelis constant K_m and maximal velocity V_{max} of the purified enzyme were determined using pectin as substrate in the range of 5-30 mg with the help of Lineweaver-Burk plot **(Lineweaver and Burk 1934)** relating 1 / [V] to 1 / [S]**.**

Amino Acid Composition

Amino acid sequences of *Bacillus sp.* pectinase was retrieved from NCBI database (http://www.ncbi.nlm.nih.gov/) in FASTA format. Amino acid composition of retrieved sequences was done using BIOEDIT software.

RESULTS AND DISCUSSION

Pectinase positive colonies were screened by formation of clear zone around colonies. Figure 1 shows the bacterium producing pectinase enzyme, a clear zone around the well as the pectin near the well is utilized. Depending upon the zone of clearance, the isolate was selected for further experimental studies. Morphologically, it is gram positive and rod shaped bacterium, 3.0 – 5.2 µm in length and 0.4 – 0.7 µm in width growing at pH 7 and temperature 37°C. It produced oxidase, catalase, lipase, protease, gelatinase and amylase (Table 1). The Microbial Identification System (MIDI) for fatty acid methyl ester (FAME) analysis is a standard method for identification of microorganisms **(Schutter *et al.*2000)**. Whole cell fatty acids are converted to methyl esters and analyzed by gas chromatography. The fatty acid composition of the unknown is compared to a library of known organisms in order to find the closest match. The list of the fatty acids composition like straight Chain fatty acids 15.63%, branched chain fatty acid 83.34%, Mono Unsaturated Fatty Acid 0.34% and oleic acid (C18:1) w9c 0.69 % was given clearly according to the GC report **(Anju Rajan *et al.* 2011).** The chromatogram obtained in this experimental analysis (Figure 2) is more descriptive and elaborative. Our experimental data matches and establish the similar result mentioned in the report of MIDI Sherlock software databases and the similarity was matched with *Bacillus-cereus-GC subgroup A*. The mostly concerned genus was *Bacillus* and it reported 0.736 similarity indexes. Based on morphological, physiological, biochemical characteristics and FAME-GC analysis it was identified as *Bacillus Cereus subgroup A*. The production of enzyme was carried out at the optimized condition 37° C for 96 h at pH 7. The enzyme was purified using 70% ammonium sulphate precipitation and dialyzed against 50mM phosphate buffer of pH 7 for overnight at 4°C. The results of purification steps of pectinase of *Bacillus Cereus* are presented in (Table 2). After the two steps of purification, the purification fold was 2.5. The pH optimization study has reported, pectinase production in *Bacillus cereus* in a modest range (pH 6 to 8) (Figure-3). There was near about loss of pectinase activity at pH values less than 4.0 and more than 9.0. As per literature study, optimal pH range (8.9 to 9.4) reported for pectinase of *Bacillus polymyxa* **(Nagel and Vaughn 1961)**; pH 9.0 for *Bacillus stareothermophillus* **(Karbassi and Vaughn 1980)** and pH 10.0 reported for *Bacillus sp.* RK9 **(Fogarty and Kelly 1983)**. The isolate was capable of utilizing a wide variety of carbon sources. However, galactose was the best carbon source in the present study (Figure-4). Six different nitrogen sources such as Beef extract, Malt extract, Peptone, Ammonium sulphate and tryptone were tested for pectinase production in *Bacillus cereus.* Among them Malt extract supported a maximum enzyme activity (Figure-5). Partial characterization study shows that *Bacillus cereus* could grow well at 37°C (optimum growth temperature), at pH 6 (Figure-6). The temperature stability profile of pectinase activity revealed that the enzyme is maximally active at moderately high temperatures ranging from 40 to 60ºC with highest activity detected at 40ºC incubation temperature for 1 hour (Figure-7). This temperature stability of *Bacillus sp.* DT7 is higher than the values reported by **(Nagel and Hasegawa 1968)** for the crude enzyme of a *Bacillus sp.* (below 30°C) and by **(Nagel and Vaughn 1961)** for the crude enzyme of *B. polymyxa* (45ºC) but less than the values reported by **(Horikoshi 1972)** for *Bacillus* (65°C) and by **(Karbassi and Vaughn 1980)** for *B. stearothermophilus* (70ºC). A further increase in the reaction temperature caused significant drop in the pectinase activity. Similarly, temperatures lower than 40°C resulted in decrease in the pectinase activity. The purified enzyme was sensitive to some of the salts tested at a concentration of 1mM, 10 mM, 50mM and 100mM. The presence of $CaCl_2$, NaCl acted as a stimulator of pectinase activity resulting in an increase of in the enzyme activity, whereas NH_4Cl decreased pectinase activity (Table-3).The K_m *and* V_{max} of pectinase towards pectin were determined. The apparent K_m *and* V_{max} of the pectinase for pectin were 8.3 mg/ml and 1428.5 IU/ml, respectively (Figure-8). The primary sequence of the enzyme was retrieved from NCBI database http://www.ncbi.nlm.nih.gov/) having GenBank accession No: AGK82226.1. The sequence obtained is given below.

MMRSSIVKLHAFSEVVIQLWLYGVSSVTADLSTPNLGLQGFATLDGGTTT
VGGLGGEIVFVRNELINALKSKNPNRPLTIYVNGTITPSNTSNSKISDKDVS
NVSILGVGTNGRLNGIGIKVIIRNIHFEGFYMEDDPRGKKYDFDYINVENS
HHIWIDHCTFVNGNDGAVDIKKYSNYITVAYNITFSHDKVSLGGSSDKEG
NSEAGHYDRNITFHHNYFKTLNSRVPARFFGKAHLCSNYFENMRTGVSG
NVFRAEMLVEHNVFENATNPLGFPIYGVAGAMGAKVHVEGFYYCKEPE
VRPVEEGKPALDPREYYDYTLDPVQDVPKIVVDGAGAGKLVFGLITIA

The molecular weight obtained from BIOEDIT data (348 amino acids) was 38304.27 Da and the amino acid composition of pectinase was mentioned in (Table 4**).** The data shows, pectinase enzyme was rich in glycine and valine (Figure-9). In concern with the use of various agricultural waste and agro-industrial by-products, in the present study suggested that mixture of orange peel and wheat bran found to be the best substrate for pectinase production by *Bacillus cereus.* The Enzyme activity of the pectinase enzyme was obtained with 0.5% of Orange peel powder and 0.5% of wheat bran powder 166 IU/ml and 333IU/ml respectively after 72 h of incubation at pH 7 and temperature 37°C whereas enzyme activity of pectinase enzyme was obtained 804 IU/ml with mixture of both the substrate after 72 h of incubation at pH 7 and temperature 37°C (Figure-10). The similar studies were also carried out by **(Silva *et.al.* 2002)**. In order to use enzyme from the isolates for commercial applications, it must have desirable biochemical, physiochemical characteristics and low cost of production. Orange bagasse, wheat bran is very cheap, abundantly available. Its dumping in nature causes pollution problem; hence its eco-friendly utilization is essential which tempted to use agro-waste for pectinase production by solid state fermentation.

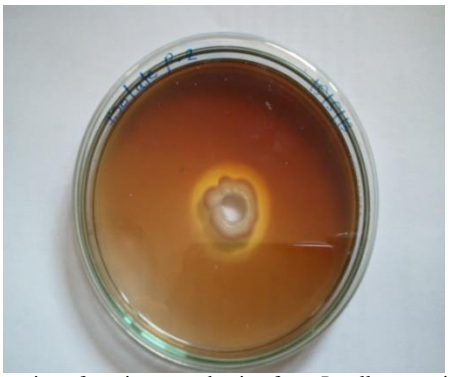

Figure 1 Screening of pectinase production from *Bacillus sp.* using pectin as substrate

Table 1 Morphological and biochemical characterization of isolated strain

Characters	Strain
Colonies	Large, irregular and flat with undulate margin
Morphology	Rod, Beta haemolytic
Gram nature	+
Motility	+
Oxidase	+
Catalase	+
pH Range	5-9
Temperature	20-60
Urease	-
Nitrate reduction	+
H_2S Production	-
Indole Production	-
Lipase	+
Protease	+
Hydrolysis of:	
Casein	+
Gelatin	+
Starch	+
Utilization of:	
Glucose	+
Lactose	-

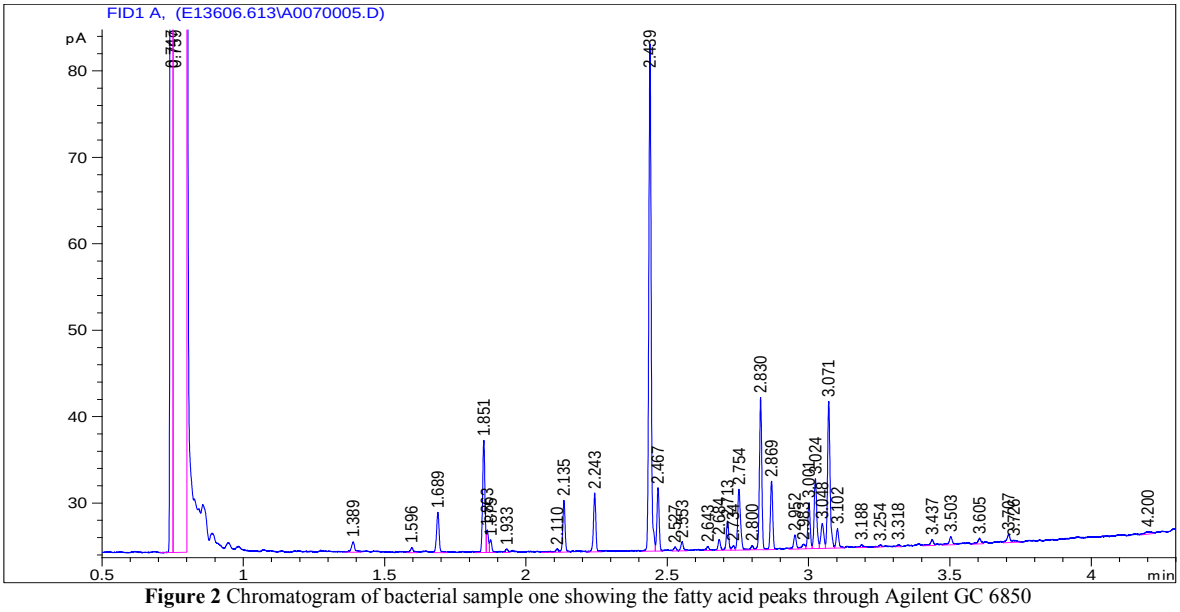

Figure 2 Chromatogram of bacterial sample one showing the fatty acid peaks through Agilent GC 6850

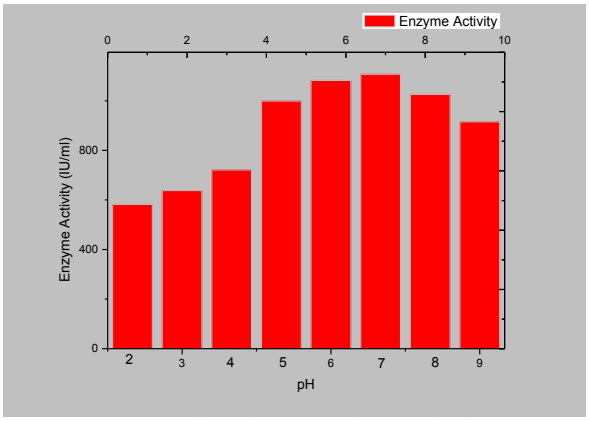

Figure 3 Effect of pH on pectinase production

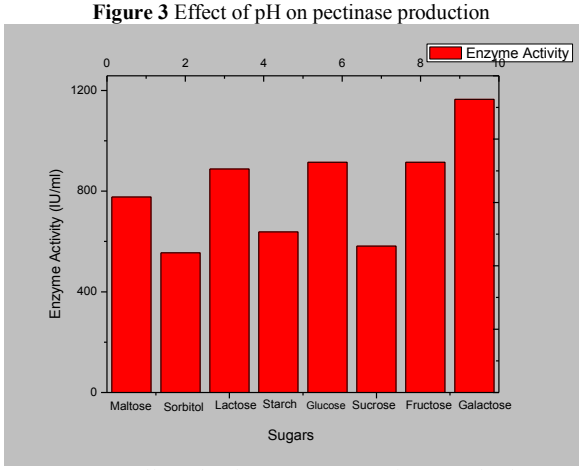

Figure 4 Effect of carbon source on pectinase production

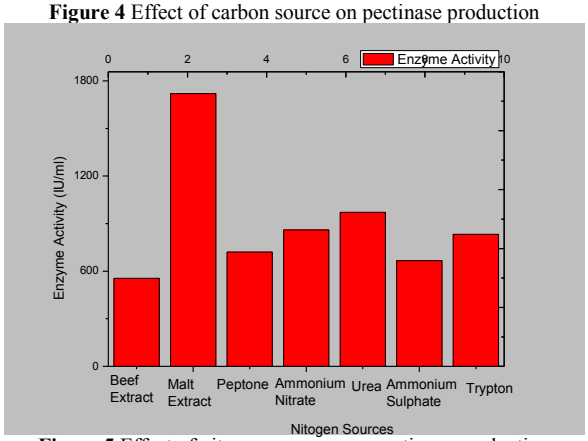

Figure 5 Effect of nitrogen sources on pectinase production

Table 2 Purification and recovery of pectinase from *Bacillus Cereus*

Steps	Pectinase activity (U/ml)	Total protein (mg/ml)	Specific activity (U/mg)	Purification fold	Recovery (%)
Crude extract	888	6	116.4	1	100
70% Ammonium sulphate precipitation	582	5	148	1.2	83.33
Dialysis	582	2	291	2.5	40

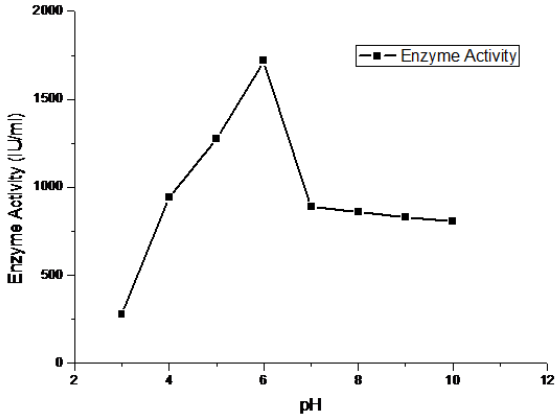

Figure 6 Effect of pH on purified pectinase

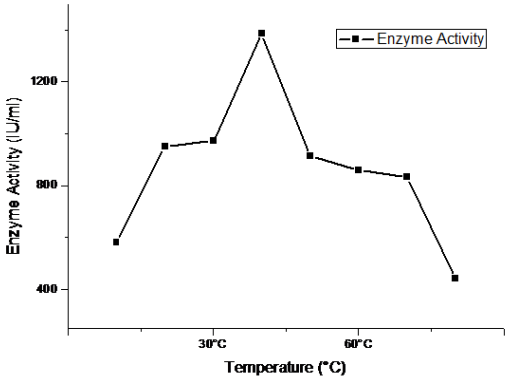

Figure 7 Effect of temperature on purified pectinase

Table 3 Effect of different salts on pectinase activity

Activator or Inhibitor	Relative Activity (%)			
	10^{-3}M	1×10^{-2}M	5×10^{-2}M	10^{-1}M
Control	100	100	100	100
NaCl	100±0.12	106±0.28	120±0.34	140±0.36
NH_4Cl	147±0.21	142±0.28	128±0.14	123±0.23
$CaCl_2$	110±0.11	147±0.06	305±0.32	324±0.16

Values are mean ± Standard deviation, n=3

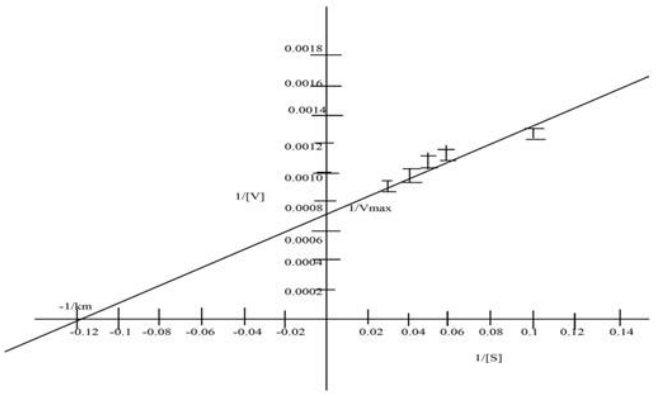

Figure 8 Determinutesation of *Km* and *Vmax* of pectinase from. *Bacillus Cereus GC subgroup A*.

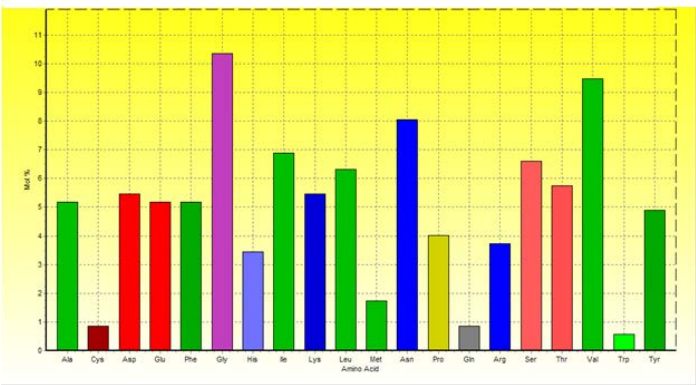

Figure 9 Amino Acid Composition Of pectinase from *Bacillus sp.*

Table 4 Amino acid contents (mole %) of *Bacillus sp.* Pectinase protein

Protein: pectinase Length = 348 amino acids Molecular Weight = 38304.27 Daltons		
Amino Acid	Number	Mole (%)
Ala A	18	5.17
Cys C	3	0.86
Asp D	19	5.46
Glu E	18	5.17
Phe F	18	5.17
Gly G	36	10.34
His H	12	3.45
Ile I	24	6.90
Lys K	19	5.46
Leu L	22	6.32
Met M	6	1.72
Asn N	28	8.05
Pro P	14	4.02
Gln Q	3	0.86
Arg R	13	3.74
Ser S	23	6.61
Thr T	20	5.75
Val V	33	9.48
Trp W	2	0.57
Tyr Y	17	4.89

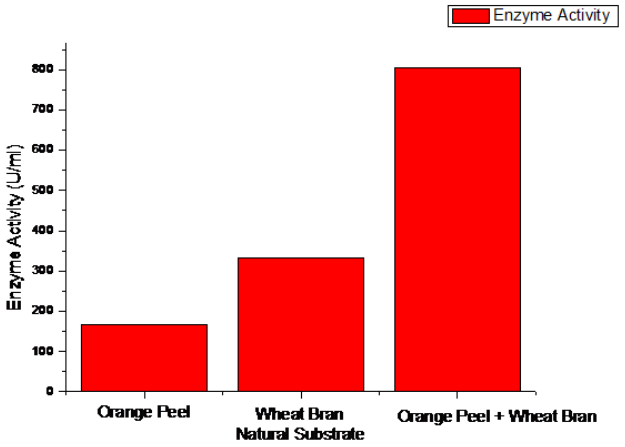

Figure 10 Effect of Natural substrate on pectinase production

CONCLUSION

In this study, the isolate *Bacillus cereus GC subgroup A* was identified by morphological, biochemical and FAME-GC Analysis. The culture conditions were optimized for pectinase production. The bacterium Bacillus *cereus subgroup-A* were produced significant amount of pectinase after 72 h of incubation in fermentation medium at 37°C and pH 7. Previous reports on the production of pectinase by *Bacillus sp.* under solid state fermentation is less. Since the Solid state Fermentation has of special economic interest of countries with large amount of agro-industrial residues such as wheat bran and orange peel can be used as carbon source for pectinase production in large scale. Large scale-up studies are needed for better output for commercial production. Some additional features like enhanced production with the addition of $CaCl_2$; shorter period of incubation for pectinase production, lesser amount of pectin in the growth medium indicate the potential of this organism to be used at commercial level for degumming of ramie, pre-treatment of waste water from fruit juice-processing industries.

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IMPLICATIONS OF ANTIBIOTIC RESISTANCES PRODUCED BY PHENOTHIAZINES IN *Mycobacterium tuberculosis*

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ABSTRACT

Several chemotherapeutic agents applied to human beings for past few decades for different ailments, have been found to possess potent antitubercular activity. Two such agents, methdilazine (Md) and promethazine (Pz) were used to select mycobacterial mutants resistant to themselves at different levels and tested to determine if such mutants simultaneously developed cross-resistance to known antitubercular drugs. Mutants were produced by application of a heavy inoculum on Lowenstein-Jensen medium containing Md (or Pz) at concentrations higher than their respective minimum inhibitory concentrations (MICs). These were then tested to find out if such mutants have changed their resistogramme pattern with respect to the test antitubercular agents. Certain first-step Md-mutants became simultaneously resistant to ethambutol and pyrazinamide; while the step-2 Md-mutants revealed further increase in resistance to these agents along with resistance to isoniazid, rifampicin and streptomycin as well. In the study with Pz it was noted that many mutants showed distinctly higher levels of resistance to all the test drugs, particularly to isoniazid, pyrazinamide and streptomycin. The levels of MIC were distinctly high in many mutants. These observations on cross-resistances seem to be best explained on the basis of a reduction in cell-membrane permeability acting in a non-specific manner. The role of such a cross-resistance may possibly be accounting for an overall increase in the MICs of many drugs against several groups of microorganisms including *Mycobacterium tuberculosis* and other species of *Mycobacterium* during the last five decades.

Keywords: Cross-resistance; *Mycobacterium tuberculosis*; phenothiazines; mutants; antitubercular agents

INTRODUCTION

Tuberculosis is a remarkably high risk communicable disease of human beings. The causative organism *Mycobacterium tuberculosis* is airborne and is often transmitted among people of lower income group suffering from malnutrition and immunological deficiencies. The World Health Organization (**WHO, 2013**) reported a total of 8.6 million new cases of tuberculosis and 1.3 million deaths due to this infection in 2012. In the treatment regime referred as Directly Observed Treatment Schedule (DOTS) isoniazid, rifampicin, ethambutol and pyrazinamide are administered for two months after presumptive diagnosis. Subsequently isoniazid and rifampicin are continued in the same patients for 4 to 7 months depending on the severity of infection. However, now there are more virulent forms designated as multi-drug resistant strains that are resistant to isoniazid and rifampicin. Apart from this many strains are found to be resistant to isoniazid, rifampicin, streptomycin, any fluoroquinilone plus any of the antitubercular injectables like amikacin/ kanamycin/ capreomycin. Such strains have evolved due to misuse or overuse of the scheduled drugs or failure in continuation of the correct therapy. These strains may also arise if the treatment schedule is allowed to continue for more than 12 months. Simultaneous application of so many drugs creates sufficient pressure for the causative organism to select multi-drug resistant mutants. Such a situation could not have occurred by a simple mode of action since different drugs have different sites of action. The modes of action of anti-tubercular drugs are very varied, although structurally similar drugs usually have the same mutated target.

The occurrence and prevalence of tuberculosis by drug resistant organisms initiated systematic search for antimycobacterial agents from various existing pharmacological agents by several groups of researchers in different parts of the world. Such studies revealed that antipsychotic and antihistaminic phenothiazines possess powerful antitubercular action. Most potent among these were thioridazine, methdilazine, trifluoperazine, chlorpromazine and promethazine (**Molnar et al., 1977; Kristiansen and Vergemann, 1986; Ratnakar and Murthy, 1993; Chakrabarty et al., 1993; Dutta et al., 2009; Crowle et al., 1992; Amaral et al.,1996; van Ingen et al., 2009; Advani et al., 2012; Kristiansen et al., 2015**). These were reported to be simultaneously active against a large number of Gram positive and Gram negative bacteria as well (**Kristiansen, 1979; Radhakrishnan et al., 1999; Mazumdar et al., 2001; Dastidar et al., 1995; Dastidar et al., 2004; Dastidar et al., 2013**).

Occurrence of cross-resistances among aminoglycosides in *Mycobacterium tuberculosis* was reported as far back as 1959 by several workers (**Torii et al., 1959; Tsukamura, 1959**). **Koseki & Okamoto (1963)** presented evidences for a significant change in resistance to viomycin caused by development of resistance to capreomycin in *Mycobacterium tuberculosis*. **Tsukamura (1969)** while trying to produce drug resistant mutants in the laboratory found that highly kanamycin resistant *M.tuberculosis* strains were resistant to capreomycin and strains moderately resistant to kanamycin were susceptible to capreomycin. He further observed that experimentally produced capreomycin resistant strains failed to develop resistance to kanamycin. Such a one way cross-resistance relationship was observed among many other aminoglycosides **(Tsukamura,1974)**. **Tsukamura and Mizuno (1975)** while trying to determine cross-resistance relationships among aminoglycosides in *M.tuberculosis* reported that resistances to several antibiotics could be produced by a single mutation to any one of the agents. In **2005 Maus et al** attempted to analyze cross-resistance to capreomycin, kanamycin, amikacin and viomycin in *M.tuberculosis* at molecular level. According to these authors mutation of the *thyA* gene confer capreomycin and viomycin resistance in *M.tuberculosis*. It is known that in mutations in the 16S rRNA gene (*rrs*) have been associated with resistance to all these four drugs (**Suzuki et al., 1998**). **Maus et al (2005)** reported three *rrs* mutations in their *M.tuberculosis* test strains each of which was associated with a particular cross-resistance pattern. They opined that when *M.tuberculosis* strains are exposed to one or two drugs phenotypic and genotypic differences can be seen in the development of antibiotic cross-resistance.

In the present study we have tried to determine the ability of antitubercular phenothiazine compounds methdilazine and promethazine to produce mutants resistant to themselves and also to detect if such mutants develop cross-resistances to known antitubercular agents.

MATERIAL AND METHODS

Strains

M.smegmatis 789, *M.phlei* L1, *M. avium 724, M.flavescens 1541, M. gordonae 1324, M.intracellulare* 1406, *M.tuberculosis* H_{37}Rv 102 and *M.tuberculosis* H_{37}Ra 16 were obtained from Dr V.M. Katoch, the then Director of National JALMA Institute for Leprosy and other Mycobacterial Diseases, Agra, India. Remaining three strains *M.tuberculosis* Bajaj 1, J15, and N23 were obtained from Dr A.N. Chakrabarty, Department of Medical Microbiology & Parasitology, Calcutta University College of Medicine, Kolkata, India. All the organisms were received as live culture slants in Lowenstein-Jensen Medium (LJM) from both the institutions and maintained in the same medium throughout the study.

Media

All the biological components were obtained from Oxoid (UK). Kirchner's Liquid Medium (KLM) and LJM were prepared as per established protocol (**Barrow and Feltham, 2003**).The growth was confirmed for *Mycobacterium* spp. after performing Z-N staining and different biochemical tests, like niacin test, nitrate reduction test and catalase test (**Kamerbeek et al., 1997**).

Overlay medium

This was prepared with 7H10 agar base, distilled water and glycerol, distributed in 2mL amounts and sterilized by autoclaving.

Inoculation

The known standard strains *M.tuberculosis* H_{37}Rv 102 and H_{37}Ra 16 along with all the other mycobacteria were grown in KLM, vortexed, diluted and standardized. Mcfarland standard 0.5 (in turbidity standard; the turbidity standard was prepared by adding 0.5 ml of a barium chloride solution to 99.5 ml of 1% H_2SO_4) was routinely taken for inoculation of all the strains and their mutants.

Media containing antitubercular drugs and other selecting agents

The agents were obtained in pure dry powder form from their manufacturers in India and stored at 4°C. To 2ml of overlay medium was added, any of the following agents: isoniazid acid hydrazide (INH), rifampicin (Rf), ethambutol (Eb), pyrazinamide (Pz), streptomycin (Sm), methdilazine (Md) and promethazine (Pz). The final concentration (µg/ml) of each of the agents were: 1,2,5,10,25,50,100, 200,400, 800, 1000 and 2000; these were added to the overlay medium before being allowed to flow over the freshly prepared LJM slants. All such bottles were inoculated for appearance of growth.

Determination of Minimum Inhibitory Concentration (MIC)

The MIC of the agents for different strains of *Mycobacterium* and their mutants were determined by inoculating the media as described above and incubating at 37°C. Appearance of growth was checked every day up to 3 weeks. Adequate strain and media controls were run throughout the tests.

Production and isolation of Md and Pz mutants

This was done by application of a heavy inoculum on LJM containing Md, the amount being higher than the MIC of the organism with respect to the agent. As the mutants developed they were designed as Step 1 mutants and were inoculated on LJM containing still higher amount of Md. The colonies developing on such a medium were designated as Step 2 mutant. In this way higher steps of mutants were selected with both Md and Pz. The relationship of the mutants with respect to the original wild types was determined with the help of various known and standardized morphological and biochemical parameters as described earlier.

RESULTS AND DISCUSSION

Sensitivity of bacteria

The minimum inhibitory concentration (MIC) of INH, Rf, Eb, Py,Sm,Md nd Pz with respect to 20 strains of *Mycobacterium* is presented on Table 1. These strains were selected on the basis of previous studies that had revealed that most of these were sensitive to the test drugs including the phenothiazines at low concentrations. The MIC of INH, Py and Sm in the recent clinical isolates *M.tuberculosis* Bajaj 1, J15 and N23 was rather high. In *M.tuberculosis* Bajaj 1, the MIC of INH and Sm was 25µg/ml. The strain N23 was even more resistant, the MIC of Py and Sm was as high as 50µg/ml. With respect to the phenothiazines, the MIC values ranged between 10 and 25µg/ml in most of the test strains; however, in case of Bajaj 1, J15 and N23 the MIC of Md and Pz was 50µg/ml.

Table 1 Minimum inhibitory concentration (MIC) of anti-tubercular drugs, methdilazine and promethazine against various strains of *Mycobacterium*

Mycobacterium	MIC (µg/ml)						
	INH	Rf	Eb	Py	Sm	Md	Pz
M.smegmatis 789	2	2	2	5	2	25	10
M.smegmatis 1546	2	2	2	5	2	10	25
M.fortuitum 1529	2	2	2	2	5	10	25
M.scrofulaceum 1323	2	1	2	2	2	10	10
M.avium 724	5	5	10	2	10	25	25
M.gordonae 1324	2	2	2	5	2	10	10
M.phlei L1	5	2	2	10	2	25	25
M.marinum 50	2	2	5	5	2	25	25
M.intracellulare 1406	5	5	5	5	2	10	25
M.flavescens 1541	2	2	5	5	2	10	10
M.terrae 1450	5	2	2	5	2	10	10
M.tuberculosis H_{37}Ra16	2	2	2	5	2	5	25
M.tuberculosis H_{37}Rv102	2	2	5	5	2	10	25
M.tuberculosis Bajaj1	25	5	5	5	25	50	50
M.tuberculosis J15	25	5	10	25	25	50	50
M.tuberculosis N23	25	5	5	50	50	50	50
M.tuberculosis K1	2	1	2	5	2	10	25
M.tuberculosis 2	2	1	5	2	2	10	25
ICRC bacillus	2	2	2	5	2	10	10
"Skinsness" bacillus	2	2	5	5	2	10	25

INH, isoniazid acid hydrazide; Rf, rifampicin; Eb, ethambutol; Py, pyrazinamide; Sm, streptomycin; Md, methdilazine; Pz, promethazine

Selection of mutants

All the 20 strains of *Mycobacterium* spp. were investigated for production of highly resistant Md mutants; of these 11 failed to produce mutants even after repeated tests. Nine strains of mycobacteria passaged on low but step-wise gradually rising concentrations of Md finally produced mutants of high resistance. The mutants were accepted only when the identification tests particularly with respect to cultural morphology in LJM coupled with acid fastness, established that they belonged to the respective wild-types. Following the same principle Pz mutants were selected. It may be pointed out here that *M. tuberculosis* Bajaj 1, J15, N23, H_{37}Rv102 and H_{37}Ra16 were able to develop fairly resistant mutants with respect to both Md and Pz. Among the others *M.phlei* L1, *M.flavescens* 1541, *M.avium* 724 and *M.gordonae* 1324 were able to produce Md- resistant mutants while 4 other strains of *Mycobacterium* could successfully develop Pz resistant mutants. The remaining 7 strains were unable to select drug resistant mutants.

Antibiotic cross-resistance patterns of Md and Pz-resistant mutants

It may be noted from Table 2 that in *M phlei* L1 the MIC of Md was 25 µg/ml, the first step mutant could be developed at 50 µg/ml of Md when the MIC of Md was found to be 100 µg/ml. From this the step 2 mutant of the same organism was developed whose MIC value of Md was 400 µg/ml. There was a gradual increase in the MIC values with respect to INH, Rf, Eb and Py while there was no change in MIC values with respect to Sm. A similar pattern was noted in *M.flavescens* 1541. However, in the Md- mutants of *M.avium* 724 and *M.gordonae* 1324, MIC values decreased in case of Sm and Py respectively. *M. tuberculosis* $H_{37}Rv$ 102 and Ra 16 being rather sensitive to test drugs, the levels of Md resistances in mutants of these strains were not so high and the changes in resistogramme pattern of mutants were not significant (Table 2). On the contrary, *M. tuberculosis* Bajaj 1, J15 and N23 being much less sensitive to the test agents, produced mutants at much higher levels of Md and simultaneously exhibited greater MICs with respect to all the antitubercular drugs except the strain N23 whose MIC value of Eb decreased in the mutants.

Table 2 Changing pattern of resistances of antimycobacterial drugs in methdilazine resistant mutants of *Mycobacterium* spp.

Mycobacteria	Types of cultures	MIC (µg/ml in LJM) of different agents for the wild type and mutant bacteria					
		INH	Rf	Eb	Py	Sm	Md
M.phlei L1	Wild Type	5	2	2	10	2	25
	Md mutant step 1 (50)	5	2	2	10	2	100
	Md mutant step 2 (200)	10	10	5	25	2	400
M.flavescens 1541	Wild Type	2	2	5	5	2	10
	Md mutant step 1 (25)	5	2	5	25	10	100
	Md mutant step 2(200)	5	2	10	25	25	500
M.avium 724	Wild Type	5	5	10	2	10	25
	Md mutant step 1 (50)	5	10	25	5	10	200
	Md mutant step 2(400)	5	10	25	5	5	100
M.gordonae 1324	Wild Type	2	2	2	5	2	10
	Md mutant step 1 (50)	2	2	2	2	2	200
	Md mutant step 2(400)	5	2	5	2	5	1000
M.tuberculosis Bajaj1	Wild Type	25	5	5	5	25	50
	Md mutant step 1 (100)	25	5	10	10	25	200
	Md mutant step 2(400)	50	5	10	25	25	500
M.tuberculosis J15	Wild Type	25	5	10	25	25	50
	Md mutant step 1 (200)	25	5	25	10	25	500
	Md mutant step 2(1000)	50	5	25	10	25	2000
M.tuberculosis N23	Wild Type	25	5	5	50	50	50
	Md mutant step 1 (200)	50	5	2	50	50	500
	Md mutant step 2(1000)	100	5	2	100	50	2000
M.tuberculosis $H_{37}Rv102$	Wild Type	2	2	5	5	2	10
	Md mutant step 1 (25)	2	2	10	5	5	100
	Md mutant step 2(200)	2	2	10	25	5	500
M.tuberculosis $H_{37}Ra16$	Wild Type	2	2	2	5	2	5
	Md mutant step 1 (25)	2	5	2	5	5	50
	Md mutant step 2(100)	5	5	2	2	5	200

*INH, isoniazid acid hydrazide; Rf, rifampicin; Eb, ethambutol; Py, pyrazinamide; Sm, streptomycin; Md, methdilazine

The step-wise mutants produced by mycobacteria against Pz showed nearly similar pattern of increase in resistances against the antitubercular drugs. However, in case of *M.intracellulare* 1406 there was a loss in the MIC value of Eb (Table 3).

Table 3 Promethazine (Pz) resistant mutants of *Mycobacterium* spp. and their effects on change of resistant patterns with respect to anti-mycobacterial drugs

Mycobacteria	Types of cultures	MIC (µg/ml in LJM) of different agents with respect to wild type and mutant bacteria					
		INH	Rf	Eb	Py	Sm	Pz
M.fortuitum 1529	Wild Type	2	2	2	2	5	25
	Pz mutant step 1 (50)	5	2	2	5	5	100
	Pz mutant step 2 (200)	5	2	5	25	10	400
M.scrofulaceum 1323	Wild Type	2	1	2	2	2	10
	Pz mutant step 1 (25)	5	1	2	25	10	50
	Pz mutant step 2(100)	5	2	10	25	25	400
M.marinum 50	Wild Type	2	2	5	5	2	25
	Pz mutant step 1 (50)	5	5	25	5	5	100
	Pz mutant step 2(200)	5	10	25	5	5	400
	Pz mutant step 3(500)	10	25	50	10	5	1000
M.intracellulare 1406	Wild Type	5	5	5	5	2	25
	Pz mutant step 1 (50)	5	2	5	5	2	200
	Pz mutant step 2(400)	5	2	10	10	5	500
	Pz mutant step 3(1000)	25	2	50	25	10	2000
M.tuberculosis Bajaj1	Wild Type	25	5	5	5	25	50
	Pz mutant step 1 (100)	50	5	5	10	25	200
	Pz mutant step 2(400)	100	5	10	25	50	1000
M.tuberculosis J15	Wild Type	25	5	10	25	25	50
	Pz mutant step 1 (100)	25	10	25	10	50	200
	Pz mutant step 2(400)	25	10	50	10	50	1000
M.tuberculosis N23	Wild Type	25	5	5	50	50	50
	Pz mutant step 1 (100)	25	5	5	50	100	400
	Pz mutant step 2(500)	100	5	10	100	100	1000
M.tuberculosis	Wild Type	2	2	5	5	2	10

H_{37}Rv102	Pz mutant step 1 (25)	5	2	5	10	2	50
	Pz mutant step 2(100)	5	2	10	25	10	400
	Wild Type	2	2	2	5	2	5
M.tuberculosis H_{37}Ra16	Pz mutant step 1 (10)	2	2	2	5	2	25
	Pz mutant step 2(50)	10	5	5	5	5	100

*INH, isoniazid acid hydrazide; Rf, rifampicin; Eb, ethambutol; Py, pyrazinamide; Sm, streptomycin; Pz, promethazine

DICUSSION

Prevalence of cross-resistances between aminoglycosides in drug-resistant mutants of *M.tuberculosis* has been studied extensively. Resistance to kanamycin in viomycin-resistant strains and resistance to streptomycin in kanamycin-resistant strains were two to four times greater than the resistant levels of the parent strains of *M.tuberculosis* (**Torii et al., 1959; Steenken et al., 1959**). **Tsukamura (1974)** isolated two types of tuberactinomycin N resistant mutants of *M.tuberculosis,* the first one was resistant to low levels of tuberactinomycin N, viomycin and capreomycin, while the other was resistant at high levels to all these three antibiotics plus kanamycin and lividomycin. With the help of an intensive study **Tsukamura and Mizuno** in **1975** proved that aminoglycoside antibiotics could be classified into three major types: streptomycin resistance, combined viomycin-tuberactinomycin N- capreomycin resistance, and the third type included resistance to kanamycin, lividomycin and paramomycin. No cross-resistance between streptomycin and any other amionoglycoside antibiotic was observed. Although there is a large number of studies on clinical isolates of *M.tuberculosis* on mutations of genes and their relatedness of resistances to specific antibiotics (**Taniguchi et al., 1996; Telenti et al., 1993; Ginsburg, 2005; Pitaksajjakul et al., 2005**) evaluation of drug-resistance in experimentally produced mutants of *M.tuberculosis* has not been reported during past several years.

Imperiale et al (2014) studied cross-resistances to isoniazid, rifampicin and levofloxacin at a molecular level in clinical isolates of *M.tuberculosis*. With the help of microplate colorimetric method they determined MIC of isoniazid, ethionamide, rifampicin, rifabutin and moxifloxacin in the clinical isolates. Mutations conferring drug resistances were detected by GenoType MTBDR plus and DNA sequences. Isoniazid and ethionamide cross resistance was detected in 95.12 % of isoniazid resistant isolates harbouring a mutation in inhAP or inhA open reading frame, but rifabutin cross-resistance was observed in 90% of clinical isolates originally shown to be resistant to rifampicin. This study highlighted that the same mutation causing resistance to the first line antitubercular drugs can be responsible for resistance to their respective structural analogs. Such findings are expected to help clinicians to decide on the treatment regime.

In this study it has been observed that Md mutants of *M.phlei* L1 developed cross-resistance to INH, Rf, Eb and Py in stepwise manner but not to Sm; with respect to *M.flavescens* 1541 the Md mutants developed cross-resistances to INH, Eb, Py, Sm and not To Rf. Wild type clinical isolates *M.tuberculosis* Bajaj 1, J15 and N23 had much higher level of MIC of all the test drugs, except Rf. Surprisingly, step-up mutants of all these 3 strains failed to develop resistance to Rf as well. On the contrary, resistance level of Eb decreased in step-two mutants of *M.tuberculosis* N23. Similar decrease in MIC of Sm was noted in *M.avium* 724 (Table 2). The Pz-resistant step-three mutants of *M.marinum* 50 and *M.intracellulare* 1406 were resistant to Pz at very high levels and increase in MIC values of Eb in these mutants was also significantly high. The resistances of wild-type and mutant mycobacteria differed in most instances by sufficiently large margins (within the limits of confidence of the test system), and therefore appeared to be truly reflective of the actual resistance of such mycobacteria. It is unlikely that the low levels of resistances exhibited by some of the mutants were due to their reduced growth rate, incubation period was kept for sufficiently long period even for slow-growers and these resistances did not seem to depend on drug-modifying enzymes; these, therefore, should not affect their MIC determination. Indeed MICs of several test drugs were found to be elevated simultaneously. Thus these observations on cross-resistance seem to be more easily explainable on the basis of a non-specific reduction in cell membrane permeability of the various Md and Pz mutants in varying degrees and selectivity with respects to different test drugs. A literature survey on the MICs of different antibiotics shows that there had been a significant rise in the highest values that characterize drug resistant strains as well as the lowest values of MICs of drugs/antibiotics (characterizing sensitive strains) with respect to almost all groups of pathogenic bacteria during the last five decades since the beginning of antibiotic era (**Garrod and O' Grady, 1971; Ray et al., 1980**). Development of such resistances has been ascribed to "intrinsic resistance" which is an evolutionary ancient phenotype and can be defined as resistance of any bacterial species to a particular drug/antibiotic that has not been acquired as a result of exposure to such agents (**Fajardo et al., 2008**). Intrinsic resistance is the result of reduced permeability of bacterial envelope and the activity of efflux pumps (**Nikaido and Zgurskaya, 1999**). This suggests that the main physiological role of the components of intrinsic resistance involves the prevention of toxic components by restricting the permeability of the cell or the active export of toxic compounds. However, intrinsic resistance of *M.tuberculosis* has been traditionally attributed to the unusual structure of its mycolic acid containing cell wall that contributes to the low permeability for antitubercular antibiotics and chemotherapeutics (**Jarker and Nikaido, 1994**). The role of efflux mechanisms has been recognized as an important factor in the natural resistances of mycobacteria against tetracyclines, aminoglycosides and fluoroquinolones (**De Rossi et al., 2006**). Even though mutations in several genes are evidently related to drug resistance in *M.tuberculosis* a large number of clinical isolates do not seem to present such classical mutations (**Almeida Da Silva and Palomino, 2011**). It is known that inadvertent and extensive use of antitubercular drugs in tuberculosis patients have continuously contributed to substantial increase in drug resistances in *M.tuberculosis* resulting in emergence of multi-drug resistant and extensively drug resistant strains.

In view of our data and other findings it may be stated that the overall elevation of MIC of antitubercular drugs could also be due to a prolonged and massive use of a vast number chemotherapeutics, many of which have undetected or unsuspected antimicrobial/antitubercular activities. A few among such drugs are several phenothiazines including Md and Pz (**Molnar et al., 1977; Kristiansen and Vergemann, 1986; Ratnakar and Murthy, 1993; Chakrabarty et al., 1993; Dutta et al., 2009; Crowle et al., 1992; Amaral et al.,1996; van Ingen et al., 2009; Advani et al., 2012; Kristiansen et al., 2015)**. It may be plausible to presume that many or all of these drugs could have selected mutants resistant to themselves and non-specifically to other antitubercular drugs all at low levels, at the therapeutic dosages at which they are used, thus contributing towards a gradual rise of the baseline of MIC of antitubercular agents. The present study provides experimental evidences for one of such possibilities.

CONCLUSION

The accelerated speed with which tuberculosis has spread in all communities throughout the world and the higher frequency of infection by multidrug and extensively drug resistant *M. tuberculosis* have resulted in an alarming situation. In this study it was observed that many strains of *M.tuberculosis* could be inhibited by both the phenothiazines methdilazine and promethazine.Thus these findings open up a new arena of treatment for multidrug and extensively drug resistant mycobacteria. It may be pointed out here that a structurally similar phenothiazine, thioridazine, has been repeatedly proved to be a potent antitubercular drug. Both the two compounds methdilazine and promethazine are routinely used as antihistaminic drugs while thioridazine is an effective antipsychotic drug. It needs to be pointed out here that since both methdilazine and promethazine are given to patients as antihistaminics and not as neuroleptics development of track resistance is not possible on the basis of prescription. Since the structurally similar neuroleptic drug thioridazine has repeatedly shown to be active against *M.tuberculosis* all such phenothiazines may be grouped together to define the results as a " class mechanism". When the antihistaminic compounds methdilazine and promethazine were allowed to produce mutants resistant to either of them at higher levels, the organisms showed elevated levels of MIC of tested antitubercular drugs. Such a phenomenon may be attributed as intrinsic resistance, which is the result of reduced permeability of bacterial envelope alongwith action of efflux pumps,which, in turn, result in prevention of toxic components into the cell. Therefore, it may be plausible that prolonged and indiscriminate use of such phenothiazines having antitubercular action, often unknowingly, may have allowed development of mutants resistant to themselves and simultaneously cross-resistances to many routinely used antitubercular drugs.

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QUANTITATIVE PROTEASE ASSAY BY SUBSTRATE-AGAROSE PLATE METHOD

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ABSTRACT

Proteases are unique class of industrial biocatalyst; occupy a key chair with respect to wide range of utility in both physiological and commercial sector. Repertoire of various enzyme assays were suggested for determining enzyme units of protease. Majority of currently reported assays are expensive, time consuming and cumbersome. In this direction, a development of speedy and sensitive quantitative enzyme assay for the characterization of proteases is highly desirable. The present investigation reported a quick method for characterization of proteases using single substrate-agarose plate method. A trypsin (obtained from bovine pancreas) and bacterial protease (serine protease of *Bacillus circulans* MTCC 7942) on subsequent interaction with proteinaceous substrate gives proteolytic zone on substrate-agarose plate. The clear zone diameter of proteolysis was directly proportional to enzyme units of protease. Furthermore, enzyme activities of protease treated with various chemical activator/inhibitor gives vis-à-vis response and yielded significant data. The biochemical characteristics of proteases are documented using rapid single substrate-agarose plate method. A rapid, sensitive and reliable improved quantitative protease assay using single substrate-agarose plate method could be used at academic, research and commercial level.

Keywords: Characterization, Protease, Proteinaceous substrate, Substrate-agarose plate

INTRODUCTION

The biotechnological applications of proteases are rapidly growing as the proteases with novel properties and wide substrate specificities were documented in recent years (**Li *et al.*, 2013**). Proteases are physiologically important, as ~2 % genes codes for proteases in each organism (**Barrett and Rawlings, 2001**). Proteases capable of functioning at extreme pH (3-11), temperature (40-80 ℃) and in organic solvents are considered as robust proteases and suitable for industrial applications. Currently, thermo-, organic solvent stable, alkaline proteases were preferred for various industrial and therapeutic applications (**Patil and Chaudhari, 2010; Li *et al.*, 2013**).

In order to evaluate the commercial utility of newly reported protease, it should be characterized. The characterization of protease from newly reported source includes (i) knowing their active pH and thermal range; (ii) revealing their functional type; (iii) understanding their robustness against oxidizing-, reducing-, bleaching agents, organic solvents and detergents. On the basis of these characteristics, the suitability of protease is determined for commercial applications. As well, type of protease secreted by pathogenic strains could offer a clue about the role of protease in the pathogenesis of infectious diseases (**Jarocki *et al.*, 2015**).

Currently proteases were assayed by arrays of quantitative approaches, which measure hydrolytic products of either proteinaceous substrate or residual fragmented peptides. These methods usually vary in their simplicity, rapidity, sensitivity, range of detection and cost per test. The routinely used quantitative assay methods for protease activity involves the use of natural or synthetic substrates by employing spectrophotometric, fluorimetric, radiometric and enzyme-linked immunosorbent assay (ELISA) (**Sumantha *et al.*, 2006**). An absorption spectroscopy is widely used for protease assay (**Mokashe *et al.*, 2015**).

Pioneering contributions of **Anson (1938)** and **Kunitz (1947)** in protease assay was further modified by several investigators (**Nakanishi *et al.*, 1974; Shimogaki *et al.*, 1991**). The majority of protease assay involves incubation of protease with proteinaceous substrate for a specific period. On subsequent trichloroacetic acid reagent (TCA) precipitation, the fragmented residual peptides were determined by (i) measuring the absorbance at 275-280 nm using tyrosine (0 -75 µg/mL) as a standard; (ii) Folin-Lowry method or (iii) analyzing the nitrogen of fragmented peptides by Kjeldahl method. Although, the continuous monitoring of time-dependent progressions of enzyme reactions make these methods attractive, but stressed handling, cumbersome additions and reagent/buffer preparations are several hurdles in the routine protease assay. In this view, there is a scope for a development of rapid and sensitive quantitative enzyme assay for the characterization of proteases.

Generally, protease producing microbes/biological source was detected by assessing the proteolytic zone on casein, gelatin, skimmed/lactose free milk agar plate (**Vermelho *et al.*, 1996; Morris *et al.*, 2012**). Furthermore, agents like Bradford reagent and bromocresol green dye were suggested for detecting very narrow proteolysis zones clearly (**Lu *et al.*, 2013; Vijayaraghavan and Vincent, 2013**). Also, **Montville (1983)** reported the dual-substrate plate diffusion assay for characterization of endopeptidases by correlating the proteolytic zone diameter and enzyme activity. Although, these approaches were commonly used for qualitative detection of the protease, few investigators have utilized this principle for quantitative protease assay (**Gallahgher *et al.*, 1986**). The method reported here describes quantitative enzyme assay by employing single substrate-agarose plate without use of a detection reagents (Coomassie Blue dye/TCA). A gel-diffusion method was legitimately described for quantitative estimation of antibiotics/vitamins (**Indian pharmacopoeia, 2014**). Such gel-diffusion method determines the potency of antibiotic/vitamin rather than its quantity. Likewise, an optimized procedure could be able to quantify the enzyme activity (units) by employing gel-diffusion principles. The method suggested in this investigation describes simple, rapid and quantitative noteworthy enzyme assay for characterizing the protease using substrate-agarose plate.

MATERIALS AND METHODS

Enzymes

The proteases used in this study were - purified alkaline serine protease of *Bacillus circulans* MTCC 7942 (**Patil *et al.*, 2016**) and commercial trypsin (EC 3.4.21.4, MP Biomedicals, USA; obtained from bovine pancreas).

Protease assay

Protease activity was assayed as per Anson-Hagihara (**Hagihara, 1958**) using buffered casein as a substrate. Bacterial alkaline serine protease assay was conducted at 60 ℃ using casein prepared in 20 mM carbonate-bicarbonate buffer (pH 10), while trypsin assay was conducted at 37 ℃ using casein; prepared in 100 mM Tris buffer (pH 7.5). One unit of bacterial alkaline protease activity was defined as the amount of enzyme required to produce peptides equivalent to 1 µg of tyrosine in the filtrate per minute per ml at pH 10 and 60 ℃. Similarly, one unit of trypsin activity was defined as the amount of enzyme required to produce peptides equivalent to 1 µg of tyrosine in the filtrate per minute per ml at pH 7.5 and 37 ℃.

Casein-agarose plate preparation

Casein-agarose plates with medium (g/L) containing skim milk, 28 and agarose 20 in 20 mM carbonate-bicarbonate buffer (pH 10) for bacterial serine protease plate assay and 100 mM Tris buffer (pH 7.5) for trypsin plate assay was used. The casein-agarose media was autoclaved at 121 ℃ for 5 min. The medium was subsequently degassed using sonication for 5 min in ultrasonic cleaning bath (33 kHz) (Equitron, Medica Instrument Mfg. Co., Mumbai) and poured (thickness - 3.1 mm) in sterile disposable square petri plate (120 mm × 120 mm). *Then* ~25 wells (diameter - 6 mm) were punched on solidified casein-agarose plates.

Substrate-agarose plate method

To optimize the substrate-agarose plate enzyme assay, a standard graph of enzyme activity versus zone of proteolysis was determined. For this, 80 µL purified protease aliquot (Bacterial serine protease 40-440 U/mL and trypsin 20-240 U/mL) was loaded on punched experimental wells in casein-agarose plate. These plates were incubated precisely for 8 h at 60 ℃ and 37 ℃, respectively. After incubation, plates were observed for the zone of proteolysis and zone diameters were determined using *vernier caliper (Mitutoyo, Digimatic Caliper, Japan)*. Each experiment was conducted in triplicates.

Enzyme characterization by Substrate-agarose plate method

The respective enzyme aliquots were further allowed to interact with various activators, inhibitors and stabilizers to characterize the biochemical nature of enzyme. The substrate-agarose plate method described as above was employed to determine the residual activity of the enzyme on subsequent treatment with activators, inhibitors, stabilizers and organic solvents. For this, various salt solutions [NaCl, KCl, $MgCl_2$, $CaCl_2$, $MnCl_2$, $ZnCl_2$, $CuCl_2$, $CdCl_2$ and $HgCl_2$ (5 mM)]; surfactants (1 % sodium dodecyl sulfate and 1 % Triton X-100), protease inhibitors (5 mM) - phenyl-methyl-sulfonyl-fluoride (PMSF), N-ethyl-maleimide, ethylene-diamine-tetra-acetic acid (EDTA) and organic solvents (acetone, benzene, butanol, chloroform, dimethyl sulfoxide, glycerol, pyridine, *n*-dodecane and toluene) were interacted with respective enzyme solutions. After pre-incubation (1 h at 27 ℃) respective mixture was loaded in labeled well of casein-agarose plate. The loaded plates were further incubated for 8 h at a respective optimum temperature of the enzyme.

RESULTS AND DISCUSSION

The caseinolytic activity of trypsin/bacterial protease was compared with the zone of proteolysis on casein-agarose plate. A standard curve of trypsin activity versus zone of proteolysis was plotted (Figure 1). Protease activities (U/mL) and zones of proteolysis on casein-agarose plate were found to be positively correlated. The Pearson correlation coefficient of standard curve for bacterial serine alkaline protease and trypsin were 0.9694 and 0.9469, respectively. Several experimental attempts have been undertaken with a view to quantitate the enzyme units by substrate-agarose assay method. Also, the F-test is used to compare variances in newly reported substrate-agarose assay and known assay (Anson-Hagihara protease assay method) to determine significant differences in precision of two methods. The calculated F value is 1.1, while the tabulated F-value is 4.28, indicating no significant difference in precision of two methods.

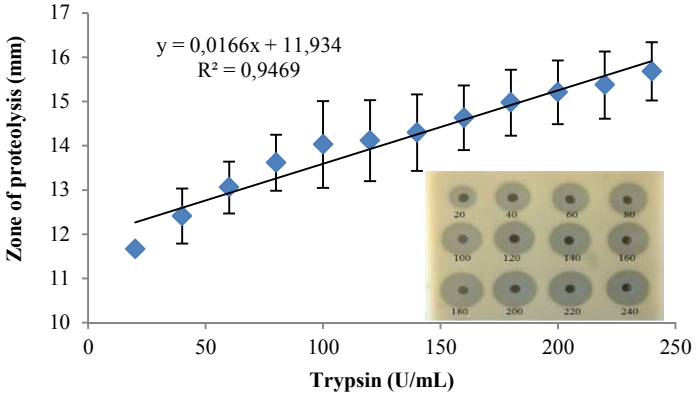

Figure 1 Standard curve for trypsin

Based on proteolysis zones formed by respective treated bacterial serine protease (Figure 2) and trypsin (Figure 2) the residual enzyme activities were determined. The equations to determine enzyme activity are as follows-

Bacterial protease activity (U/mL) = (Proteolysis zone in mm - 9.93) /0.0119.

Trypsin activity (U/mL) = (Proteolysis zone in mm - 11.93) /0.0166.

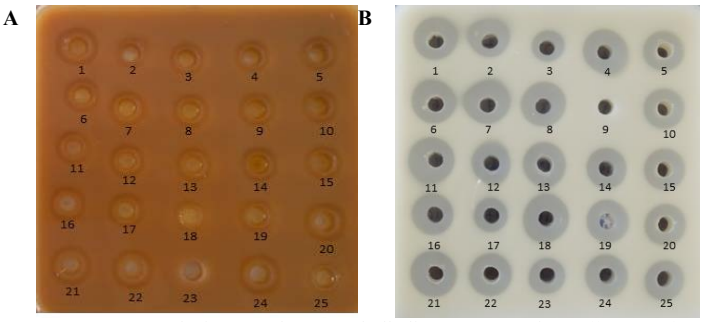

Figure 2 (A) Proteolysis zones by treated alkaline protease of *Bacillus circulans* MTCC 7942 on single substrate-agarose plate (pH 10): 1) Control, 2) PMSF (5 mM), 3) Pyridine, 4) Benzene, 5) $MgCl_2$, 6) NaCl (5 mM), 7) KCl, 8) Butanol, 9) *n*-dodecane, 10) NaCl (150 mM), 11) Glycerol, 12) SDS, 13) DMSO, 14) $ZnCl_2$, 15) $CuCl_2$, 16) $CdCl_2$, 17) $HgCl_2$, 18) Toluene, 19) Chloroform, 20) $CaCl_2$, 21) Triton X-100, 22) EDTA, 23) PMSF (10 mM), 24) N-ethyl maleimide, 25) Acetone, **(B)** Proteolysis zones by treated trypsin on single substrate-agarose plate (pH 7.5): 1) Control, 2) $HgCl_2$, 3) Pyridine, 4) Benzene, 5) $MgCl_2$, 6) NaCl (5 mM), 7) KCl, 8) Butanol, 9) PMSF (10 mM), 10) NaCl (150 mM), 11) Glycerol, 12) SDS, 13) DMSO, 14) $ZnCl_2$, 15) $CuCl_2$, 16) $CdCl_2$, 17) PMSF (5 mM), 18) Toluene, 19) Chloroform, 20) $CaCl_2$, 21) Triton X-100, 22) EDTA, 23) *n*-dodecane, 24) N-ethyl maleimide, 25) Acetone.

The enzyme activities determined using substrate-agarose assays were in agreement with Anson-Hagihara protease assay method. Also, these findings were in agreement with previous reports on characterization of bacterial serine protease from *Bacillus circulans* MTCC 7942 (Table 1) and trypsin (Table 2) (**Blanco *et al.*, 2014; Patil *et al.*, 2016**).

Table 1 Proteolytic zones on single substrate-agarose plate and corresponding protease activities of treated serine alkaline protease of *Bacillus circulans* MTCC 7942

Sr. No.	Activator/ inhibitor/organic solvents/salts	Alkaline serine protease of *Bacillus circulans* MTCC 7942	
		Zone of proteolysis (mm)	Protease activity (U/mL)
1.	Control	13.93	335.88
2.	PMSF (5 mM)	10.12	15.71
3.	Pyridine	11.24	109.83
4.	Benzene	11.29	114.03
5.	$MgCl_2$	12.82	242.61
6.	NaCl (5 mM)	12.76	237.56
7.	KCl	12.72	234.20
8.	Butanol	11.45	127.48
9.	*n*-dodecane	12.36	203.95
10.	NaCl (150 mM)	11.93	167.82
11.	Glycerol	12.4	205.63
12.	SDS	11.84	160.25
13.	DMSO	12.4	207.31
14.	$ZnCl_2$	11.63	142.61
15.	$CuCl_2$	11.67	145.97
16.	$CdCl_2$	12.07	179.58

17.	$HgCl_2$	11.61	140.92
18.	Toluene	10.57	53.53
19.	Chloroform	10.98	87.98
20.	$CaCl_2$	14.22	360.25
21.	Triton X-100	13.97	339.24
22.	EDTA	13.95	337.56
23.	PMSF (10 mM)	6.04	0
24.	N-ethyl maleimide	14.44	378.74
25.	Acetone	10.83	75.38

'-' No proteolysis; SDS: sodium dodecyl sulphate; PMSF: phenyl-methyl-sulfonyl-fluoride; EDTA: ethylene-diamine-tetra-acetic acid (EDTA); DMSO: dimethyl sulfoxide

To optimize the respective protease assay, several factors which could contribute to improve the efficiency of protease assay were reviewed. The proteinaceous substrate for protease assay must be completely soluble in buffer for accurate analysis; otherwise their compact native conformation and insensitivity to protease action may inadvertently generate errors. Casein starts precipitating below pH 6; hence, it is used to assay neutral or alkaline proteases while haemoglobin is suitable for acidic proteases.

In current assay, agarose (2 %) was used as a solidifying agent; it forms porous gel which allows the diffusion of protein molecules. Agarose has several advantages over agar- (i) the zone of proteolysis on casein-agarose plate is clear and transparent as compared to a zone of proteolysis developed on substrate-agar plate, (ii) the proteolysis zone can easily perceived without a detection reagents and (iii) agarose has neutral charge and less chemically complex and less likely to interact other biomolecules. The impurities associated with agar might interfere and restrict with the development of clear zone.

Table 2 Proteolytic zones on single substrate-agarose plate and corresponding protease activities of treated trypsin

Sr. No.	Activator/ inhibitor/organic solvents/salts	Trypsin	
		Zone of proteolysis (mm)	Protease activity (U/mL)
1.	Control	14.32	143.73
2.	$HgCl_2$	11.13	-
3.	Pyridine	11.94	-
4.	Benzene	15.06	188.31
5.	$MgCl_2$	13.54	96.75
6.	NaCl (5 mM)	14.37	146.75
7.	KCl	14.27	140.72
8.	Butanol	14.76	170.24
9.	PMSF (10 mM)	11.23	-
10.	NaCl (150 mM)	13.36	85.9
11.	Glycerol	14.32	143.73
12.	SDS	12.47	32.29
13.	DMSO	14.06	128.07
14.	$ZnCl_2$	13.58	99.16
15.	$CuCl_2$	13.77	110.6
16.	$CdCl_2$	13.24	78.67
17.	PMSF (5 mM)	11.93	-
18.	Toluene	15.43	210.6
19.	Chloroform	14.01	125.06
20.	$CaCl_2$	13.92	119.64
21.	Triton X-100	14.63	162.41
22.	EDTA	13.39	87.71
23.	*n*-dodecane	6.02	-
24.	N-ethyl maleimide	14.03	126.27
25.	Acetone	12.79	51.57

'-' No proteolysis; SDS: sodium dodecyl sulphate; PMSF: phenyl-methyl-sulfonyl-fluoride; EDTA: ethylene-diamine-tetra-acetic acid (EDTA); DMSO: dimethyl sulfoxide

CONCLUSION

Although, **Montville (1983)** reported the dual-substrate (casein and gelatin) plate diffusion assay for quantitative estimation of endopeptidase units, the simultaneous degradation of gelatin with casein could alter the composition and solubility of medium. The present report describes novel technique to quantitate the enzyme units using single substrate-agarose plate diffusion method. The sophisticated protocols can be pursued for characterizing other protease variants. Presently, various applications of proteases in food, pharmaceutical and biotechnological sectors are continuously explored. This newly suggested method could be much more appropriate to characterize the protease for academic, research and commercial purpose. The rapid, sensitive and reliable protease assay method could serve the purpose of selection and screening of several proteases for their suitable exploration in the industrial sector. This substrate-agarose plate protease assay method could be efficiently useful to characterize the proteases obtained from other microbial, animal or plant sources.

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LACCASE PRODUCING *STREPTOMYCES BIKINIENSIS* CSC12 ISOLATED FROM COMPOST

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ABSTRACT

Lignin degradation by actinobacteria is more attractive than well known white rot fungal system by the fact of their higher survival rate, sporulating ability, economic production of useful chemicals rather than evolution of CO_2 as in white-rot fungi. One of the important enzymes in ligninolysis is laccase, which oxidize a broad range of substrates, preferably phenolic and non-phenolic compounds. Among the laccase producing microbes in nature, white rot fungi have been characterized much, very little is known about the laccase producing actinobacteria. The present investigation aims to isolate and screen active laccase producing actinobacteria from diverse environments. Fifteen isolates of actinobacteria were isolated from various organic substrates *viz.,* coir compost, groundnut shell compost, fully and partially decomposed municipal solid waste and identified based on molecular characterization. With initial screening using ρ-anisidin and Poly-R 478, seven actinobacterial isolates had more efficient ligninolytic activity than others. Of which, isolate CSC_{12} secreted highest laccase and was identified as *Streptomyces bikiniensis* CSC12. Laccase from *S.bikiniensis* CSC12 was partially purified as monomer having molecular mass ~69 kDa based on SDS-PAGE and had pH and temperature optima of 6 to 7 and 50 to 60 °C respectively.

Keywords: Actinobacteria, compost, laccase, *Streptomyces*

INTRODUCTION

Lignin is the most abundant natural aromatic polymers in the biosphere after cellulose. It comprises 20-30 per cent in woody plant cell walls by forming a matrix surrounding the cellulose and hemicelluloses, and provides strength and protection against biodegradation (**Boerjan et al., 2003**). Lignin exists in close association with cellulose and other polysaccharides in plant tissues that limit the efficiency and extent of utilization of those compounds to fuels and chemicals through microbial conversion (**Wang et al., 2011**). Low molecular weight lignins are released into the environment as a major waste product in the pulp industry and its disposal has decreased over recent years in spite of increased pulp production (**Magalhaes and Milagres, 2009**). Several microorganisms that include a variety of filamentous fungi are known to attack lignin to various extents through secretion of oxidoreductases, such as laccases that offer most promise because of their high redox potential. However, lignin degradation by filamentous bacteria requires detailed study because of their definite role in biosphere carbon cycle and production of value added products from lignocellulose biomass/waste. Even though the ligninolytic potential of actinobacteria has been established long before, the studies on lignin degrading enzymes from this group are still in its infancy compared to fungi. Laccase activity has been demonstrated in a few species of *Streptomyces* such as *S. cyaneus* (**Berrocal et al., 1997**), *S. lavendulae* (**Suzuki et al., 2003**) and *S. coelicolor* (**Machczynski et al., 2004**). Economic production of useful chemicals by actinobacteria rather than evolution of CO_2 as in white-rot fungi, sporulating property, and survival are said to be advantageous characters of actinobacteria. Hence the present study was undertaken to isolate ligninolytic actinobacteria associated with different wastes and their enzyme systems for biomass conversion more specifically for lignin bioconversion eventually this will lead to accelerated lignin bioconversion, which could be used for production of humic polymers from lignocellulosic biomass/waste.

MATERIALS AND METHODS

Isolation of actinobacteria

Compost samples like coir compost, groundnut shell compost, fully decomposed municipal solid waste and partially decomposed municipal solid waste were collected from Coimbatore and Namakkal (Tamil Nadu, India). Actinobacteria were isolated from these compost samples by adopting serial dilution and plating technique using Kuster's agar medium (**Jayashree *et al.*, 1991**). One of our earlier actinobacterial isolate, *Streptomyces violaceusniger* (MTCC 3959) lignin degrader deposited at Institute of Microbial Technology, Chandigarh, India was used as a reference culture. *S. violaceusniger* and other actinobacterial isolates were maintained in Crawford's agar slants and slopes of mineral medium containing indulin (2 %) (**Crawford *et al.*, 1982a**).

Screening for ligninolytic activity

The isolated cultures and *S. violaceusniger* were tested for ligninolytic activity on Crawford's agar medium containing different indicators *viz.,* 0.02 per cent Poly R-478 and 10 mM ρ-anisidine. ρ-anisidine was added to sterilized Crawford's medium where as PolyR-478 was filter sterilized and added to the medium. The actinobacterial cultures were streaked over the plate and incubated for 7 days at room temperature. Cultures which show positive reaction for ligninolytic activity exhibit initial browning and later clearing zone in the ρ-anisidine medium and clearing zone around the colony in Poly-R medium were selected for further studies (**Rittstieng *et al.*, 2002**).

Decolorization assay

Filter sterilized Poly-R, the polymeric dye was added to the liquid medium as an aqueous minimal medium inoculated with all the actinobacterial cultures to a final concentration of 0.02 per cent with gentle swirling. At 1, 2, 3, 4, and 5 days of intervals, 0.1 ml of the cell free culture medium was diluted 10-fold with water and measured absorbance ratio A_{518}/A_{346} of visible absorption spectra and routine absorbance measurements of the dye were determined on the spectrophotometer (**Glen and Gold, 1983**).

Enzyme production and assay for laccase

The enzyme in the cell-free culture medium obtained after 7 days of growth (450 ml) of ligninolytic culture was precipitated at 60 per cent saturation of $(NH_4)_2SO_4$ and the resulting precipitates were collected by centrifugation. The precipitates were dissolved in a minimal volume of buffer 0.1M sodium phosphate buffer, pH

6.8 and dialyzed against the same buffer (**Arias *et al*, 2003**). All the purification steps were carried out at 4 ºC. Cell free culture supernatants obtained from 7 days old culture was used as an enzyme source. The reaction mixture consists 1.5 ml of 0.1 mM syringaldazine in 50 mM sodium phosphate buffer pH (6.0) and 0.1 ml of enzyme extract. The change in absorbance of the reaction mixture was recorded at 530 nm at 30 sec interval for 3 min at room temperature (28 ± 2 °C). The enzyme activity was expressed as change in absorbance of the reaction mixture per $min^{-1}ml^{-1}$ of culture extract (**Chefetz *et al.,* 1998**).

Temperature and pH optima of laccase

To optimize the conditions for maximum laccase activity, the culture supernatant from actinobacteria culture grown in Crawford's broth with 0.1 percent glucose at room temperature was used an enzyme source and the enzyme activities were determined by measuring change in absorbance at 530nm at different temperature conditions (The reaction mixture contained 0.4 ml of the enzyme sample, 1 ml of 0.1 mM syringaldazine and 50 mM sodium phosphate buffer). To estimate the optimum pH, enzyme activity was monitored at pH values from 3 to 10 using buffers *viz.,* 50 mM citrate buffer for pH 3 to 5; 50 mM PO_4 buffer for pH 6 to 8; 50 mM and Tris buffer for pH 8 to 10 (The reaction mixture contained 0.4 ml of enzyme sample, 1 ml of 0.1mM syringaldazine and 50 mM sodium phosphate buffer (**Chefetz *et al*., 1998**).

SDS-PAGE analysis of laccase protein profile

To determine the purity of the protein and its molecular weight, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 10% polyacrylamide gel containing 0.1% SDS. Samples (10 µg of protein) were treated before they were loaded onto the gel with 0.5% SDS and 5% ß-mercaptoethanol and boiled at 100 °C for 10 min. The protein bands were visualized after staining the gel with coomassie blue and compared with molecular weight markers (**Chefetz *et al.,* 1998**).

Identification of laccase producing *Streptomyces CSC12*

The genomic DNA from the actinobacterial isolates was carried out using Ctab method (**Azadeh and Meon, 2009**). The 16SrRNA target gene amplification was performed using 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (5'-GGT TAC CTT GTT ACG AC TT-3') primers. PCR amplification was performed with an initial denaturation step at 95 °C for 4 min; followed by 35 cycles consisting of denaturing at 95 °C for 30 s, annealing at 55 °C for 30 s and elongation at 72 °C for 1 min; final extension at 72 °C for 10 min, and end at 4 °C. The PCR product was purified (Qiagen, Germany) and sequencing reaction was performed using ABI prism terminator cycle sequencing ready reaction kit and electrophoresis of the products were carried out on an Applied Biosystems (Model 3100) automated sequencer.

RESULTS AND DISCUSSION

Isolation and screening of actinobacteria for ligninolytic activity

Until present much of the information on lignin degradation using laccase produced by basidiomycetous fungi serves as a model organism in lignin degradation and extensive research has yielded some understanding about the biochemistry and enzymology of ligninolysis (**Martínez et al., 2009**). Unfortunately laccases from fungal origin show lower activity and stability in alkaline pH or at high temperature (**Singh and Chen, 2008**), while bacterial laccases are highly active and stable at high temperature or high pH value (**Jordaan, 2005**). After the first report (**Crawford and Sutherland, 1979)** on the isolation and characterization of lignocellulose degrading actinobacteria, few attempts were made to assess the ligninolytic activity **(Kalaichelvan, 1987; Sivakumar, 1991, Karthikeyan, 2001 and Sivakumar *et al*., 2004**) of these filamentous bacteria. *Streptomyces* spp. are promising ligninolytic enzyme producers having great application under extreme conditions. Laccase from *Streptomyces lavendulae* are stable at 70 °C (**Suzuki et al., 2003**) and withstand a pH from 3.0 to 9.0 (**Dube et al., 2008**). In the present study, fifteen isolates of actinobacteria isolated from various organic substrates were identified as actinobacteria based on molecular identification by 16S rRNA gene sequences. The isolates were tested for growth, polymerization and depolymerization activity on ρ-anisidin and poly-R enriched agar plates. Besides one of our earlier isolate *S. violaceusniger* which has been deposited at IMTECH (MTCC 3959), Chandigarh, was obtained and used as a reference culture. Based on the oxidation of ρ-anisidin and clearing zone on poly-R in Crawford's medium, seven isolates *viz.,* CC_1, CC_2, CC_7, CSC_{12}, NPS_4, $CFYMC_8$, and $CFYMC_9$ were found as more efficient ligninolytic organisms (Table 1). Initial growth of seven actinobacteria on ρ-anisidin caused oxidation and upon further incubation showed a clearing zone around growth which might be due to the utilization of oxidized products by the organism during the later stage of growth. The initial polymerization and later depolymerization by the phenol oxidizing enzyme (laccase) indicates the positive ligninolytic activity (**Sivakumar, 1991**). *Streptomyces* spp. produce intermediate lignin derived water soluble low molecular weight compound called APPL (Acid Precipitable Polymeric Lignin), instead of mineralization to CO_2 by *P. chrysosporium*. This intermediate compounds are much of interest in economic point of view to produce value added chemicals from the ligninocellulosic materials rather than converting them to CO_2. Only, a few *Streptomyces* sp. have the ability to degrade lignocellulosis (**Crawford, 1978**), and the conversion efficiency is lower than *P. chrysosporium*.

Table 1 Screening of different actinobacteria isolates for ligninolytic activity

Designation	Source	ρ-anisidin	Poly R- 478
CC_1	Coir compost	+++	++
CC_2	″	+++	+++
CC_4	″	-	+
CC_7	″	++	+++
NFS_5	Partially decomposed municipal compost	+	-
CSC_7	Groundnut shell compost	-	-
CSC_8	″	+	-
CSC_2	″	-	+
CSC_{12}	″	+++	+++
NPS_4	Fully decomposed municipal compost	++	+
NPS_5	″	+	-
NPS_{10}	″	+	+
NPS_{12}	″	+	-
$CFYMC_8$	Farmyard manure compost	+++	+
$CFYMC_9$	″	+++	++

+ + + : Highly efficient, + + : Moderately efficient, + : less efficient - : no activity

Decolourization of polymeric dye

Degradation of lignocellulosics, decolourization of polymeric dye (Poly-R), polymerization and depolymerization activity on ρ-anisidin plates and APPL production were the criteria used for selection of efficient strains of actinobacteria for ligninolytic activity. Poly-R decolourizing ability of the organism is considered as an useful indicator of ligninolytic activity in *Phanerochaete chrysosporium.* It is necessary to record decolourization as a ratio of two peaks because cell adsorption and degradation reduce the absorbance of the dye (**Ball *et al.,* 1989**). To confirm the ligninolytic efficiency of the isolates, quantitative test *viz.,* decolourization of polymeric dye, Poly-R by the isolates was carried out. It was measured as the ratio between the absorbance at 518 nm and 346 nm. In this study, *S. violaceusniger* was used as reference culture for comparing ligninolytic efficiency. Among the seven isolates CSC_{12} performed better than the other isolates and the reference culture *S. violaceusniger* (Table 2). Present study also showed a decrease in the absorbance level due to inoculation of actinobacteria cultures. On further evaluation with the reference culture *S. violaceusniger,* the isolate, CSC_{12} was found better than the other isolates based on the decolourization of polymeric dye, Poly-R 478. **Hernandez *et al.* (1994)** studied the decolourization of paper mill effluent by 50 actinobacteria strains isolated from lignocellulosic substances. The colour reactions with synthetic dye were more easily detectable and thereby detect more laccase positives, and thus these compounds can reliably be used for the screening of laccase activity (**Kiiskinen *et al.,* 2004**).

Table 2 Decolourization of polymeric dye (Poly-R) by actinobacteria isolates (Change in OD_{518}/OD_{346} ratio)

Actinobacteria isolates	Number of days				
	1	2	3	4	5
CSC_{12}	0.79	0.71	0.66	0.63	0.61
CC_2	1.11	1.09	1.07	1.02	0.99
NPS_4	1.18	1.11	1.06	1.00	0.98
CC_7	1.15	1.05	0.99	0.96	0.95
$CFYMC_9$	1.09	0.98	0.91	0.83	0.81
CC_1	0.99	0.94	0.89	0.80	0.78
$CFYMC_8$	1.02	0.98	0.97	0.80	0.78
S.v	0.82	0.79	0.76	0.72	0.69
SEd	0.032	0.030	0.030	0.027	0.027
CD(0.05)	0.069	0.064	0.064	0.058	0.057

Laccase activity of actinobacteria isolates

In the present investigation, the activity of laccase was assayed and their activities were expressed as change in absorbance $min^{-1}ml^{-1}$ enzyme preparation as described by **Chefetz *et al.* (1998)** and **Mayer *et al.* (1965)**. The results revealed that there was a gradual increase in laccase activity of cultures upto 14th day of incubation and later a slow decline was noticed. The isolate, CSC_{12} recorded higher activity compared to other isolates. On 14th day, this culture exhibited maximum activity of 1.56 $min^{-1}ml^{-1}$ cell extract, which was significantly higher than other cultures but lower than the reference culture S. *violaceusniger* (Fig. 1).

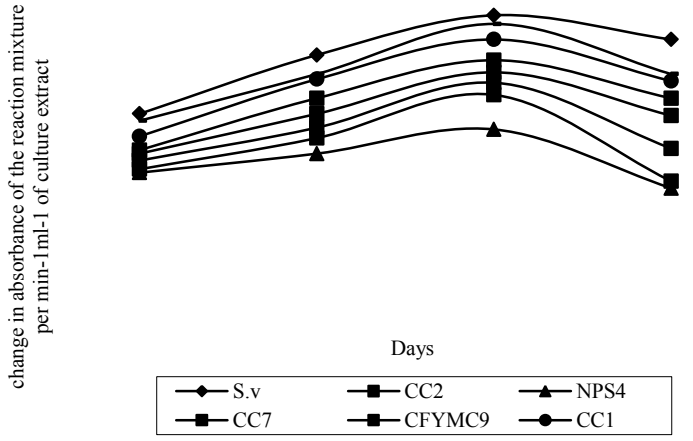

Figure 1 Production of laccase by actinobacterial isolates

One unit of enzyme activity was expressed as change in absorbance $min^{-1}ml^{-1}$ culture extract (Chefetz *et al.,* 1998)

The phenol oxidizing enzymes such as laccase is frequently found in the culture broths of lignin degrading microbes, play a significant role in lignin degradation. It is possible that laccase may cause initial demethylation of phenol rings and the O-quinone moieties left after demethylation can be reduced to catechol, compounds which are known to be substrates of ring cleaving oxygenases (**Ishiraa, 1980**). This primary role of laccase may explain not only its early appearance but also the dependence of lignin degradation on laccase (**Ander and Eriksson, 1976**). To support this statement, our results on laccase activity of actinobacteria showed that the enzyme synthesis initiated 3rd day and extended upto 21st day, such early synthesis and extended production of laccase can be exploited for commercial production of laccase using lignocellulosic biomass/agricultural wastes. The independent action of laccase in lignin biodegradation by white-rot fungi in the absence of ligninase (Lip) and Mn-peroxidases (Mn-P) has been reported by **Eggert *et al.,* (1997)**. **Ardon *et al.* (1998)** reported the maximum ligninolytic enzymes of *Pleurotus ostreatus* on 8th day of incubation in cotton stalk. **Castillo *et al.* (1997)** showed that lignin and manganese peroxidase activity in extracts from straw solid substrate fermentation was maximum at 6th day of inoculation. **Eggert *et al.* (1997)** reported that the white-rot fungus, *Pycnoporus cinnabarinus,* an excellent organism to elucidate the controversial role of laccase in lignin degradation and indicated that laccase is absolutely essential for lignin degradation. Earlier, **Kalaichelvan and Ramasamy (1989)** reported the presence of ligninolytic enzyme *viz.,* peroxidase, polyphenoloxidase, laccase and ligninase in *Streptomyces* sp. RK-1 and laccase hyper producing strains were also developed by **Sivakumar et al., (2004)**. **Ramasamy *et al.* (1989)** reported that peroxidase, laccase and phenol oxidases of *Pleurotus ostreatus* were associated with the lignolysis of coir dust. **Pasti *et al.* (1991)** reported that extracellular peroxidases of both *S. chromofuscus* and *S. viridosporus* appear extracellularly after cell cease to grow and nutrients depleted from the medium.

Temperature optima for laccase produced by *Streptomyces* CSC2

The optimal temperature range for the activity of laccases obtained from the actinobacteria *viz.,* CSC_{12} and *S. violaceusniger* reached a maximum between 50 and 60 °C (Fig. 2). The enzymes were inactive below 20 °C and above 70°C determined during 10 min reactions. Laccase from actinobacteria isolate CSC_{12} and *S. violaceusniger* had an optimal activity at 50 to 60 °C. **Chefetz *et al* (1998)** reported that laccase of *Cheatomium thermophilum* had an optimum temperature range of 50 to 60 °C. The laccase purified from *Trametes sanguinea* and *Botrytis cinerea* showed increased activity with increasing temperature (**Slomczynski *et al.,* 1995**).

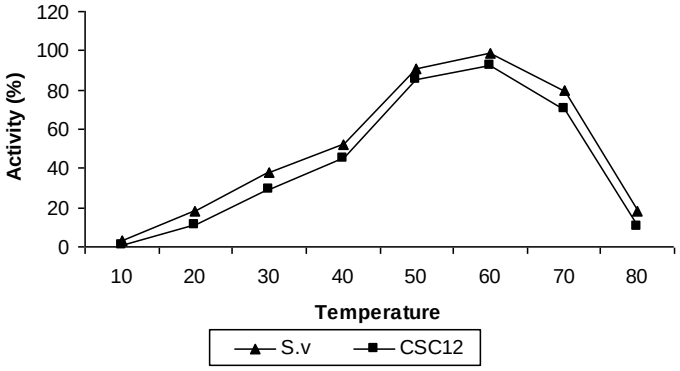

Figure 2 Influence of different temperature on the activity of laccase produced *Streptomyces* CSC12

pH optima for laccase produced by *Streptomyces* CSC12

The pH of laccase will be one of the key parameters to affect oxidation rates. Hence the influence of different pH on the activity of laccase enzyme was studied. The results obtained in the present study indicate that the pH optima of laccase (with syringaldazine oxidation) by *Streptomyces* CSC12 and *S. violaceusniger* was pH 6.0-7.0 (Fig.3). Most of the fungal laccases are active at pH values 3.0-5.0 (**Slomczynski *et al.,* 1995**). But studies on laccase of *C. thermophilum* showed maximum activity at pH 6.0 to 8.0 (**Chefetz *et al.,* 1998**).

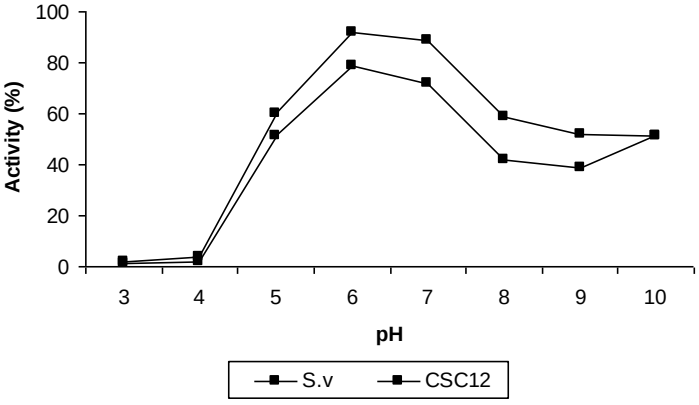

Figure 3 Influence of different pH on the activity of laccase from *Streptomyces CSC12*

SDS- PAGE profile of laccase from actinobacteria

Cell free culture supernatant obtained from both *S. violaceusniger* and *Streptomyces* CSC_{12} was analyzed in 12 per cent SDS-PAGE and size of these proteins ranged from 14 to 97 kDa. The results indicated that the banding pattern in the protein profile was almost similar in these two isolates and majority of the proteins have the molecular size between 60 and 75 kDa. However, among the isolates, variations were observed in the protein profile at same molecular sizes. Molecular weight of most fungal laccase proteins fall between 43 to 110 kDa (**Yoropolov *et al.,* 1994**). Majority of laccases from basidiomycetous fungi were reported to have molecular weights in the range of 55 to 72 kDa. Based on in-gel laccase activity assay, laccase from the isolate CSC_{12} and *S. violaceusniger* was found to have a molecular weight of 69 kDa (Fig 4). The *Streptomyces* CSC_{12} and *S. violaceusniger* laccase appears to occur in only one single, isoform, an unusual feature among laccases especially fungal laccases. The purified laccases of *Streptomyces cyaneus* produced one band on SDS-PAGE gel at a molecular mass of approximately 75 kDa. Hence the band obtained from the protein profile of cell free cultures of CSC_{12} and *S. violaceusniger* is with molecular weight of 69 k Da, it may probably a laccase enzyme which was confirmed with zymogram obtained from native PAGE. This preliminary finding paves the way for expanding our dimensions on various biotechnological applications of laccase on energy and environment.

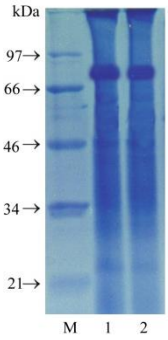

Figure 4 SDS PAGE Profile of laccase from actinobacteria (M- Molecular weight marker, 1-CSC 12, 2-S.v)

Identification of the actinobacteria

The 16S rRNA gene sequence of the actinobacteria was aligned with homologous regions from various actinobacteria, and the phylogenetic tree was constructed using neighbor-joining method (**Saitou and Nei, 1987**). A bootstrap confidence analysis was performed on 1000 replicates to determine the reliability of the distance tree topologies obtained (**Felsenstein, 1985**). The graphic representation of the resulting tree was obtained using MEGA software (version 5.05). Based on the phylogenetic data obtained from the isolate CSC_{12} showed a maximum similarity (98 per cent) with *Streptomyces bikiniensis* (Fig.2).

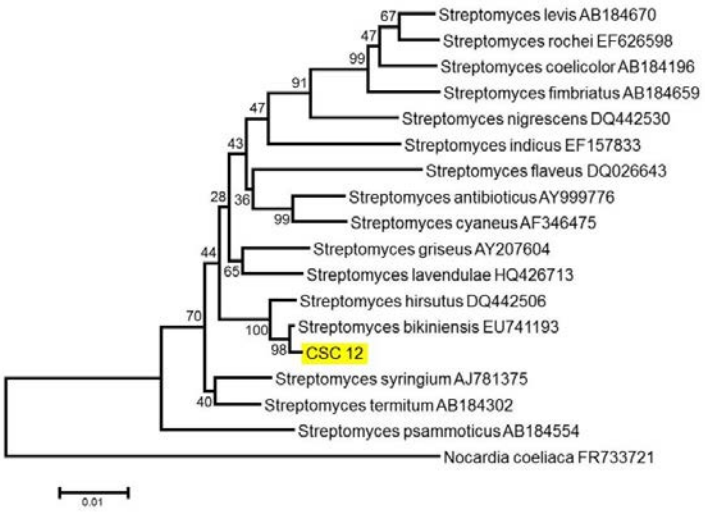

Figure 5 Phylogenetic relationship of CSC12 based on 16S rRNA gene sequences. The tree was constructed using the NJ method. Branch support is recorded at the nodes as a percentage of 1,000 bootstrap iterations

CONCLUSION

This study revealed that organic substrates *viz.,* coir compost, groundnut shell compost, fully and partially decomposed municipal solid waste serve as a source of laccase producing actinobacteria. Initial screening using ρ-anisidin and Poly-R 478 yielded seven potential actinobacterial isolates that were more efficient in ligninolytic activity. Among them, one potential isolate CSC_{12} secreted laccase at comparative levels with reference culture *S. violaceusniger* MTCC 3959 and was identified as *S.bikiniensis* CSC12. Laccase from S.*bikiniensis* CSC12 had pH and temperature optima of 6 to 7 and 50 to 60 °C, respectively and the partially purified monomer reported molecular mass of ~69 kDa based on SDS and Native PAGE.

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MICROBIAL LIPASES – PROPERTIES AND APPLICATIONS

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Review

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ABSTRACT

Owing to wide spectrum of catalytic reactions both in aqueous and non-aqueous media, microbial lipases occupy an unquestionable position among the biocatalysts. The chemo-, regio- and enantio-specificities of lipases have contributed to their versatile applications in biotechnology. As far as global scenario is concerned, microbial lipases - especially from bacteria and fungi - contribute to the choice of interest to meet the commercial needs. At this context, this review critically looks into the major domains of microbial lipases with an industrial perspective, which include: properties, secretion and industrial applications with appropriate illustrations. Due to great specificity and versatility of the reactions catalyzed, lipases claim unique applications in various process and products industries engaged in food, dairy, fats and oils, detergency, tannery, pharmaceutics and cosmetics.

Keywords: Hydrolysis, *trans*-esterification, interfacial activation, active site, applications

INTRODUCTION

Enzymes are considered as the biocatalysts of nature. The increasing concerns about the environmental pollutions and stringent government regulations over the world have turned the attention of industries toward green technologies. Majority of the industrial enzymes are of microbial origin. Until the 1960s, the total sale of enzymes was only a few million dollars annually; since then, the market was broadened up spectacularly. At present, more than 200 microbial enzymes are used commercially and approximately 20 types are produced on truly industrial scale (**Li *et al.* 2012; Pandey *et al.* 1999**). Most of the enzymes produced by the industry are hydrolytic in nature; of which lipolytic enzymes draw enormous attention, because of their immense biotechnological potentials. Lipids constitute an abundant biomass on earth, and lipolytic enzymes play the pivotal role in the conversion of these hydrophobic compounds to simpler units **(Benjamin & Pandey 1998)**.

Lipases or triacylglycerol hydrolases (E.C. 3.1.1.3) are ubiquitous enzymes mediating the hydrolysis and synthesis of esters formed by the conjugation of glycerol and long-chain fatty acids **(Hasan *et al.* 2006)**. Physiologically lipase hydrolyzes triglycerides into diglycerides, monoglycerides, fatty acids and glycerol. They are abundant in animals, plants, bacteria and fungi; where they play the crucial role in lipid metabolism. For the past few decades, lipases have gained much attention due to their versatile activities toward extreme temperature, pH, organic solvents; and chemo-, regio- and enantio-selectivities **(Benjamin & Pandey 1996)**. In contrast to esterases, lipases are activated only when adsorbed to an oil-water interface *i.e*., lipase possesses a unique property of catalyzing the hydrolysis of ester bonds at the interface between an insoluble substrate phase and the aqueous phase, where the enzyme remains dissolved **(Benjamin & Pandey 2000; Priji *et al.* 2014)**. For the past two decades, the world-wide production and consumption of microbial lipases have increased considerably owing to their fascinating industrial applications, which summarize the lipases as the third largest group of enzymes after proteases and amylases. In fact, the panorama of lipase utilization encompasses many industries like dairy, food, detergents, textile, pharmaceutical, cosmetic, biodiesel, *etc*. **(Schmid & Verger 1998)**.

Upon this background, this review examines the advancements in the various domains of microbial lipases such as production, properties, secretion and industrial applications.

MICROBIAL SOURCES OF LIPASES

Lipases are produced by several microorganisms, namely bacteria, fungi, archea, eucarya; as well as animals and plants - among which bacteria, fungi and yeasts yield the majority of commercial lipases. The commercial significance of microbial lipases is mainly attributed to their vast variety of catalytic activities, ease of genetic manipulation and high yield, coupled with exponential growth of the producing microbes in inexpensive media and absence of seasonal fluctuations **(Benjamin & Pandey 1996)**. Moreover, most of the microbial lipases do not require cofactors for their activation, and they exhibit broad range of substrate specificity and high enantioselectivity **(Kirk *et al.* 2002)**. Many species of bacteria such as *Pseudomonas, Bacillus, Serratia, Alcaligens, etc*., fungi such as *Aspergillus, Penicillium,* and yeast *Candida, etc*. are known to produce lipases (Table 1). The potential for the production of lipases enable these microbes to utilize the non-conventional carbon sources such as lipids that cannot directly pass through the cell membrane and have to hydrolyze partially to release free fatty acids prior to the cellular uptake **(Najjar *et al.* 2011)**. Even though, a vast variety of microbial species are known for lipase production, only a few are utilized commercially and most of them are extracellular inducible enzymes. *i.e*, they are synthesized within the cell in the presence of inducers of long chain fatty acids such as vegetable oils, oil industry wastes, surfactants, triglycerides, *etc*. and are secreted to the external environment.

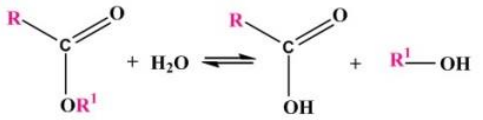
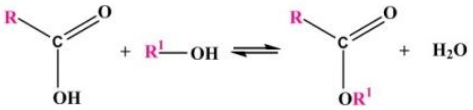
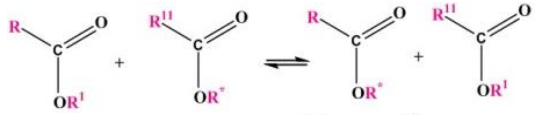
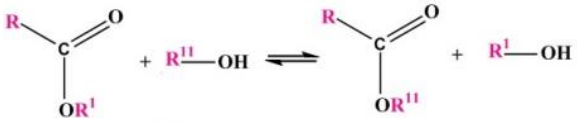
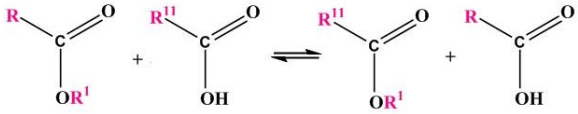
Table 1 Some bacteria and fungi producing lipase, their sources of isolation, and yield of lipase using different inducers

	Organism	Source	Substrate	Yield	Reference
Bacteria	*Pseudomonas aeruginosa*	Wastewater at sidi bel abbes, Algeria	Olive oil	41.6 U/ml	**(Zouaoui & Bouziane 2011)**
	Pseudomonas sp. 3AT	Contaminated soil and water samples	Olive and sunflower frying oils	2.748 U/ml	**(Haba *et al.* 2000)**
	Pseudomonas aeruginosa ATCC 111	Contaminated soil and water samples	Olive and sunflower frying oils	1.7038 U/ml	**(Haba *et al.* 2000)**
	Bacillus sp.	Olive mill wastewater	Tributyrin	168 U/ml	**(Ertuğrul *et al.* 2007)**
	Bacillus sp.	Setapak hot spring	Olive oil	4.58 U/ml	**(Hamid *et al.* 2003)**
	Pseudomonas aeruginosa KM110	Wastewater of an Oil processing plant	Olive oil	0.76 U/ml	**(Mobarak-Qamsari *et al.* 2011)**
	Staphylococcus warneri	Thai fish sauce	Olive oil	90.12 U/ml	**(Kanlayakrit & Boonpan 2007)**
	Staphylococcus saprophyticus M36	Seawater	Olive oil	42 U/ml	**(Fang *et al.* 2006)**
	Burkholderia sp.	Soil sample	salad oil	1.720 U/ml	**(Matsumiya *et al.* 2007)**
	Bacillus strain THL027	Oil-contaminated area	Rice bran oil	7.8 U/ml	**(Dharmsthiti & Luchai 1999)**
	Bacillus coagulans BTS-3	Kitchen waste	Olive oil	1.16 U/ml	**(Kumar *et al.* 2005)**
	Bacillus thermoleovorans CCR11	Hot springs	Olive oil		**(Castro-Ochoa *et al.* 2005)**
	Burkholderia multivorans	Compost by enrichment in oil containing medium	Palm oil	58 U/ml	**(Gupta *et al.* 2007)**
	Bacillus sp. RSJ-1	Hot springs	Cottonseed oil	10.5 U/ml	**(Sharma *et al.* 2002)**
	Bacillus thermoleovorans IHI-91	Icelandic hot spring	Olive oil	0.300 U/ml	**(Markossian *et al.* 2000)**
	Bacillus sphaericus	Soil sample	Olive oil	0.42 U/ml	**(Hun *et al.* 2003)**
	Streptomyces rimosus		Triolein	19 U/ml	**(Abramić *et al.* 1999)**
Fungi	*Penicillium camembertii* Thom PG-3		Soybean meal (fat free), Jojoba oil	500 U/ml	**(Tan *et al.* 2004)**
	Colletotrichum gloesporioides	Brazilian savanna soil	Olive oil	27.7 U/ml	**(Colen *et al.* 2006)**
	Rhizopus oryzae KG-5	Soil contaminated with lipids, oils, and decaying organic matter	Olive oil	48.66 I.U.	**(Shukla & Gupta 2007)**
	Aspevgillus niger MTCC 2594	Curd	Gingelly oil cake	236.6 U/g	**(Kamini *et al.* 1998)**
	Aspergillus niger NCIM 1207	-	Wheat bran and olive oil	630 IU/g	**(Mahadik *et al.* 2002)**
	Candida sp. 99-125 (mutant)	-	Soy bean oil	8060 U/ml	**(Tan *et al.* 2003)**
	Penicillium restrictum	Waste of oil industry	Babassu oil cake	30.3 U/g	**(Gombert *et al.* 1999)**
	Colletotrichum gloeosporioides	Oil seeds	pongamia oil cake	983 U/g	**(Balaji & Ebenezer 2008)**
	Candida cylindracea		Olive-mill wastewater	21.6 U/ml	**(Brozzoli *et al.* 2009)**
	Pseudozyma hubeiensis HB85A	Phylloplane of *Hibiscus rosa-sinensis*	Soy oil	0.386 U/ml	**(Bussamara *et al.* 2010)**
	Fusarium oxysporum		Olive oil	16 U/ml	**(Rifaat *et al.* 2010)**
	Aspergillus niger mutant 11T53A14	Contaminated butte	Soapstock	62.7 U/g	**(Damaso *et al.* 2008)**
	Penicillium wortmanii	Soil	Olive oil	12.5 U/ml	**(Costa & Peralta 1999)**
	Rhizopus homothallicus		Sugarcane bagasse and olive oil	826 U/g	**(Rodriguez *et al.* 2006)**
	Mucor sp.	Palm fruit	Palm oil	57 U/ml	**(Abbas *et al.* 2002)**
	Botryosphaeria ribis	Eucalyptus citriodora tree	Stearic acid	316.7 U/ml	**(Messias *et al.* 2009)**
	Mucor hiemalis f. hiemalis	Palm fruit	Rape seed oil	97 U/ml	**(Hiol *et al.* 1999)**
	Penicillium simplicissimum		Babassu cake	90 U/g	**(Gutarra *et al.* 2009)**
	Mutant Yarrowia lipolytica		Olive oil	1100 U/ml	**(Fickers *et al.* 2006)**
	Rhizopus oryzae	Palm fruit	-	120 U/ml	**(Hiol *et al.* 2000)**
	Aspergillus sp.	Oil cakes and seeds	Wheat raw and olive oil	1934 U/g	**(Adinarayana *et al.* 2004)**
	Trichoderma viride	Soil	Olive oil	7.3 U/ml	**(Kashmiri *et al.* 2006)**

REACTIONS CATALYZED BY LIPASE

Lipases catalyze a variety of reactions, which are primarily determined by the availability of water. Principally, they catalyze the hydrolysis of triglycerides at the aqueous-non aqueous interface, and favour the synthesis of esters from alcohols and long chain fatty acids when the water activity is low **(Aravindan *et al.* 2007)**, *i.e*, lipases can catalyze esterification, inter-esterification, and *trans*-esterification reactions in non-aqueous environments (Table 2). Thus, the versatility in activities makes lipases a suitable choice of catalyst in many industries.

Table 2 The reactions catalyzed by lipase

Nature of reaction	Name of reaction	Reaction
Hydrolysis	Hydrolysis	
Synthesis	Esterification	
Synthesis	Inter-esterification	
Synthesis	Alcoholysis	
Synthesis	Acidolysis	

PROPERTIES OF LIPASE

The pH

Most of the microbial lipases are neutral or acidic at their optimum activity. Alkaline lipases, which are stable at pH range of 8-10 offer promising applications in many upcoming bio-based industries such as textile, detergent, *etc*. Lipase from *Aspergillus carneus, Bacillus thermoleovorans, Bacillus stearothermophilus, Fusarium oxysporum* were found stable at the pH range 8-10 **(Castro-Ochoa *et al.* 2005; Prazeres *et al.* 2006; Sinchaikul *et al.* 2001)** whereas lipase produced by *Rhizopus oryzae, Cryptococcus sp. showed the maximum activity at the pH range 7-8* ***(Hiol et al. 2000; Kamini et al. 2000)***.

Temperature

Generally, most of the microbial lipases are mesophilic in nature, *i.e.*, the optimum temperature for their maximum activity ranges from 30 to 50 ^{o}C, but the increased demand for thermotolerant lipases in industries has led to the exploration of many microbial species producing thermophilic lipases, most of which retained 70-100 % of the activity even at the temperature range 50-70 ^{o}C **(Bora & Bora 2012; Gohel *et al.* 2013; Khalil 2013)**; for instance, the lipase from *Bacillus* sp. (an isolate from the hot spring) retained 90% activity at 60 ^{o}C and 70% of activity at 70 ^{o}C for 1h **(Bora & Bora 2012)**, whereas the lipase *from Pseudomonas* sp. strain ZBC1 showed the optimum activity at 80 ^{o}C **(Xing *et al.* 2013)**. Cold active lipases are active at 10-25 ^{o}C, and they facilitate gentle and efficient industrial applications by significantly reducing energy consumption. Many microbes such as *Rhodococcus cercidiphylli* BZ22, *Penicillium expansum*, *Yarrowia lipolytica*, *Stenotrophomonas maltophilia*, *Pseudoalteromonas* sp. *etc*. isolated from the harsh environments produced lipases that are active at low temperatures **(Mohammed *et al.* 2013; Park *et al.* 2013; Sathish Yadav *et al.* 2011; Wang *et al.* 2012; Yu & Margesin 2014)**.

Effectors

Usually metal ions can alter the efficacy of an enzyme either by enhancing or by inhibiting the activity. Divalent metal cations such as Ca^{2+}, Cd^{2+}, Fe^{2+}, *etc.* enhance the activity of lipases, among which Ca^{2+} plays a critical role in stabilizing the enzyme under detrimental conditions (**El Khattabi *et al.* 2003; Verma *et al.* 2012)**. Some other cations such as Co^{2+}, Zn^{2+}, Mn^{2+}, and Mg^{2+} have mild to strong inhibitory effect on lipase activity **(Kumar *et al.* 2005)**. Generally, detergents such as tween 80, tween 20 and sodium dodecylsulphate (SDS) were shown to have inhibitory effects on lipase activity; whereas triton X-100 enhanced the activity **(Castro-Ochoa *et al.* 2005)**.

Specificity

Specificities of lipases play a crucial role in their possible applications in analytical and industrial purposes, especially in pharmaceutic industry. Majority of the lipases show substrate or regio- or enantio-specificites, which are highly determined by the size, shape and hydrophobicities of the binding pockets located in the active site. Some of the lipases, specifically act on tri-, di-, mono-glycerides and other esters. Non-specific lipase completely hydrolyze the triglycereides to fatty acids and glycerol, but most of the extracellular lipases are regiospecific especially at 1, 3 positions. Lipase from *Burkholderia cepacia* found applications in organic synthesis due to its enantiospecificities, preferably the (*R*)- enantiomer over the (*S*)- forms **(Jaeger *et al.* 1999)**.

Interfacial activation

Lipase exhibits a characteristic property, called interfacial activation which makes it a suitable catalyst in water-oil medium, *i.e.*, the activity of the enzyme is highly increased when the substrates form emulsion in the reaction media. In aqueous medium, the active site of enzyme is covered by a loop of peptide called '*lid*', but contact with the interfacial area induce drastic conformational changes on to the active site, so that the lid moves aside facilitating the enzyme- substrate reaction **(Dheeman 2011)**.

Active site of lipase

The crystalline structures of many bacterial lipases have been elucidated to date, among which most of them shared a common folding pattern known as α/β hydrolase (Figure 1). Generally, α/β hydrolase consists of α helices (αA- αF) packed on either sides of a central beta sheet. The central β sheet is made of 8 parallel strands (β1- β8), except the second strand which is in antiparallel direction. The active site of lipase consists of 3 catalytic residues (the triad), a nucleophilic residue, a catalytic acid residue and a histidine residue. In lipases, the nucleophile is invariably serine, whereas the catalytic acid is either an aspartate or glutamate. The topological position of the nucleophilic residue is often after β5 strand, the Asp/Glu residue is after β7 strand and the histidine residue is after β8 strand **(Jaeger *et al.* 1999)** (Figure 1). In most of the lipases, the '*lid/hood*' or the flexible fragment is made of one or two alpha helices, which covers the active site at the inactive state of the enzyme. In the presence of hydrophobic substrates, the enzyme undergoes interfacial activation so that the conformational changes at the active site make the lid open, so as to facilitate the entry of substrates to the catalytic residues **(Pleiss *et al.* 1998)**.

The geometry of the active sites of lipases varies widely and determines the biochemical properties of the enzyme. Generally, it is a deep hydrophobic pocket which exactly fits scissile fatty acids of substrates into it. According to the shapes of the bindings sites, lipase can be categorized into three; lipase with crevice-shaped (*Rhizomucor* and *Rhizopus*), funnel-shaped (Pancreatic lipase, lipase B from, *Candida antarctica*, *Pseudomonas),* and tunnel-shaped (*C. rugosa, Geotrichum candidum*) **(Pleiss *et al.* 1998)** (Figure 2).

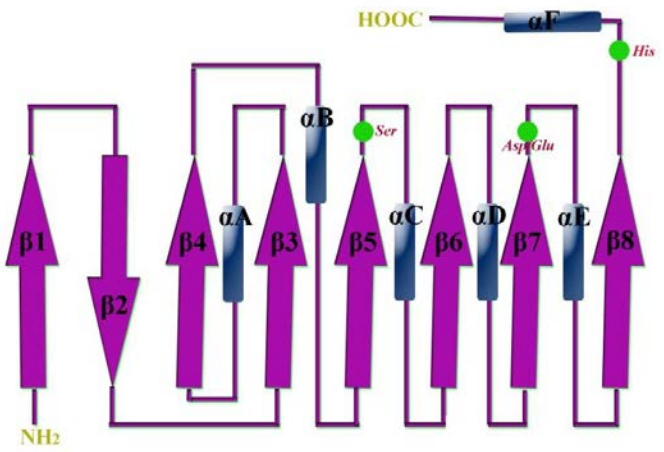


Figure 1 Structure of α/β hydrolase

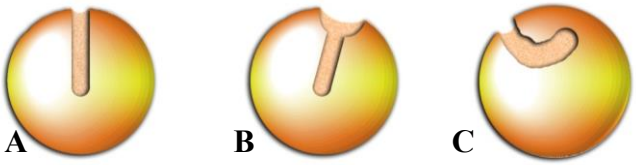


Figure 2 Different shapes of lipase active site A) tunnel –like B) funnel –like C) crevice-like

CLASSIFICATION OF LIPASE

Most of the lipases possess a consensus sequence of Gly-x-Ser-x-Gly around the serine residue situated at the active site. But advances on their crystalline studies and sequence analyses revealed the existence of other motifs also. Thus, based on their sequence homology and functional properties, lipases are classified into 8 major groups, among which the first largest family is again classified into 6 subfamilies **(Arpigny & Jaeger 1999; Bornscheuer 2002)** (Table 3).

Table 3 Classification of lipases

Family	Subfamily	Microorganism
I	1	*Pseudomonas aeruginosa*
		Pseudomonas fluorescens C9
		Vibrio cholerae
		Acinetobacter calcoaceticus
		Pseudomonas fragi
		Pseudomonas wisconsinensis
		Proteus vulgaris
	2	*Burkholderia glumae*
		Chromobacterium viscosum
		Burkholderia cepacia
		Pseudomonas luteola
	3	*Pseudomonas fluorescens SIK W1*
		Serratia marcescens
	4	*Bacillus subtilis*
		Bacillus pumilus
	5	*Bacillus stearothermophilus*
		Bacillus thermocatenulatus
		Staphylococcus hyicus
		Staphylococcus aureus
		Staphylococcus epidermidis
	6	*Propionibacterium acnes*
		Streptomyces cinnamoneus
II		*Aeromonas hydrophila*
		Streptomyces scabies
		Pseudomonas aeruginosa
		Salmonella typhimurium
		Photorhabdus luminescens
III		*Streptomyces exfoliatus*
		Streptomyces albus
		Moraxella sp.
IV		*Alicyclobacillus acidocaldarius*
		Pseudomonas sp. B11-1
		Archaeoglobus fulgidus
		Alcaligenes eutrophus
		Escherichia coli
		Moraxella sp.
V		*Pseudomonas oleovorans*
		Haemophilus influenzae
		Psychrobacter immobilis
		Moraxella sp.
		Sulfolobus acidocaldarius
		Acetobacter pasteurianus
VI		*Synechocystis* sp.
		Spirulina platensis
		Pseudomonas fluorescens
		Rickettsia prowazekii
		Chlamydia trachomatis
VII		*Arthrobacter oxydans*
		Bacillus subtilis
		Streptomyces coelicolor
VIII		*Arthrobacter globiformis*
		Streptomyces chrysomallus
		Pseudomonas fluorescens SIK W1

Source: **(Arpigny & Jaeger 1999)**

LIPASE SECRETION PATHWAYS

Lipases are extracellular enzymes, and are to be transported across the cell membrane to reach their final destination. In Gram-positive bacteria, secreted enzymes have to cross just a single cytoplasmic membrane whereas in Gram-negative bacteria, it has to be translocated through the periplasm and outer membrane **(Jaeger *et al.* 1999)**. A series of complex transporter proteins play crucial roles in the transportation of lipase through the membrane. Two major secretory pathways have been identified (represented as type I and II), of which lipases can utilize at least one of them **(Jaeger & Eggert 2002)**. Type I pathway is mainly mediated by ABC transporters, whereas secreton mediates the type II.
Lipases produced by *P. fluorescence* and *Serratia marcescens* display a C-terminal secretion signal located in the last 60 amino acids, and is not cleaved during secretion. The signal sequence specifically recognizes the ABC protein, triggering the assembly of the functional trans-envelope complex **(Delepelaire 2004).** Type I secretory pathway *via* ABC transporters generally consists of three major membrane proteins, *i.e*., the inner membrane ATPase [ATP-binding cassette (ABC) superfamily], the second protein named membrane-fusion-protein (MFP) anchored to the inner membrane with a large hydrophilic domain facing the periplasm, and a C-terminal domain presumably interacting with the outer membrane. The third component is an outer membrane protein (OMP) (Figure 3). The assignment of different proteins as these exporters may vary depending on the species. *S. marcescens,* the most studied bacterium for type I secretion of lipase, possess a signal peptide that is rich in glycine and comprises of nine-residue sequence Gly-Gly-X-Gly-X-Asp-X-U-X (where X is any amino acid and U is a large hydrophobic amino acid). It utilizes LipB as ABC protein, LipC as the MFP component which mediates contact with both the inner and the outer membrane, and LipD as the OMP component **(Akatsuka *et al.* 1995; Jaeger & Eggert 2002)**. AprD, AprE and AprF are necessary proteins acting as ABC, MFP and OMP, respectively for the efficient secretion of lipase by *P. fluorescence* **(Duong *et al.* 1994)**. The exporter constituted by these proteins forms a multiprotein complex across the periplasm for tranlocating lipase from the cytoplasm to extracellular space without forming any active periplasimic enzyme intermediates. Over expression of the ABC exporter provides a considerable increase in the secretion of the lipase, and therefore an increased yield of extracellular lipase protein **(Ahn *et al.* 2001)**.

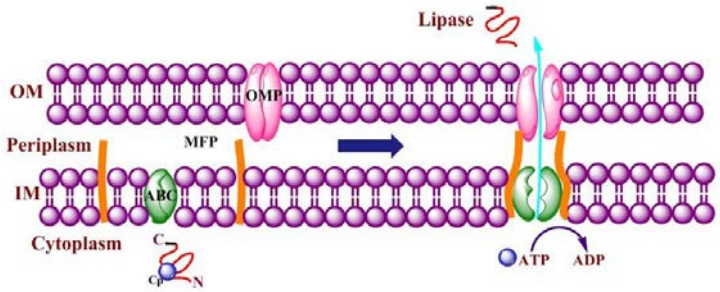


Figure 3 Type I secretory pathway of lipase *via* ABC transporters

The type II secretory pathway is generally known as sec-dependent pathway, which was found involved in the secretion of lipase in bacteria like *P. aeruginosa* and *Bacillus* spp. Many lipases of both Gram-positive and Gram-negative bacteria possess an N-terminal signal sequence and are secreted *via* type II pathway. It consists of two steps: proteins are first translocated across the inner membrane by the general secretory pathway **(Cao *et al.* 2014)** or twin-arginine translocation (*Tat*) pathway, and subsequently transported from the periplasm to the exterior of the outer membrane in an extremely short period (Figure 4). In Gram-positive bacteria, secreted enzymes have to cross just a single cytoplasmic membrane. Usually, these proteins contain a signal sequence, which directs their translocation *via* the *Sec* or *Tat* translocase, the multi-subunit proteins identified

in *Bacillus* spp. However, lipases from Gram-negative bacteria do have to cross a second barrier constituted by the outer membrane. In *P. aeruginosa*, the prolipase exported to the periplasmic space by *Sec* machinery, fold in the periplasm into an enzymatically active conformation with the help of specific intermolecular chaperones called *Lif* proteins (lipase-specific foldases). Subsequently, they are transported through the outer membrane by means of a complex machinery called secreton, consisting of up to 14 different proteins. Similar multi component secretons have been identified in lipase produced by *P. alcaligenes, Aeromonas hydrophila*, *Xanthomonas campestris* and *Vibrio cholera* **(Jaeger & Eggert 2002)**.

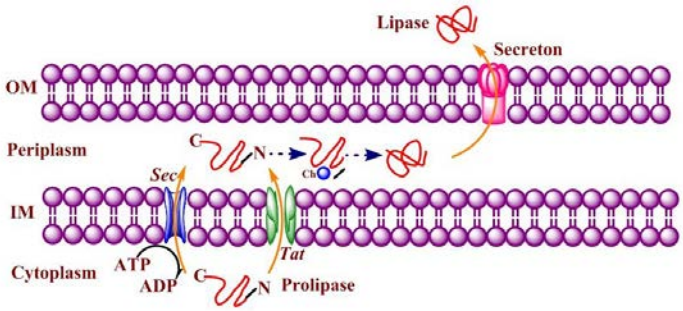


Figure 4 Type II secretory pathway of lipase mediated by *Sec* or *Tat* translocase

In *P. aeruginosa*, autotranspoters also mediate the transport of enzymes across the outer membrane. These autotransporters form channels spanning in the outer membrane, which is usually made up of a *β*-barrel composed of nearly 14 *β*-sheets and hold the enzyme in close contact to, or even firmly bound to the surface of the cell exposing its catalytic domain **(Rosenay & Jaeger, 2000)**.

APPLICATIONS OF LIPASE

Microbial lipases claim a wide variety of industrial applications due to the ease for mass production and versatile specificities. Based on total volume of sales, lipases occupy the third largest group of enzymes next to proteases and carbohydrases. The commercial use of lipases is a billion-dollar business and their applications are highly dependent on specificities, optimum pH, temperature, tolerance to organic solvents, *etc*. Lipases form an integral part of the industries ranging from food and fats, dairy, organic chemicals, pharmaceuticals, leather, environmental management, agrochemicals, detergents, oleo-chemicals, tea, cosmetics, and in several bioremediation processes **(Verma *et al.* 2012)**.

Food and dairy industry

Lipases are widely used in food industries for the hydrolysis or modification of biomaterials. Egg white is an important ingredient of many bakery products such as cakes, bread, *etc*. Contamination of egg white with lipids decreases the desirable foaming ability of the product. Therefore, lipase is used to remove the lipid contamination and to improve the quality of dough and to achieve an even, light-coloured crust on the products and a soft texture. Treatment of egg yolk with phospholipase hydrolyzes egg lecithin and iso-lecithin, thereby improving emulsifying capacity and heat stability, which can be used to make myonnaise, custards, baby foods, *etc.* **(Buxmann *et al.* 2010)**. Lipases can enhance the flavor of bakery products by synthesizing esters of short-chain fatty acids and alcohols **(Alves Macedo *et al.* 2003)**. Lipases can also be used in degumming of vegetable oils during the process of refining. The degumming process removes the phospholipid impurities from the crude vegetable oils which may otherwise pose many problems for the storage and processing of vegetable oils from soybean, sunflower and rape seed; for instance, lipase produced by genetically modified *Aspergillus oryzae* (Lecitase® Ultra) was used for the degumming of rapeseed and soybean oils, which removed more than 90% of phospholipids within 5 h at 50 °C **(Yang *et al.* 2006)**. Biolipolysis is being used to make fat free meat and fish. The controlled application of lipase to wheat flour produces different variations of quality; thereby noodles and pasta are given an even and intense color, and their stickiness when overcooked is reduced **(Menzi 1970; Søe *et al.* 2005)**. Genetically engineered baker's yeast (*Saccharomyces cereviciae*) with bacterial lipase gene LIP A resulted in higher productivity of lipase and found application in bread making as a technological additive **(Sánchez *et al.* 2002)**.
Lipases are extensively used in dairy industry for the hydrolysis of milk fat to improve the flavor of cheese, butter, fat and cream. Enzyme modified cheese technology is now gaining importance for making a variety of cheese with desired flavor and aroma; in this process lipase is generally used under controlled conditions in combination with other hydrolytic enzymes such as protease and amylase. It reduces the bitterness as well as ripening time and modifies flavor intensity **(Aminifar & Emam-Djomeh 2014)**. For instance, commercial microbial lipase (Piccantase A) enhances the flavor development during the ripening stage of Talum cheese **(Yilmaz *et al.* 2005)**. Extracellular lipase produced by *Cryptococcus flavescens* 39-A releases short-chain fatty acids (C_4 - C_6) in milk fat during the mozzarella cheese-making process and produce a favorable cheese flavor **(Mase *et al.* 2013)**. *Penicillium roqueforti*, the lipolytic activities of which contributes to the characteristic flavor and blue-green veined appearance to blue cheese **(Cao *et al.* 2014)**. Likewise, lipases enhance the flavors of natural milk fat producing volatile flavoring compounds (Omar *et al*. 2015). Lipases are widely used to produce novel fats through the process of hydrolysis, esterification and inter-esterification. Lipases modify the properties of lipids by altering the positions of fatty acid chains or by adding or removing one or more fatty acids to the glycerides. The position, chain length and degree of unsaturation greatly influence not only the physical properties, but also the nutritional and sensory value of a given triglyceride as well. Thus, high value fats and oils can be synthesized from cheap resources. For example, cocoa butter fat often used in bakery foods, is often in short supply and the price can fluctuate widely. Lipase from *Mucor miehei* can effectively be utilized to produce cocoa butter like fat from palm olein and distillate from palm oil refinery which contribute to the flavor of chocolate, caramels, toffees and butter creams (Mohamed 2012). In coffee whiteners, lipases assist in imparting a rich creamy flavor **(Godfrey, 1982)**.

Processing of fats and oil

Processing of fats and oils by the enrichment of specific fatty acids or hydrolysis of triglycerides to release fatty acids or by altering the location of fatty acids is an important application of lipase. It enables the commercial exploitation of naturally produced renewable raw materials such as oils from corn, rapeseed, sunflower, palm, coconut, olives, rice bran, and a wide range of animal fats. Wax esters, esters of long chain fatty acids and alcohols (C ≥12) are widely used in lubricant, pharmaceutical, cosmetic and plasticizer industries, which are usually extracted from expensive spermaceti oil and jojoba oil for commercial applications. Lipase mediates the synthesis of wax esters from cheap oils, for instance, immobilized lipase from *Candida* sp. synthesizes the wax ester, cetyl oleate, from oleic acid and cetyl alcohol with over 96% purity **(Deng Li *et al.* 2011)**. Lipase catalyzes the hydrolysis of salmon oil to increase its omega-3 polyunsaturated fatty acids (PUFA) content. It was observed that lipase from *C. rugosa* increased omega-3 PUFA content by 2.5 folds **(Kahveci *et al.* 2010; Kahveci & Xu 2011)**. Similarly, microbial lipases, from *C. rugosa*, *C. cylindracea*, *Mucor javanicus* and *Aspergillus niger* were used for the enzymatic hydrolysis of sardine oil to increase the content of omega-3 PUFAs by 10-35 % **(Okada & Morrissey 2007)**. Production of biodiesel consisting of methyl esters (methanolysis) of long chain fatty acids is yet another promising application of lipase which, has been widely exploited all over the world. In such cases immobilized lipase was used, which offers repeated usage of the enzyme without losing its specificity. The enzymatic production of biodiesel by methanolysis of cottonseed oil was studied using immobilized lipase from *C. antarctica* as catalyst in *t*-butanol solvent, in which the ester yield was about 95-97% **(Royon *et al.* 2007)**. Lipase producing whole cells of *Rhizopus oryzae* was employed for the production of biodiesel employing the low cost non-edible oil from the seeds of *Jatropha curcas.* A variety of low cost vegetable oils such as sunflower oil, soybean oil, karanj oil, etc. can effectively be used to produce biodiesel by lipase mediated hydrolysis **(Dizge *et al.* 2009; Kaieda *et al.* 2001; Modi *et al.* 2007; Noureddini *et al.* 2005)**. Recently, it is demonstrated that lipase from *Bacillus* spp. can be used for the biotransformation of fungal lipids into cost-competitive biodiesel **(Abd-Alla *et al*. 2015)**.

Detergents

Now-a-days enzymes have become the key constituents of detergent formulations, of which lipases play an important role for the removal of tough fatty stains such as butter, oil, *etc*. from the fabric that are hard to remove under normal washing. For the last two decades, detergent industry has become one of the biggest markets of microbial lipases, because of their functional importance for the removal of fatty residues in laundry, dishwashers and for cleaning of clogged stains. Addition of these enzymes not only improves the performance of detergents but also offers better ecological acceptance and produce effluent with lower COD and corrosive nature **(Nerurkar *et al.* 2013)**. Standard wash liquids contain anionic and nonionic surfactants, oxidants, and complexing agents at a pH of about 10 and temperatures around 35-45 °C, which is a rather hostile environment for enzymes. As a result, massive screening is required to find out suitable enzymes exhibiting low substrate specificity, stability under alkaline pH (8-11), elevated temperature (30-60 °C), and also compatibility with other ingredients of formulations such as metals, oxidants, surfactants *etc*. Bacterial lipase from *Staphylococcus arlettae* JPBW-1 isolated from the rock salt mine has been assessed for its use in laundry formulations which exhibited good stability towards surfactants and oxidizing agents, and removed about 62 % of olive oil from cotton fabrics **(Chauhan *et al.* 2013)**. A novel alkaline lipase from *Burkholderia cepacia* RGP-10 exhibited better stability towards commercial detergents and oxidizing agents than Lipolase® **(Rathi *et al.* 2001)**. Application of cold active lipase in detergent formulations allows laundering at low temperatures and reduces the energy expenditure. Recently, many cold-active lipases have been reported in bacteria such as *Pseudoalteromonas* sp. NJ 70

(Wang *et al.* 2012), *Bacillus sphaericus* **(Joseph & Ramteke 2013)**, *Microbacterium luteolum* **(Joseph *et al.* 2012)**, *Pichia lynferdii* **(Park *et al.* 2013)**, *etc*. Most of them are active at temperature ranging from 0-30 ^{o}C, and showed good tolerance to salt, synthetic surfactants and oxidizing agents.

Lipolase, the first industrial lipase was obtained from *Humicola lanuginose*, which was marketed by Novozymes (Denmark). Later on three genetically modified commercial lipases such as LipoPrime, Lipolase Ultra, Lipex were also marked by expressing the lipase gene of *Humicola lanuginose* in *Aspergillus oryzae,* a fungus. Many other detergent lipases are available on the market and some of them are listed in Table 4.

Table 4 Commercial lipases used in detergent industry

Trade name	Source	Supplier	Expression
Lipolase®	*Humicola lanuginosa*	Novozymes	*Aspergillus oryzae*
LipoPrime™	Protein engineered variant of lipolase	Novozyme	*Aspergillus oryzae*
Lipolase Ultra®	Protein engineered variant of lipolase	Novozymes	*Aspergillus oryzae*
Lipex®	Protein engineered variant of lipolase	Novozymes	*Aspergillus oryzae*
Lipomax™	*Pseudomonas alcaligenes*	Genencor Inc.	*Pseudomona alcaligenes*
Lipase P	*Pseudomonas fluorescens*	Amano Pharmaceutical Co. Ltd	*Pseudomonas fluorescens*
Luma fast	*Pseudomonas mendocina*	Genencor, USA	*Bacillus* sp.

Leather industry

These days, enzymes are widely used for the processing of hides and skins in leather industry. Lipase and proteases are the most important enzymes which found applications during bating, soaking, dehairing and degreasing of skin **(Dayanandhan *et al.* 2003; Hasan *et al.* 2006)**. During bathing, the enzymes enhance water uptake, loosen the scud and disperse fats and oils together with dirt and other materials present on the skin. Lipase specifically degrades fat but do not damage the leather which is proteinaceous in nature, it hydrolyses the fat on the outside of the hides and skins as well as inside the skin structure. Thus, lipase assisted treatment of leather gives the leather with far better quality and finish with uniform color and cleaner appearance as compared to conventional chemical agents. Lipase also improves the production of hydrophobic waterproof leather, which represents an environment friendly method of leather processing. It was found that the lipase produced by *B. subtilis* can be used for the degreasing process, thereby removing all the fat within 8-12 h of incubation by maintaining natural skin colour **(Saran, 2013)**. NovoLime, a protease/lipase blend for enzyme-assisted liming of hides and skins, and NovoCor AD, an acid lipase for degreasing of hides and skins, are some of the commercially available lipases for the leather industry.

Pharmaceutics

Applications of lipase for the synthesis of chiral drugs, kinetic resolution of racemic alcohols, acids, esters or amines are well established. Synthesis of diltiazem hydrochloride, a calcium antagonist (a coronary vasodilator), using lipase mediated asymmetric hydrolysis of *trans*-3-(4-methoxyphenyl) glycidic acid methyl ester [(±)-MPGM] was found to be a more efficient process compared to the conventional chemical synthesis, for which lipase *from Serratia marcescens* was generally employed **(Matsumae *et al.* 1993)**. Lipase from *C. rugosa* immobilized on a nylon scaffold was used to synthesize lovastatin, a drug lowering the serum cholesterol levels, by the regioselective acylation of a diol lactone precursor with 2-methylbutyric acid in mixtures of organic solvents **(Yang *et al.* 1997)**. Lipase from *Pseudomonas* sp. AK mediated the kinetic resolution of the chiral silane reagents used for the synthesis of a potent antitumor agent called epothilone A **(Zhu & Panek 2001)**.

Pulp and paper

The paper industry utilizes huge amount of lignocellulose every year. Historically, the enzymatic applications in paper industry was confined to the treatment of raw starch; but, later in since 1990s, lipase mediated removal of pitch has become an essential process of large-scale paper making process. 'Pitch' or 'resin stickies' is a term used to collectively describe the hydrophobic components of wood such as triglycerides, waxes, *etc.* **(Farrell *et al.* 1997)**. Pitch and related substances, which usually create major problems to the machines and cause holes and spots in the final paper, are common in paper mills. It may reduce the production levels and increase equipment maintenance as well as operation costs. Nippon paper Industries, in Japan, have developed a pitch control method that uses the *C. rugosa* fungal lipase to hydrolyze up to 90% of the wood triglycerides **(Arpigny & Jaeger 1999, Jaeger & Reetz 1998)**. Lipase in paper industry decreases chemical usage thereby reducing pollution level of waste water. It provide prolonged equipment life as lipase removes sticky deposits in the paper machines, save energy and time and reduce composite cost **(Farrell *et al.* 1997)**. The addition of lipase from *Pseudomonas* sp. (KWI-56) to a deinking composition for ethylene oxide-propylene oxide adduct stearate improved whiteness of paper and reduced residual ink spots **(Fukuda *et al.* 1990, Gandhi 1997)**. Lipase from *Thermomyces lanuginosus* was immobilized on the resin coated with chitosan along with pectinase, which reduced the pitch deposits in white water by 74% **(Liu *et al.* 2012a)**. Similarly, alkaline lipase was found to be efficient for removing pitch from the recycled fiber pulping waste water **(Liu *et al.* 2012b)**.

Cosmetics

Recently, lipases found applications in producing many cosmetic ingredients such as retinol, natural dyes, *etc*. For the cosmetic industry, the natural products are always of interest as people demands those products which improve not just the appearance of the skin, but the health of the skin as well. Vitamin A (retinol), vitamin C (ascorbic acid), and derivatives combat many skin disorders including photoaging, psoriasis and acne **(Adamczak & Bornscheuer 2009)**. Lipase catalyzed synthesis of retinyl esters has become popular as the chemical synthesis meets some serious defects and offers mild reaction conditions, high catalytic efficiency, inherent selectivity, and much purer products **(Maugard & Legoy 2000, Maugard *et al.* 2000, Moreno-Perez *et al.* 2013)**. Lipase from *C. antarctica* efficiently catalyzes the *trans*-esterification between glycerides and vitamins to produce retinyl/ ascorbic esters **(Lerin *et al.* 2012; Moreno-Perez *et al.* 2013; Reyes-Duarte *et al.* 2011; Sun *et al.* 2013)**. Immobilized lipase catalyzes the synthesis of retinyl L-lactate by the *trans*-esterification reaction between retinol and L-methyl lactate, and the synthesis of ascorbyl L-lactate by the *trans*-esterification of ascorbic acid with L-methyl lactate, with yield over 90% and 80%, respectively **(Maugard *et al.* 2000)**. Lipase from *C. antartica* also mediated the *trans*-esterification between olive oil and ascorbic acid to produce liposoluble ascorbyl oleate, which is widely used as an antioxidant **(Moreno-Perez *et al.* 2013)**.

Lipases also found applications in the production of natural dyes such as indigo and its derivatives, water soluble dyes of interest for cosmetics. Lipase releases indoxyl from istan B which in combination with isatan C can be processed to produce indigo **(Maugard *et al.* 2002)**. Aroma esters consisting of short chain fatty acids and alcohol are synthesized by the direct application of lipase, which provides natural fragrance to the cosmetics. In 2001, Gatfield *et al.* reported a method to produce natural ethyl (E,Z)-2,4-decadienoate, the compound of pear, by lipase mediated *trans*-esterification of stillingia oil with ethanol **(Gatfield *et al.* 2001)**.

CONCLUSIONS

Global enzyme market is expected to rise by 7 % in 2015. Growing trends in the world market of biocatalysts indicate that, developed countries in North America would be the largest consumers followed by Western Europe. Their stringent government rules and regulations made them to adopt green technologies in industries to address the environmental issues as well as to improve the product quality and acceptability. Industrial processes demand enzymes with unique specificities and high performance which attracts the attention of researchers and industrialists to produce novel enzymes to minimize the cost. Application of lipases is broadening up rapidly, due their remarkable potential for accomplishing innumerable novel reactions, both in aqueous and non-aqueous environments. Hence, the demand for the production and characterization of new lipases is still increasing significantly. Though many microbial lipases have been explored for their mode of actions, the high cost of production and purification hinders its world-wide commercialization. Moreover, it is necessary to elucidate the reaction mechanism of lipases - both general and type-wise in tune with the specific need in industry, mode of control and regulated expression to meet the future needs and to hit the anticipated level of commercial demand.

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COMPARISON OF PREVALENCE AND GENETIC DIVERSITY OF *ESCHERICHIA COLI* O157:H7 IN CATTLE AND SHEEP

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ABSTRACT

In this study the prevalence of *Escherichia coli* O157:H7 was detected by immunomagnetic separation (IMS) based cultivation technique and polymerase chain reaction (PCR) in feces and/or colon tissue of cattle (n= 282) and sheep (n= 218) at slaughterhouse. The major virulence genes, intimin variants, Shiga toxin variants and antibiotic resistance genes of the isolates were examined by PCR and genomic diversity of the cattle and sheep *E. coli* O157:H7 isolates were assessed using pulsed field gel electrophoresis (PFGE). In the present study the prevalence of *E.coli* O157:H7 was found higher in sheep (6.4 %) than in cattle (3.9 %). All the *E.coli* O157:H7 isolates were detected as positive for at least one *stx* gene and positive for other virulence genes. Twelve (29.3 %) and one (2.4 %) of the cattle isolates carried stx_2 and stx_1 gene, respectively. However 11 (17.7 %) of the sheep *E. coli* O157:H7 isolates carried stx_2 and five (8.1 %) of the isolates harbored stx_1 gene only. At least one antibiotic resistance gene was detected from 35 isolates. *E. coli* O157:H7 isolates from four sheep and three cattle harbored *tetB* gene. From three cattle and one sheep samples *strA* carrying *E. coli* O157:H7 were isolated. Among them, isolates from 2 cattle and one sheep samples were carried both *tetB* and *strA*. Isolates were grouped into six different clusters. From a cattle and a sheep, two different *E. coli* O157:H7 which have different PFGE patterns, were isolated. It can be concluded that sheep pose a risk as cattle for STEC O157:H7 contamination in Turkey.

Keywords: *E. coli* O157:H7; virulence genes; *stx* variants; antibiotic resistance; PFGE

INTRODUCTION

Escherichia coli O157:H7 has emerged as a pathogen of considerable public health importance which causes a spectrum of illnesses ranging from hemorrhagic colitis to hemolytic-uremic syndrome (HUS) worldwide. Gastro-intestinal system of the ruminants is the primary reservoir of this organism **(Meng *et al.*, 2001)**. Many studies showed that both dairy and beef cattle were the primary source and the carrier of the bacterium **(Chapman *et al.*, 1993; Byrne *et al.*, 2003; Shin *et al.*, 2014)**. Limited number of studies reported sheep as a reservoir of this emerging pathogen **(Söderlund *et al.*, 2012)**.
Several virulence factors have been described in *E. coli* O157:H7, the major ones are known as Shiga toxins (stx_1 and stx_2) which are responsible for life threatening illnesses like HC and HUS. *E. coli* O157:H7 produces putative virulence genes such as intimin (encoded by *eaeA*) which is necessary for attaching and effacing adhesion on host cell membrane, and hemolysin (encoded by *hly*). There are several intimin variants identified and they were shown to affect tissue tropism and colonization site **(Mundy *et al.*, 2007)**. In addition, *espA* (*E. coli* secreted protein A) and *lpf* (Long polar fimbria) are the virulence genes which are important in tropism, attachment, persistence and virulence of *E. coli* O157:H7, were identified in recent years **(Torres *et al.*, 2009)**.
Antibiotic treatment in *E. coli* O157:H7 infections in human is not always possible because of lysis of the cells and increased expression and release of the Shiga toxins (Stx) in the intestinal tract **(Wong *et al.*, 2000)**. However using some antimicrobials in the early stage of infection may be protective against heamolytic uremic syndrome (HUS) progression **(Ikeda *et al.*, 1999)**. Due to extensive use of antibiotics in veterinary medicine for prophylaxis or growth promotion in animal production in several studies, resistant *E. coli* O157:H7 strains were reported to various antibiotics **(Schroeder *et al.*, 2004; Goncuoglu *et al.*, 2010)**. In a report 79.8% of the *E. coli* O157:H7 isolates were found to carry one or more antibiotic resistance genes **(Srinivasan *et al.*, 2007)**.
The objectives of this study were to determine the prevalence of *Escherichia coli* O157:H7 by immunomagnetic separation (IMS) based cultivation technique and PCR in feces and/or colon tissue of cattle and sheep, to detect the major virulence genes, intimin variants, Shiga toxin variants and antibiotic resistance genes of the isolates by polymerase chain reaction (PCR) and to determine the genomic diversity using pulsed field gel electrophoresis (PFGE).

MATERIAL AND METHODS

Sample collection

A total of 282 cattle consisting 207 beef and 75 dairy cattle and 218 sheep (with a total of 500 animals) feces and/or colon tissue samples were collected from a slaughterhouse within 28 visits in Ankara province. Samples were taken into sterile filtered bags and taken into laboratory in an ice box and analyzed within 2 hours.

Microbiological analysis

IMS based cultivation technique was used for the isolation of *E. coli* O157 **(Byrne *et al.*, 2003)**. Ten grams of samples were weighed to a sterile bag and suspended with 90 ml EC broth (Oxoid CM0853, Hampshire, UK) containing novobiocin (20 µg/L; Sigma N-1628, St. Louis, USA) and incubated at 37°C at 100 rpm/min for 18 h in a shaking incubator (Bellco Shel Lab Shaking Incubator S16R, Oregon, USA). Then, IMS was performed with 20 µl of magnetic beads (Dynabeads anti *E. coli* O157, Dynal, Norway) according to the manufacturer's protocol.
Following to IMS procedure, 100 µl of resuspended suspension was plated on Cefixime-tellurite (Oxoid SR0172) supplemented Sorbitol MacConkey Agar (Oxoid CM0813) and incubated at 42°C for 24 h. After incubation, sorbitol negative colonies were tested for the O157 antigen by latex agglutination (Oxoid DR0620) and up to five positive colonies were picked for PCR analysis.

PCR analysis for the detection of virulence genes

DNA extraction was performed by Chelex-100 (Bio-Rad, Hercules, CA, USA) resin based technique, using proteinase K (20 mg/ml; AppliChem GmbH, Darmstadt, Germany). Virulence genes including; *stx*$_{1,}$ *stx*$_2$, *eaeA*, *hly* and *fliC*$_{h7}$ **(Fratamico *et al.*, 2000)** were detected by multiplex PCR; *espA* **(McNally *et al.*, 2001)** and *lpfA*1-3 **(Torres *et al.*, 2009)** genes were detected by PCR. *E. coli* O157:H7 ATCC 43895 (*stx*$_1^+$, *stx*$_2^+$, *eaeA*$^+$, *hly*$^+$, *lpfA1-3*$^+$, *espA*$^+$) was used as positive control.

Detection of intimin and Shiga toxin variants of *E. coli* O157:H7 isolates

Intimin variants α1, α2, β, β1, β2, γ1 and γ2/θ were tested in *eaeA* gene detected intimin harboring isolates by previously published primer pairs and PCR conditions **(Blanco *et al.*, 2004)**. *E. coli* O157:H7 ATCC 43895 (*eae*γ_1^+) was used as positive control.
E. coli O157:H7 isolates were subjected to consecutive multiplex and conventional PCR assays for determination of *stx*$_1$ variants (*stx*$_{1c}$ **[Zhang *et al.*, 2002]** and *stx*$_{1d}$ **[Bürk *et al.*, 2003]**) and/or *stx*$_2$ variants (*stx*$_{2c}$, *stx*$_{2d}$, *stx*$_{2e}$, *stx*$_{2f}$ **[Osek, 2003]** and *stx*$_{2g}$ **[Leung *et al.*, 2003]**). *E. coli* O157:H7 ATCC 43895 (*stx*$_{1c}^+$), strains *E. coli* O157:NM 137/98 (*stx*$_{2c}^+$), *E. coli* O62:H$^-$ 551/98 (*stx*$_{2d}^+$), *E. coli* O139:K12 107/86 (*stx*$_{2e}^+$), *E. coli* O$^-$:H18 214/125 (*stx*$_{2f}^+$) and *E. coli* O2:H25 S86 (*stx*$_{2g}$) were used as positive controls.

Detection of antibiotic resistance genes by multiplex PCR

Antimicrobial resistance genes encoding for the tetracycline efflux pump (*tetA*, *tetB*, *tetC*, *tetD*, *tetE*, and *tetG*); streptomycin phosphotransferases (*strA* and *strB*); aminoglycoside adenyltransferase (*aadA*); chloramphenicol transporter nonenzymatic chloramphenicol-resistance protein (*cmlA*); florfenicol export protein (*floR*); dihydropteroate synthetase type I (*sulI*); dihydropteroate synthetase type II (*sulII*); and beta-lactamase-ampicillin resistance (*ampC*) in *E. coli* O157:H7 isolates were determined by PCR according to **Srinivasan *et al.*, (2007)**.

Genomic characterization by PFGE

Escherichia coli O157:H7 isolates were sub-typed by PFGE technique of CHEF electrophoresis described by **Harsono *et al.*, (1993)**. Genomic DNA was digested in agarose plugs with *XbaI* (Promega) as recommended by the manufacturer. The resulting DNA fragments were resolved by CHEF- PFGE with a CHEF-DR III apparatus (Bio-Rad Laboratories, CA, USA) at 200 V for 19 h at 14°C and switch times from 1 to 60 s. Low-range lambda concatemers (Promega) were used as DNA size standards. The fragments were visualized by a gel documentation system (Syngene Ingenius). GeneTools software (version 3.08.01; Syngene, United Kingdom) was used for processing the gel image. PFGE results were ascertained by the presence, absence and similarity of restriction fragments and the subtypes were coded as A, B, C, D, E and F.

RESULTS AND DISCUSSION

Fourteen of 218 sheep and 11 of 282 cattle were determined as positive for *E. coli* O157:H7. The prevalence of *E.coli* O157:H7 was found in sheep as 6.4 % and in cattle as 3.9 %. Among cattle samples, dairy cattle prevalence of *E. coli* O157:H7 was higher than beef cattle samples with a ratio of 5.3 % and 3.4 %, respectively. Most of the isolates (20/25, 80 %) were determined in warm months (spring and summer). *E. coli* O157:H7 was isolated from 54.5 % (6/11) of all positive cattle samples in the July. Other one and four *E. coli* O157:H7 positive cattle samples were recovered in May and October, respectively. Seasonal distribution of *E. coli* O157:H7 is similar in sheep with cattle samples. Ten out of 14 (71.4 %) *E. coli* O157:H7 positive samples were taken in summer; three positive samples were detected in spring. Only in one (9.1 %) sheep winter sample *E. coli* O157:H7 was found (Tab 1).

Table 1 Seasonal distribution of *E. coli* O157:H7 in cattle and sheep feces and/or colon tissue samples

Season	Months	Number of visits	Number of samples	Number of positive samples	% Seasonal distribution
Spring	April	3	5C+9S	2S	6.3%C, 16.7%S
	May	4	11C+9S	1C+1S	
	June	5	30C+68S	-	
Summer	July	6	54C+50S	6C+3S	6.1%C, 5.9%S
	August	4	15C+52S	7S	
Autumn	September	2	25C	-	6.6%C
	October	2	36C	4C	
	December	3	61C+30S	1S	
Winter	January	1	35C	-	3.3%S
	February	1	10C	-	

C: Cattle; **S:** Sheep

In order to find the *E. coli* O157:H7 contamination with multiple strains, up to five colonies were picked from the positive samples for further molecular characterization. For this purpose a total of 103 colonies (41 cattle and 62 sheep isolate) were isolated from these positive animals (14 sheep and 11 cattle). All of the 103 colonies carried at least one *stx* gene and 74 of them (71.8 %) were found to carry both of toxin genes. Additionally, all *E. coli* O157:H7 isolates carried *hly*, *eaeA*, *lpf* , *espA* genes and harbored *eae-γ1* as an intimin variant (Tab 2).
When we compared the cattle and sheep isolates, 12 (29.3 %) of the cattle isolates carried *stx*$_2$ and one (2.4 %) *stx*$_1$ gene only. However 11 (17.7 %) of the sheep *E. coli* O157:H7 isolates carried *stx*$_2$ and five (8.1 %) harbored *stx*$_1$ gene only. In the study, 28 of 41 (68.3 %) cattle *E. coli* O157:H7 colonies and 46 of 62 (75.4 %) sheep *E. coli* O157:H7 colonies harbored *stx*$_1$ and *stx*$_2$. Six (5.8 %) and 23 (22.3 %) of 103 isolates carried *stx*$_1$ and *stx*$_2$, respectively. In *stx*$_1$ and *stx*$_2$ positive isolates *stx*$_{1c}$ and *stx*$_{2c}$ variants were detected. In general, colonies that were isolated from the same sample harbored the same toxin profile except a cattle (coded as C3 in Tab 2) and a sheep (coded as S4 in Tab 2) sample. In sample C3 5 *E. coli* O157:H7 were picked during isolation; although 4 of them had both *stx*$_{1c}$ and *stx*$_{2c}$ Shiga toxin genes, one colony had only *stx*$_{2c}$. Similarly in sample S4, 4 of the colonies had both *stx*$_{1c}$ and *stx*$_{2c}$, but one colony had only *stx*$_{2c}$. This means a sheep and cattle that we sampled, carried at least two different *E. coli* O157:H7 strains.

Table 2 Virulence and antibiotic resistance gene distribution and genomic diversity of the *E. coli* O157:H7 sheep and cattle isolates

Sample no [a] (number of colonies)	H7	*hly*	*lpf*	*espA*	*stx*$_1$ variant	*stx*$_2$ variant	Intimin variant *eae-γ1*	Antibiotic resistance genes	PFGE group
S1 (5)	+	+	+	+	*stx*$_{1c}$	*stx*$_{2c}$	+	*tetB*	A
S7 (4)	+	+	+	+	*stx*$_{1c}$	*stx*$_{2c}$	+		A
S8 (4)	+	+	+	+	*stx*$_{1c}$	*stx*$_{2c}$	+		A
S9 (5)	+	+	+	+	-	*stx*$_{2c}$	+		A
S10 (5)	+	+	+	+	*stx*$_{1c}$	*stx*$_{2c}$	+	*tetB, strA*	A
S12 (2)	+	+	+	+	*stx*$_{1c}$	*stx*$_{2c}$	+	*tetB*	A
C4 (4)	+	+	+	+	-	*stx*$_{2c}$	+	*tetB, strA*	A
S3 (5)	+	+	+	+	*stx*$_{1c}$	*stx*$_{2c}$	+		B
S5 (5)	+	+	+	+	*stx*$_{1c}$	-	+		B
S13 (4)	+	+	+	+	*stx*$_{1c}$	*stx*$_{2c}$	+		B
C2 (5)	+	+	+	+	-	*stx*$_{2c}$	+		B
C5 (2)	+	+	+	+	-	*stx*$_{2c}$	+		B
C1 (5)	+	+	+	+	*stx*$_{1c}$	*stx*$_{2c}$	+	*tetB, strA*	C
S2 (5)	+	+	+	+	*stx*$_{1c}$	*stx*$_{2c}$	+	*strA*	D
S4 (4)	+	+	+	+	*stx*$_{1c}$	*stx*$_{2c}$	+		E

S4 (1)	+	+	+	+	-	stx_{2c}	+		-
S14 (5)	+	+	+	+	-	stx_{2c}	+		E
C3 (4)	+	+	+	+	-	stx_{2c}	+	*strA*	E
C3 (1)	+	+	+	+	stx_{1c}	stx_{2c}	+	*tetB*	-
C6 (5)	+	+	+	+	stx_{1c}	stx_{2c}	+		E
C7 (5)	+	+	+	+	stx_{1c}	stx_{2c}	+		E
C8 (5)	+	+	+	+	stx_{1c}	stx_{2c}	+		E
C9 (1)	+	+	+	+	stx_{1c}	stx_{2c}	+		E
C10 (1)	+	+	+	+	stx_{1c}	-	+		E
C11 (3)	+	+	+	+	stx_{1c}	stx_{2c}	+		E
S6 (4)	+	+	+	+	stx_{1c}	stx_{2c}	+		F
S11 (4)	+	+	+	+	stx_{1c}	stx_{2c}	+	*tetB*	F

[a] **S:** Sheep, **C:** Cattle.

Thirty five of 103 isolates (34 %) carried at least one antibiotic resistance gene. *E. coli* O157:H7 isolates from four sheep and three cattle harbored *tetB* gene. From three cattle and one sheep samples *strA* carrying *E. coli* O157:H7 were isolated. Among them, isolates from 2 cattle and one sheep samples were carried both tetB and strA genes. From *tetB* and *strA* were the only detected antibiotic resistance genes from the positive samples. Within the 14 antibiotic resistance genes carrying isolates (9 cattle and 5 sheep) both *tetB* and *strA* were determined while from 12 (1 cattle and 11 sheep) and nine *E. coli* O157:H7 (4 cattle and 5 sheep) only *tetB* or *strA* were detected, respectively (Tab 2). Representative PCR results are shown in the figure 1.

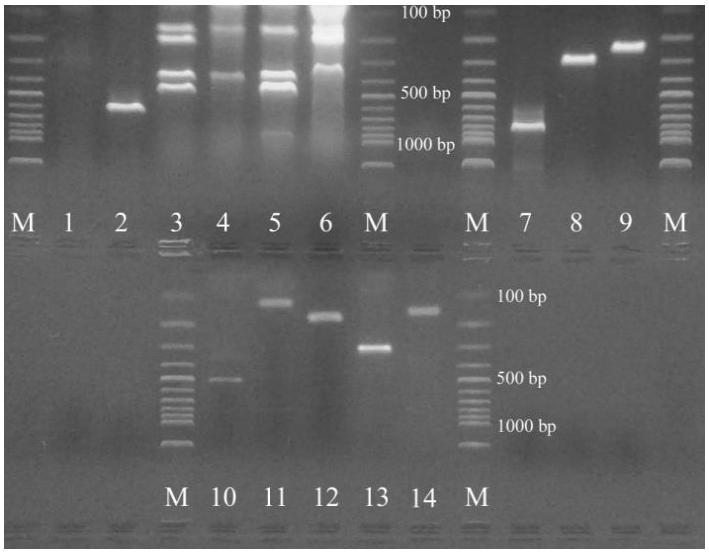

Figure 1 Representative PCR gel electrophoresis of virulence genes detected positive control strains and isolates

Lane M: 100 bp DNA marker; **Lane 1:** Negative control; **Lane 2:** *E. coli* O157:H7, ATCC 43895 ($fliC_{h7}^+$); **Lane 3:** *E. coli* O157:H7, ATCC 43895 (stx_1^+, stx_2^+, $eaeA^+$, hly^+); **Lane 4:** *E. coli* O157:H7, NCTC 12900 (stx_1^-, stx_2^-, $eaeA^+$, hly^+); **Lane 5:** *E. coli* O157:H7 isolate (stx_1^-, stx_2^+, $eaeA^+$, hly^+); **Lane 6:** *E. coli* O157:H7 isolate (stx_1^+, stx_2^-, $eaeA^+$, hly^+); **Lanes 7:** *E. coli* O157:H7 isolate ($eae\gamma_1^+$); **8:** *E. coli* O157:H7 isolate ($espA^+$); **9:** *E. coli* O157:H7 isolate ($lpfA1\text{-}3^+$); **Lane 10:** *E. coli* O157:H7, ATCC 43895 (stx_{1c}^+); **Lane 11:** *E. coli* O157:NM, 137/98 (stx_{2c}^+); **Lane 12:** *E. coli* O62:H$^-$, 551/98 (stx_{2d}^+); **Lane 13:** *E. coli* O139:K12, 107/86 (stx_{2e}^+): **Lane 14:** *E. coli* O$^-$:H18, 214/125 (stx_{2f}^+)

PFGE data indicated that there were six distinct restriction endonuclease digestion profiles (REDP) among the 103 isolates examined and coded as A, B, C, D, E and F (Tab 1). In pattern A, 29 isolates belonged to six sheep (25 isolates) and one cattle (4 isolates). Pattern A isolates were achieved from four different collection visits. Four sheep samples (18 isolates) that were from same collection date showed different toxin profile. Group B isolates (21 isolates) were belonged to five different animals (3 sheep and 2 cattle) and isolates showed quite distinct toxin profiles. Five isolates from one cattle and five isolates from one sheep were taken part in group C and D, respectively. Both isolates harbored two of the toxin genes.

In group E, nine (36 %) of the 25 positive cattle (7) and sheep (2) colonies showed the same genomic profile. Group E isolates were recovered from four different visits. A total of 35 isolates (25 from cattle and 10 from sheep) showed different toxin profiles some of them were harbored stx_1 and stx_2 together (23 isolates). One isolate was carried stx_1 while nine were stx_2. As it is shown in Table 2, remarkable results of the study were seen in group E. One isolate from cattle (coded as C3) and one from sheep (coded as S4) showed different genomic profiles from others 4 sub-colonies which were isolated the same samples. They had different toxin genes from their positive animal sub-colonies. The isolate of concern from cattle was detected as stx_1 and stx_2 positive on the other hand other colonies (4 colonies) from the same cattle harbored stx_2 only. Also this isolate carried *tetB* where the rest of the isolates from the same cattle carried *strA* as an antibiotic resistance gene. Likewise isolate of concern from sheep harbored only stx_2 although others (4 colonies) from the same sheep harbored both of the toxin genes together. These results indicated that this cattle and sheep were simultaneously contaminated with two different *E. coli* O157:H7.

Two sheep isolates (8 isolates) were grouped as F and isolates harbored both of the toxin genes. Four isolates from one sheep carried *tetB* and *strA* which the isolates from other positive sheep did not.

In this study which is an important data on the presence of *E. coli* O157:H7 in sheep in Turkey, the prevalence was found as 6.4 %. Lower than our results, 5.4 % of sheep faeces samples in Ethiopia **(Mersha *et al.*, 2010)** and 1.8 % of the Swedish sheep (**Söderlund *et al.*, 2012)** were found to carry *E. coli* O157:H7 while in North Wales in none of the sheep fecal samples this pathogen was detected **(Alhelfi *et al.*, 2013)**.

In the present study the prevalence of *E.coli* O157:H7 was found higher in sheep (6.4 %) than in cattle (3.9 %).**Van Donkersgoed *et al.*, (1999)** reported that prevalence of *E. coli* O157:H7 in fecal samples of cattle at slaughter level in Canada was 7.5 % by IMS. In another study from 1.5 % of fecal and/or tissue samples of healthy cattle in the USA *E. coli* O157:H7 was isolated **(Byrne *et al.*, 2003)**. Different from the present study only 3 of 1,300 (0.2 %) fecal samples collected from adult cattle using IMS technique in Norway were found to be contaminated with *E. coli* O157:H7 **(Johnsen *et al.*, 2001)** In the study, the PFGE analysis revealed two different PFGE profiles among 3 isolates. The toxin profiles between these groups were showed differences such as two isolates in the same group have stx_2, *eae* and *fliC*, the other isolate from the other group has both stx_1 and stx_2 with *eae* and *fliC* **(Hancock *et al.*, 1997)**.

In the study, the prevalence of *E. coli* O157:H7 in feces of healthy (3.7 %) and diarrheic cattle (4.3 %) were nearly the same. This can be explained that cattle can carry *E. coli* O157:H7 without showing any symptoms of disease **(Meng *et al.*, 2001)**. It was found that out of 207 beef cattle, and 75 dairy cattle samples, seven (3.4 %), and four (5.3 %) were found to be contaminated with *E. coli* O157:H7, respectively. However it is widely believed that dairy herds are the primary reservoirs of *E. coli* O157:H7 **(Hancock *et al.*, 1997)**.

The seasonal distribution of *E. coli* O157:H7 were 11.8, 5.9, 6.6 and 0.7 % during the spring, summer, autumn and winter, respectively. Similar to the previous studies **(Van Donkersgoed *et al.*, 1999; Johnsen *et al.*, 2001)** our results showed that the prevalence of *E. coli* O157:H7 in cattle and sheep in tested samples was higher in warm months (6.6 %) than in cold months (2.5 %).

In the present study, all 27 isolates from 15 sheep and 12 cattle which 25 of them were isolated from different samples and two of them have different toxin profile from the other sub-colonies isolated from same samples, were found to carry at least one toxin gene (stx_1 or stx_2). Eighteen (18/27; 66.7 %) of the isolates were positive both for stx_{1c} and stx_{2c}, seven of them (7/27; 25.9 %) were positive for only Shiga toxin 2 variant stx_{2c}, and two of them (2/27; 7.4 %) were positive for alone Shiga toxin 1 variant stx_{1c}. Importantly, it was reported that in most of human *E. coli* O157:H7 cases stx_2 gene was more important in generating illness than stx_1 gene harboring strains on the epidemiological study **(Boerlin *et al.*, 1999)**. Also in four different REDP, most of the isolates (72.7 %) were shown the same toxin profiles in the same genomic groups. In a study performed in Turkey, where differs from our results that 9 of the 13 *E. coli* O157:H7 isolates from cattle feces harbored only stx_2 and 2 of the isolates were found to carry stx_1 and stx_2 toxin genes **(Yilmaz *et al.*, 2006)**. In a previous study, presence of *stx1, stx2* and *stx2* variants in *E. coli* isolated from asymptomatic individuals or patients of clinical manifestations of either HUS or diarrhea without HUS was compared and they found out that the presence of stx_{2c} can more likely cause HUS while presence of stx_{2d} may manifest a milder case **(Friedrich *et al.*, 2002)**. In a different study, a higher *in vitro* cytotoxicity was also reported for stx_{2c} carrying *E. coli* O157:H7 than stx_1-stx_2 or stx_1-stx_{2c} carrying strains **(Lefebvre *et al.*, 2009)**. According to these studies it can be concluded that *E. coli* O157:H7 positive cattle and sheep were contaminated with highly virulent strains and this may pose potential public health risk.

In the present study, among the tested antibiotic resistance genes, only *tetB* and *strA* were detected. In a previous study, 25 (26.0 %) of the 96 *E. coli* O157:H7 cattle isolates harbored al least one antibiotic resistance gene. Twenty six out of 102 *E. coli* O157:H7$^+$/H7$^-$ (25.5 %) were carrying one or more tested tetracycline resistance genes. In the study, *tetC, tetA* and *tetB* were detected with a ratio of

14.7 %, 12.7 % and 4.9 % respectively. In addition to *tet* genes; *sulI*, *strA*, and *strB* were detected from 12.7 %, 4.9 % and 4.9 % of the isolates **(Ayaz *et al.*, 2015)**. **Srinivasan *et al.*, (2007)** reported that, 4 of 129 (3.1 %) *E. coli* O157:H7 isolates were resistance to tetracycline and all were harbored both *tetA-tetC* but other tetracycline resistance genes were not detected from the isolates. **Srinivasan *et al.*, (2007)** revealed that 8 of 9 streptomycin resistant *E. coli* O157:H7 carried *strA* and *strB* along with *aadA*. Also in a different study, it was reported that both *strA* and *strB* genes have to be present together in order to obtain functional streptomycin resistance **(Lanz *et al.*, 2003)**. Although in the present study detection of these resistance genes did not give information about the phenotypic antibiotic resistance of the isolates, it will be important to investigate the dissemination potential of the resistance genes to other pathogens and environment.

CONCLUSION

In the present study *E.coli* O157:H7 was more prevalent in sheep (6.4 %) than in cattle (3.9 %) therefore it can be concluded that, sheep pose a great risk as cattle for STEC O157:H7 contamination in tested samples. Most of the cattle and sheep feces and/or colon tissue *E. coli* O157:H7 isolates were carrying stx_{2c} gene which makes the isolates highly virulent for human. Also tetracycline and streptomycin resistance genes were detected from some of the isolates. In the study, *tetB* and *strA* genes were detected only a few of the cattle and sheep isolates. So due to the presence of antibiotic resistance genes, there was not any significant difference between cattle and sheep *E. coli* O157:H7 isolates. The presence of the same clone from different animals may represent the persistence of a certain strain in a limited geographical area. The PFGE results showed that, cattle and sheep is possible to contaminate with clonal *E. coli* O157:H7 isolates. On the other hand, different REDP of *E. coli* O157:H7 isolated from samples in different dates showed that no cross contamination and sort of contact was occurred between farms in Turkey.

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ISOLATION AND CHARACTERIZATION OF TANNASE PRODUCING BACTERIA FROM THE GUT OF *GRYLLOTALPA KRISHNANI*

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ABSTRACT

Gryllotalpa krishnani mainly eats the degradable food waste, so the insect gut which may contains an interesting diversity of microorganism not usually found elsewhere. The tannase enzymes have been reported for its wider applications in food, feed, beverage, pharmaceutical, and chemical industries. In the present study,microorganism were isolated from the gut of *Gryllotalpa krishnani* and characterized for tannase activity. A total number of 40 bacterial strains were isolated and checked for tannase activity. Among them, 4 strains produced high level of tannase activity in plate assay. Further, to confirm the identify of bacterial isolates strains were subjected biochemical and 16S rDNA sequencing analysis. The results confirmed the identity if the isolates belonging to *Enterobacter cloacae* (15), *Bacillus subtilis* (25), *Enteroinbacteriaceae* bacterium (39) and *Bacillus cereus* (40). The four bacterial strain where subjected for the substrate utilization. Interestingly, all the isoaltes showed clearing zone upto 2% tannic acid. Tannic acid degrading microbiota detected in the present study may endow the insect with some ecological advantages by enabling them to overcome the anti-nutritional effects of plant tannins.

Keywords: Tannase enzyme, FT-IR, Insect, *Gryllotalpa krishnani*

INTRODUCTION

Insects are one of the most diverse groups of living organisms on earth (**Chapman, 2006; Erwin, 1982**). Due to their diverse behaviors and feeding habits almost no terrestrial food source can escape the consumption by one or more insect species. Despite the diversity the highly interdependent and well regulated symbiotic interactions with microorganisms seem to be an important common property for different insect species (**Brauman *et al.*, 1992**). The role of microorganism is insects has been recognized by the distribution of microbial community in the insect gut has been reported by (**Campbell, 1989**). Insect gut microorganism plays an important role in the host nutrition, development, resistance to pathogens and reproduction (**Moran *et al.*, 2005**). Insect anchorage gut microbial communities range from simple to complex community (**Handelsman, 2005; Liburn *et al.*, 2001; Vasanthkumar *et al.*, 2006**). The gut community study gives the understanding about the function of microbial consortium and insect biology (**Leadbetter *et al.*, 1999**).
In some cases, loss of microorganisms often effect in unusual development and reduces the survival of the insect host (**Fukastu, 2002**). Some specific roles of microorganism in the insect guts have been disclosed, including cellulose and lignocelluloses digestion, etc., the bacteria in the insect gut of some specialized niche feeders, such as termites and aphids, have attracted wide attention because of the microbial digest enzymes achieving particular biochemical transformations (**Brauman *et al.*, 1992; Chen, 1997; Warnecke *et al.*, 2007**). Though, relatively little is known about insects feeding on foliage, where no strict symbiotic interaction has been proposed so far. In fact, most of them are herbivores (**Carter, 1984; Daly, 1998**) and their gut content (food bolus) is not sterile (**Dillon, 2004**). Indigenous insect gut bacteria of insects have been found to detoxify harmful secondary metabolites (**Morrison *et al.*, 2009**) and to protect the host against the colonization of pathogens. The mole cricket genus *Gryllotalpa* belongs to subfamily of *Gryllotalpinae*, in total, 65 species of *Gryllotalpa* has been recorded (**Ma, 2010**). In India five species of *Gryllotalpa* namely *G. africana, G. hirusta, G. minuta, G. orientatlis* and *G. ornata* are known (**Chandra, 2011**). Further studies on the Indian *Gryllotalpa* gut microbes are needed (**Chopard, 1969**). Tannase (tannin-acyl-hydrolase, E.C. 3.1.1.20) is an industrially important microbial enzyme that catalyses the hydrolysis of ester bonds (galloyl ester of an alcohol moiety) and despised bond galloyl ester of gallic acid, ester bonds involving a screen microorganism from different sources, for novel and competent tannase scattered with the tannin degrading enzymes for their benefit (**Raghuwanshi *et al.*, 2011**). Gallic acid, the major hydrolytic product of tannic acid, is used in food, cosmetics, adhesives and in the synthesis of potent antioxidant, propyl gallate (**Aithal and Belur, 2013**) such as bacteria, yeast and filamentous fungi are known as tannase producers. A foremost problem in the utilization of fungal strains for industrial applications is that degradation by fungi is relatively slow and difficult to genetic manipulation because of their genetic complexity (**Beniwal *et al.*, 2013**).
The major role of tannase producing insect gut microbes such as bacteria like *Citrobacter* sp, *Klebsiella pneumoniae* and *Enterobacter* sp. Microorganism such as fungi, yeast also efficiently producing tannase enzymes more significant due to their potential role in the tannase is also largely applied in textile and tannery industries for treatment of their effluents, to overcome serious environmental pollution (**Arijit *et al.*, 2014; Jian *et al.*, 2014**) and widely used in the fine chemical industry, pharmaceutical, leather industry and food industries. So far, the main application of the tannase is instant tea, corn liquor, as well gallic acid production from plant materials high in gallotannins. Tannin acyl hydrolase is also used as clarifying agent in juice and flavoured coffee soft drinks (**Aguilar, 2001; Banerjee, 2007; Belmares *et al.*, 2004**). The rumen microbial population presents a rich and, until recently underutilized source of novel enzymes with tremendous potential for industrial application (**Kang *et al.*, 2015**). The aim of the present study is to identify the bacteria producing tannase from the insect gut of *Gryllotalpa krishnani.*

MATERIAL AND METHODS

The insect was collected in wet soil with near to a kitchen waste, Salem district, Tamil Nadu, India. The soil was buried up to 10-15 cm depth by a digger and a colony of this species was found beneath the soil. The insect was collected dissection in our laboratory conditions (latitude: 11.6500 °N, longitude: 78.1600 °E; elevation: 154 ft (46.7 m).
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Strains and growth conditions

These strains were originally isolated from or and culture were grown on medium for **tryptone soya agar.** All bacteria were incubated at 37° C in 24hrs.

Culturing of the gut microbiota and tannase plate assay

The dissected gut was suspended in 10mM sterile phosphate-buffered saline (PBS) **Sambrook *et al*., (1989)**. The guts were sonicated (50/60 Hz,117 V, 1.0 Amps; Branson Ultrasonics, Danbury, CT) for 30 Sec, macerated with a plastic pestle, and vortexes at medium speed for 10 sec to separate bacterial cells from the gut suspension were cultured immediately on nutrient agar plates and plates were incubated for 24 hours at 37 °C. Screening of the potent tannase producing bacterial isolates were screened for tannase activity by hydrolysis tannin test. After incubation plate was flooded with thereafter, the isolation of tannase-producing bacteria was carried out on nutrient agar plates supplemented with tannic acid (2%; filter sterilized). Addition of tannic acid to nutrient agar forms tannin–protein complex; cleavage of this complex by bacteria producing tannase forms a zone around the colonies.

Genomic DNA extraction of bacteria

The genomic DNA was extracted from the isolated bacteria colonies were by using the slightly modified protocol described **Broderick *et al*., (2004)** the 12hrs cultures of bacterial isolates were taken in the micro centrifuge tube. The tube was centrifuged at 10,000rpm for 10min. the pellet was collected and 90 µl of 10% SDS was added. The tubes were incubated at 37 °C for 1 hrs. After incubation, addition 150 µl of 5M NaCl was added prior to the addition of 100µl of 10% Cetyltrimethyl ammonium bromide (CTAB). The sample was mixed thoroughly and incubated at 65 °C kept in a water bath for 30min, after incubation, add phenol, chloroform and isoamylalcohol in the ration of 25:24:1 (Vol/Vol/Vol). The tube was centrifuged at 13,000rpm for 15min and the aqueous layer was separated into a fresh tube. Then it was precipitated with 70% ethanol and centrifuged at 7000rpm for 5min. Pellets were suspended in 30µl of TE buffer. The DNA sample was separated according to their molecular weights under electrophoresis system. Finally the DNA band was visualized under gel documentation system (Lark, Germany). The DNA concentration was determined by measuring the absorbance at the ratio 260/280nm and the DNA suspension was stored -20 ºC it is used for further analysis.

PCR amplification of 16S rDNA gene

The selected bacterium was identified on the basis of its 16S rDNA sequence. DNA from the bacterial cells was isolated using QIAamp DNA Purification Kit (Qiagen, Japan) and electrophoresed in agarose gel. Fragment of 16S rDNA gene was amplified by PCR upto 30 cycles (using the following profile: initial denaturation, 95 ^{o}C for 2min; final denaturation, 94 ^{o}C for 30s; annealing, 52 ^{o}C for 30s; extension,72 ^{o}C for 90s; final extension,72 ^{o}C for 10min). Amplified PCR product was purified using Qiagen Mini elute gel extraction kit (Qiagen, Japan). Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with 27F AGAGTTTGATCCTGGCTCAG and 1492 R GGTTACCTTGTTACGACTT primers using BDTv3.1 Cycle sequencing kit on (ABI3730xl) Genetic Analyzer (**Maity *et al.*, 2011**). A single discrete PCR amplicon band of 1500bp was observed when resolved on 1.2% agarose gel.

Phylogenetic analysis

The reference sequences required for comparison were obtained from the NCBI database (http://www.ncbi.nlm.nih.gov/Genbank). The aligned sequences were then manually checked for gaps in each row and saved in molecular evolutionary genetics analysis (MEGA) format using MEGA v.2.1 software. Pairwise evolutionary distances were computed using the Kimura 2-parameter model (**Kimura, 1980**). To obtain confidence values, the original dataset was resampled 1,000 times using the bootstrap analysis method. The bootstrapped dataset was used directly for constructing the phylogenetic tree with the MEGA program or for calculating multiple distance matrixes. The multiple distance matrix obtained was then used to construct phylogenetic trees using the neighbor-joining method of **Saitou and Nei (1987)**. All of these analyses were performed using MEGA v.2.1 (**Kumar *et al.*, 2004**).

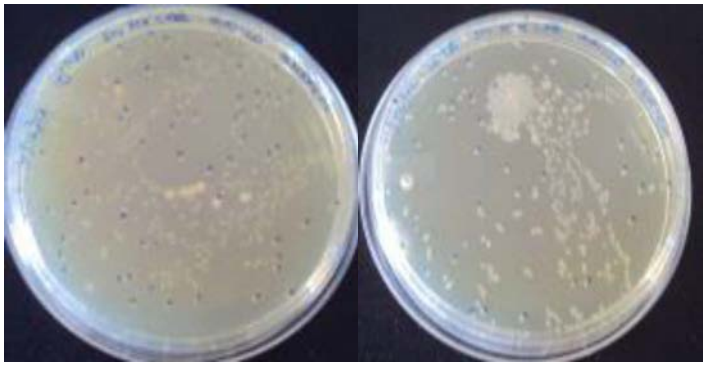
FT-IR analysis

In determining the possible functional groups FT-IR analysis was performed using Perkin Elmer's most power, which is used to detect the characteristic peaks and their functional groups. The vibration pattern that appears in the infrared spectra provides information about the chemical functional group of the sample. Tannase enzymes prepared and 500µl of 1% tannin was mixed together and incubated at 37 °C for 30min. A fraction of sample was encased directly in sample holder and spectra were scanned from 500-4000cm^{-1}.

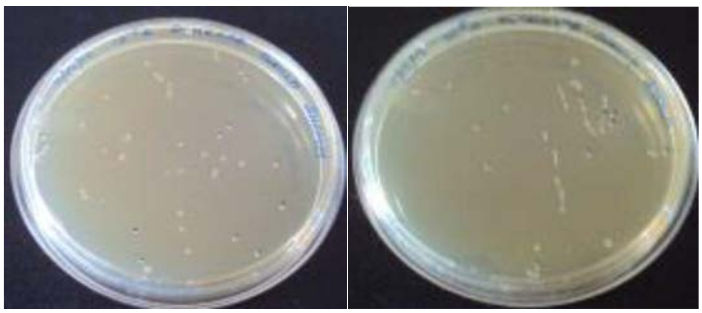
RESULTS

G. krishnani was dissected in the laboratory conditions and plated in the nutrient agar. Enumeration of microbial flora in the GI tracts of *G. krishnani* studied revealed that in general the number of cultured aerobic bacteria was higher in the gut region (Figure 1). Maximum number of microbiota 8.2×10^{6} CFU/ml was found in the gut region of *G. krishnani*. Forty bacterial strains shows tannase activity and further screening revealed 4 strains with different morphological appearances showed more tannase activity (Figure 2). The tannase secreting microbes were found growing at a fast pace despite the presence of antimicrobial tannic acid in the media. The four tannase producing bacteria were subjected to different concentration of tannic acid. The entire four strains showed clearing zone in 0.5%, 1%, 1.5% and 2% of tannic acid and the results confirmed that bacterial strains were also able to resist 2% tannic acid (Figure 3). *G. krishnani* is the mainly concerned as much to seek in its digestive systems for the search of beneficial microbes which is responsible for the digestive a large amount of tannase producing microbes.

Figure 1 Dissection of insect *Gryllotalpa krishnani* midgut



A **B**



C **D**

Figure 2 Enumeration of gut microbes of different dilution from insect *G. krishnani* maintained at 37 °C. Serial dilution of bacterial growth A-10^{4}/ ml ; B-10^{5}/ml; C-10^{6}/ml and D-10^{7}/ml .

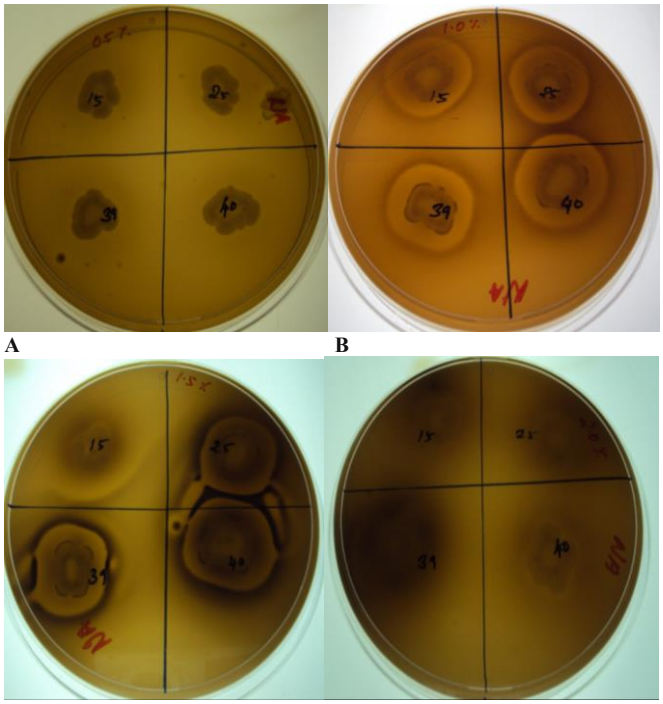


Figure 3 Efficiency of tannase-producing bacteria in different concentration tannic acid. A-0.5% Tannic acid; B-1.0% Tannic acid; C-1.5% Tannic acid; D-2% Tannic acid

A sequence analysis of ribosomal operons is a method of choice to determine the phylogenetic relationship among organisms. 16S rRNA sequence analysis has been used to distinguish the species of the organism and to delineate the lineage. The 1.5kb amplified fragment was subjected to sequence analysis (Figure 4). The results confirmed the identity of isolates belonging to *E. cloacae* (15) *B. subtilis* (25), *E. bacterium* (39) and *B. cereus* (40). A phylogram was constructed based on the UPGMA with 1,000 bootstrap samplings. The analysis was performed with the reference sequences of the representative organism belonging to *E. cloacae* (3), *B. subtilis* (2), *B. cereus* (2) and *E. bacterium* (2) (Table 1). The phylogram was generated for thirteen sequences including our isolates which resulted as in four distinct clusters (Figure 5). However, the respective isolates shared their sequence similarity with their own groups but found distinct from the others. These results clearly suggest the prevalence of genetic diversity among the bacterial strains isolated from the gut of *Gryllotalpa krishnani*. Interestingly, the present investigation revealed that all four isolates were from the same environment but showed high degree of functional and genotypic diversity among them. Finally, it concludes that the highly similar organism we can keep as a reference model and further research to our target model.It shows that formation of two major clades one is grouped with *Enterobacter* genera and another one *Bacillus* genera. The FT-IR spectrum was used to identify the functional chemical group of the active components based on the peak value in the region of infrared radiation. FT-IR spectra of tannin after treatment with tannase enzymes of strain T1 *E. cloacae* and T2 *B. cereus* for 1 hrs at 37 ºC was preformed (Figure 6).

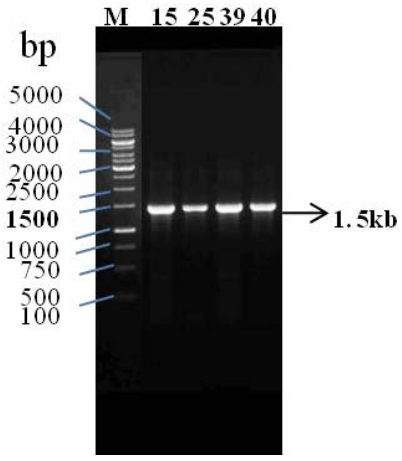


Figure 4 PCR amplification of 16S rDNA gene from insect gut microbes of *G. krishnani*.

Table 1 List of tannase producing bacteria identified by 16S rRNA sequence analysis

Strain No	Organism Name
15	*Enterobacter cloacae*
25	*Bacillus subtilis*
39	*Enterobacteriaceae bacterium*
40	*Bacillus cereus*

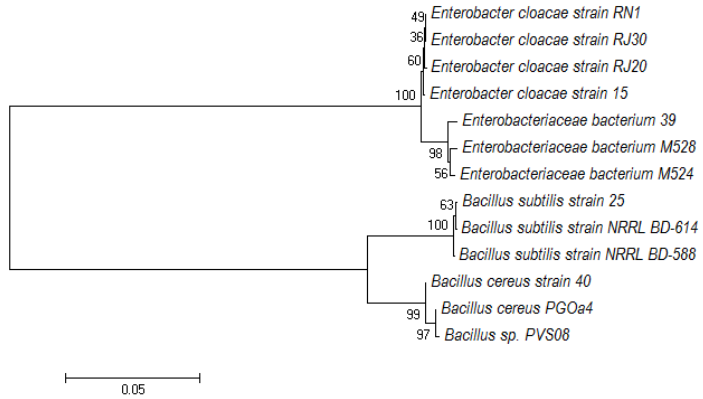


Figure 5 Phylogenetic tree based on 16S rDNA sequences for the gut microbes isolated from insect *G. krishnani* .
The tree was constructed using the neighbour joining method. To obtain confidence values, the original dataset was resampled 1,000 times using the bootstrap analysis method.

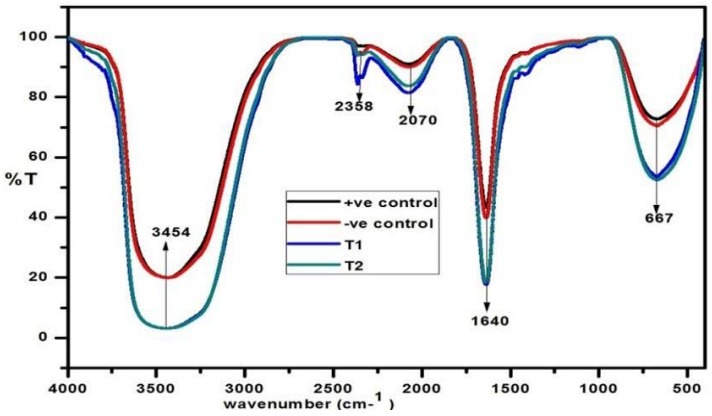


Figure 6 FT-IR analysis of tannin degradation

DISCUSSION

The insect gut of animals exhibiting a natural adaptation to dietary resources containing tannins could harbor valuable microbes and enzymes as augment livestock production (**Singh *et al.*, 2008**). Tannins have several important biological activities, such as a mechanism of defense against diseases caused by fungi, bacteria, and viruses. Bitter taste of tannin helps to protect plant tissues from the attack of insects and herbivores (**Arijit *et al.*, 2014**). Moreover, gut anaerobes have also been isolated from non-ruminants which can dissociate hydrolysable tannins HT-protein complexes, but not the complexes of proteins with condensed tannins (**McSweeney *et al.*, 2001**). *E. ludwigii* GRT-1 isolate was found to grow as scattered cells when grown on culture media containing different levels of tannic acid (**Nelson *et al.*, 1995; Goel, 2007**).Tannin-degrading bacteria such as *Streptococcus caprinus*, *Streptococcus gallolyticus, Streptococcus macedonicus, Selenomonas ruminantium* has been earlier (**Brooker *et al.*, 1994; O'Donovan, 2001; Goel, 2005; Hiura *et al.*, 2010**).Tannase activity is reported in *Enterobacter* sp isolated from soil. The fiber-degrading bacteria and fungi usually adhere to the surface of plant cell walls, and a lack of understanding of how to exploit this process may be one of the reasons for the difficulty in establishing inoculant microorganisms in the rumen (**Wang *et al.*, 2013**). The study suggests that the tannase could be useful to synthesize molecules of pharmaceutical interest, and that tannase and the *Enterobacter* sp, itself could be used to protect grazing animals against tannins (**Sharma, 2011**). This is the first report on tannase activity of *E. cloacae* (15), *B. subtilis* (25) and *E. bacterium* (39), *B. cereus* (40) isolated from gut region *G. krishnani* insect. However, the present work is a preliminary study, and relevant

to screening of tannase plate assay microbial degradation of hydrolysable tannins (HT), and the physiological role of their metabolites in these animals requires further investigation. The FT-IR spectrum of tannin samples was described by five main modes with maximum absorbance peak near 3500, 2500, 2000, 1500, 500cm-^{1}. The peaks at 3454 cm-^{1} am O-H bond stretch which the peaks at 1640 cm-^{1} recognized to be amide C=O stretch group peaks at 2070 cm-^{1}, 2358 cm-^{1}, corresponded to the overflow of aromatic and carboxyl group 667 cm-^{1} substitutions in aromatic rings. The variations in the absorbance at 3454, 2358, 2070, 1640, 667 state that the tannase enzymes degradable to tannin in the tannin compound (**Hoong *et al*., 2009**). The FT-IR result showed control tannin treated to tannin with enzymes exhibited a different pattern. This assumption is logical when one realizes that most industrial enzymes will necessarily have originated from the small percentage that has been cultured because of the poor ability to culture environmental microorganisms (**Yeung, 2012**).The result show that tannase producing bacteria have been degraded tannins substrate to simple molecules of activity was notably presented in digestive system of many insects (**Terra *et al*., 1996**). In insects the digestive of food by gut associated bacteria has been demonstrate in crickets mole, germ free crickets have much less hydrolytic enzymes activity a conventional cousins and these bacteria help in utilization a wide range of food substrate (**Santo Domingo *et al*., 1998**).

CONCLUSION

Screening of gut microbiome from the insect *G. krishnani* revealed the presence of diverse group of microbes producing tannase enzyme. Tannase are a family of esterases that catalyze the hydrolysis of the galloyl ester bond in hydrolyzable tannins to release gallic acid. The tannase enzymes have reported for its wider applications in food, feed, beverage, pharmaceutical, and chemical industries. Nevertheless, the tannase enzyme possesses several interesting phenomenon to be explored, however little is known about them at the molecular level, including the details of the catalytic and substrate binding sites in the tannase enzyme. Hence, recent biotechnological intervention, genetic manipulation through bioinformatics prediction approaches must be made to enhance the functional property of tannase enzymes to exploit them for various industrial applications. With regards to tannase much of it activity, especially in the insect world, seems get to be explored.

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IN VITRO EFFICACY OF BRACKET FUNGI FOR THEIR POTENTIAL ANTIMICROBIAL ACTIVITY

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ABSTRACT

This study was conceptualized to reveal the anti-microbial potential of bracket fungi, *viz*. - *Ganoderma lucidum* (Curtis) Karst. and *Polyporus officinalis* (syn. *Laricifomes officinalis*) (Vill.) Fr. extracts through *in vitro* approach. The aqueous and ethanolic extracts were assessed against four phytopathogenic fungi (*Alternaria solani*, *Curvularia lunata*, *Aspergillus terreus* and *Fusarium oxysporum*) along with bacteria (*Escherichia coli* and *Bacillus subtilis*). Ethanolic extract of *G. lucidum* had significant growth inhibition effect against *A. solani*, *C. lunata*, *A. terreus* and *F. oxysporum* at 500ppm and 1000ppm concentrations. On the other hand, aqueous extract showed complete inhibition at 1000ppm and 500ppm concentrations against *A. terreus* and *F. oxysporum*. Ethanolic extract of *P. officinalis*, complete fungal growth inhibition was observed against *A. solani* and *A. terreus* at 1000ppm concentration while for *C. lunata* and *F. oxysporum*, complete inhibition was observed at 1000ppm and 250ppm concentration. Similarly, the aqueous extract of same bracket fungus, showed maximum inhibition of *A. solani*, *A. terreus* and *F. oxysporum* at 1000ppm concentration but *C. lunata* had maximum inhibition at 250ppm concentration. The antibacterial action of ethanolic extract of *G. lucidum* was observed against *E. coli* having inhibitory zone (0.09 mm) at 1000 ppm concentration and *B. subtilis* had inhibitory zone (0.05mm) at 250ppm concentration. But no inhibitory zones were observed in *E. coli* and *B. subtilis* with aqueous extract of *G. lucidum*. Whereas, the ethanolic extract of *P. officinalis* showed a maximum inhibitory zone (10 mm) at 1000 ppm concentration for *E. coli* and a remarkable inhibitory zone (0.2 mm) at 250 ppm concentration for *B. subtilis*. While an inhibitory zone (0.3 mm) was observed in *E. coli* at 250 ppm concentration of aqueous extract, but no inhibitory zone was observed for *B. subtilis* at any concentration of the aqueous extract of *P. officinalis*. Based on the study, it can be concluded that *G. lucidum* and *P. officinalis* are having considerable potential as anti-fungal and anti-bacterial action, respectively.

Keywords: Antimycotic activity, Antibacterial action, Bracket macro-fungi, Minimum inhibitory concentration, Percent fungal growth inhibition

INTRODUCTION

Human use of fungi for food preparation or preservation and other purposes is extensive and has a long history. Mushroom farming and gathering are large industries in many countries. Many fungi are producers of antibiotics such as penicillin, cephalosporin. Widespread use of these antibiotics for the treatment of bacterial diseases such as tuberculosis, syphilis, leprosy, and many others began in the early 20th century and continues to play a major part in anti-bacterial chemotherapy.

Polypores and bracket fungi are members of the Aphyllophorales, a group of morphologically complex, terrestrial basidiomycetes. Many of these fungi are saprobic wood decayers and as such, these fungi are most often found on logs, stumps, or other dead wood. These fungi also exhibit medicinal properties and used in remedies of various human ailments. There are some reports that revealed their medicinal usages and biological activities. **Johnston (2005)** reported that *Ganoderma lucidum* is used in TCM (Traditional Chinese Medicine) for the treatment of cancers. **Sliva (2006)** and **Stanley *et al.* (2005)** also mentioned that *G. lucidum* is popular medicinal mushroom and used in TCM in Asian countries over the past two millennia and preserve human vitality and promote longevity. *G. lucidum* is one of the most used "*herbs*" in Asia and preclinical studies have established that the polysaccharide fractions have potent effects **(Chen *et al.* 2006)**. **Lin *et al.* (2006)** referred *G. tsugae* Murrill as the Chinese mushroom '*Songshan lingzhi'*, which is cultivated in Taiwan and used extensively to treat diseases. **Stanley *et al.* (2005)** have demonstrated that *G. lucidum* induces apoptosis, inhibits cell proliferation and suppress cell migration of highly invasive human prostrate cancer cells PC-3. **Pero *et al.* (2005)** reported that combination of extracts of *Cordyceps sinensis* (Berk.) Sacc., *Grifola blazei* Gray, *G. frondosa* (Dicks.) Gray, *Trametes versicolor* (L.) Lloyd and *G. lucidum* into a formulation designed to optimise different modes of immuno-stimulatory actions and yet that would avoid metabolic antioxidant competition. The activities of hypertension, hyperglymecia, hepatitis, chronic bronchitis, bronchical asthma, liver protection and others have been demonstrated from the fruiting bodies and cultured mycelia of *G. lucidum* **(Yuen and Gohel, 2005)**. **Shieh *et al.* (2001)** concluded that the hepatic and renal protective mechanism of *G. lucidum* might be because of its superoxide scavenging effect. Later, **Lakshmi *et al.* (2006)** studied the antimutagenic activity of the methanolic extract of the fruiting bodies of *Ganoderma lucidum* occurring in South India. The activity was assayed by Ames *Salmonella* mutageniticity test using histidine mutants of *Salmonella typhimurium* tester strains. The result revealed that *G. lucidum* extract restored antioxidant defence and prevented hepatic damage. Beside this, several compounds with Biomedicinal properties like triterpenoids **(Kim and Kim, 1999)** and polysaccharides **(Bao *et al.*, 2002)** have been isolated from *Ganoderma* species.

The extract of *Ganoderma lucidum* also showed inhibitory actions against pathogenic fungi and bacteria. **Wang and Ng (2006)** isolated 'Ganodermin', an antifungal protein from fruiting bodies of *G. lucidum* (Curtis) Karst. Ganodermin inhibited the mycelial growth of *Botrytis cinerea* Pers., *Fusarium oxysporum* Schlecht. and *Physalaspora piricola* Nose. **Wang *et al.* (2006)** isolated an antifungal polypeptide from fresh fruiting bodies of *Polyporus alveolaris* (DC.) Bond. & Sing.. The antifungal polypeptide, designated as alveolarin, had demonstrated an inhibitory action on mycelial growth in *B. cinerea* (De Bary) Whetzel, *Fusarium oxysporum* Schlecht., *Mycosphaerella arachidicola* Jenkins and *Physalospora piricola* Nose.

According to **Hleba *et al.* (2014),** the methanolic fungi extracts of both fungi *Ganoderma lucidum* and *Trametes versicolor* showed the strongest antimicrobial activity against *Saccharomyces cerevisiae*. Equally, lower antimicrobial activity of fungi extracts against Gram-positive microorganisms was detected by them. But they didn't find antimicrobial activity of fungi extracts against Gram-negative bacteria and *Candida albicans*. On the other hand, antibacterial activity has been observed against Gram positive bacteria from the basidocarp extract of *G. lucidum* **(Kim *et al*., 1993)** and *G. orogonense* Murr. **(Brian, 1951)**. **Sudirman and Muziyati (1997)** observed that seven Indonesian *Ganoderma* species inhibit the growth of *Bacillus subtilis*. **Coletto and Mondino (1991)** noted that methanolic extract of the mycelial and culture extract of *G. recinaceum* Boud and *G. lucidum* inhibited *Bacillus subtilis* Cohn. *G. recinaceum* also inhibited *Staphylococcus aureus* Rosen. Ethanolic extract from *G. lucidum* mycelium demonstrated significant anti-inflammatory effects **(Kendrick, 1985)**.
There are also some studies on inhibitory and metabolic activities of polypores and bracket fungi in India. **Quereshi *et al.* (2010)** studied the antimicrobial activity of various solvent (aqueous, ethanol, methanol and acetone) extracts (40µg/ml) of *Ganoderma lucidum* against six species of bacteria, *viz*. *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Bacillus subtilis Salmonella typhi* and *Pseudomonas aeruginosa*. **Sheikh *et al*. (2015)** have reported the potential antioxidative role of two mushrooms *G. lucidum* and *Trametes hirsuta* in free radical systems. **Bains *et al.* (2015)** evaluated the potential of methanolic and ethyl acetate extracts of *Agaricus* sp. *Morchella* sp. and *Canthrellus* sp. against four bacterial strains *K. pneumonia*, *P. aeruginosa*, *S. aureus* and *E. coli*. Similarly, **Sivaprakasam *et al.* (2011)** tested aqueous and methanolic extracts of *T. hirsuta* fruit body against five pathogenic fungi (*Penicillium* sp., *Aspergillus fumigatus*, *Aspergillus niger*, *Aspergillus flavus* and *Mucor indicus* and five bacterial stains (*E. coli*, *P. aeruginosa*, *Salmonella typhi*, *S. aureus* and *Streptococcus mutans*).
Polypores and bracket fungi are the major source of biologically active natural products extracted from the species of the diverse fungal phylum Basidomycota, furnishing a rich variety of active secondary metabolites and polysaccharides **(Zjawiony, 2004)**. In the search for active compounds from *Ganoderma* species, the majority of research has been performed on extracts from the fruiting body and there have been fewer studies on extracts from the liquid cultivated mycelium **(Russell and Paterson, 2006)**. It appears that there are a number of biologically active compounds to be found in the mycelium and the benefits of liquid cultivation over solid cultivation include: the ability to manipulate the cultivation medium to optimise mycelia growth; a shorter cultivation time; and less contamination. In fact, the reason that some of the Ganoderma preparations are not yet available as medicines may be from difficulties relating to mass production **(Smith *et al.*, 2002)**.

Considering the previous research reports on this antimicrobial aspects of *Ganoderma lucidum* and other species of bracket fungi and the fact that there is little or very scarce work on the antimycotic and antibacterial activities of extracts of *G. lucidum* and *Polyporous officinalis*, the present work was undertaken with an aim to find out the antimicrobial potential of these two bracket fungi against phytopathogenic fungi and bacteria through *in vitro* approach.

MATERIALS AND METHODS

Material collection

The fresh fruiting bodies of bracket fungi were collected from forest area near Solan district (situated between 76.42 and 77.20 degree East longitude and 30.05 and 31.15 degree north latitude) of Himachal Pradesh, India and sample was identified and confirmed with the help of Fungal Herbarium/Museum at National Centre for Mushroom Research and Training at Solan, Himachal Pradesh and Department of Plant Pathology, University of Horticulture and Forestry, Nauni, Solan, Himachal Pradesh, India.

Preparation of aqueous extracts

Aqueous extracts were prepared by adding 0.01g of dried powdered fruit bodies of *Ganoderma lucidum* and *Polyporus officinalis* in 10 ml of distilled water. The aqueous extract containing cellular debris was filtered through a fine muslin cloth to get a fine aqueous extract and then centrifuged at 1000 rpm and the supernatant was filtered through the vacuum filter unit to remove any pathogenic fungi or bacteria and then transferred to a sterilized glass container. It was considered as pure 1000 ppm solution and leveled as stock solution and from which respective concentrations (500 ppm and 250 ppm solutions) prepared by adding required amount of distilled water. These extracts were used for further investigation.

Preparations of ethanolic extracts

These were also prepared in the similar way as mentioned above but ethanol was used instead of distilled water.

Test pathogenic fungi used

Efficacy of fruiting bodies of two bract fungi as mentioned above on the four fungi *i.e. Aspergillus terreus, Alternaria solani, Fusarium oxysporum* and *Cavularia lunata* were investigated by using standard method **(Nene and Thapliyal, 1993)**.

Determination of antimycobiotic activity

For each fungi *i.e. Alternaria solani, Aspergillus terreus, Curvularia lunata, Fusarium oxysporum* three Petri plates were used. The technique used was poisoned food technique **(Parkash *et al.*, 2005)**. All the glassware in use, are sterilized properly by autoclaving for 15 min at 121 °C. Medium was poured in the Petri plates mixed with different concentrations (*i.e.* 1000ppm, 500ppm, 250ppm and control) of sample extracts under sterile conditions. Mycelia disc were taken from pure cultures of test fungi previously grown on PDA and were placed in the centre of the plates aseptically. Suitable negative control was also kept where the mycelia disc were grown under same conditions on PDA medium without supplementation of any sample compound. For positive control, Actidion (Cycloheximide) at 1000 ppm (0.01%) was used in the medium as standard antifungal drug for comparison of antifungal action. The plates were grown at 27 °C. The efficacy in each case was determined by measuring the additional mycelial growth each time after a proximal three days. The radial growth of the colony was measured in four directions at right angle to each other and average was taken. The percentage inhibition of the fungal extract was calculated by using formula:

$$Percentage\ inhibition\ (\%) = \frac{Control - Treated}{Control} \times 100$$

where C is control and T is treatment. Data was also analyzed statistically for SEm and CV. The experiment was carried out in triplicate replications

Test bacterial strains used

The bacterial strains used were *Bacillus subtilis* (APR-4) and *Escherichia coli* (EC-1). These bacterial strains were procured from Kurukshetra University, Kurukshetra and Himachal Pradesh University, Shimla and were maintained on nutrient agar for further experiment.

Determination of antibacterial activity

The antibacterial susceptibility test was carried out using agar well diffusion test **(Perez *et al*., 1990)**. The bacteria were cultured overnight at 35 °C in nutrient agar **(Zhang *et al*., 2004)**. The final cell concentrations of bacterial rods were in the range of 10^6-10^7 CFU ml^{-1}.
The activity was checked against two pathogens *i.e. Escherchia coli* (EC-1) and *Bacillus subtilis* (APR- 4) and quantified using MIC (Minimum Inhibitory Concentration). Four nutrient agar plates (two for ethanolic and aqueous culture of each bacterium) were prepared and the media was made to settle down for 10 minutes. Wells were punched in the plates with the help of borer of size 8mm to have uniform wells but the medium was not removed. The bacterial culture of respective strains were spread on the nutrient agar medium with the help of cotton swab and left for 5 minutes. Later, the medium from the wells was removed and the aqueous and ethanolic extracts of different concentrations (*i.e.*1000ppm, 500ppm, 250ppm) were poured into the different wells in the respective Petri plates with the help of micropipette. No extract was added in to the negative control well. For positive control, Ciprofloxacin (0.05% = 500ppm) was used in a single well as standard antibacterial drug for comparison of antibacterial action in a separate Petri plate. The plates were incubated at 37^0C for overnight and inhibition zone were recorded. The effect of fungal extract was expressed in terms of average diameter of the zone of inhibition measured in millimeter. Each test was carried out in triplicate replications.

RESULTS AND DISCUSSION

Effect of *Ganoderma lucidum* (ethanolic extract) on test fungi

There was complete inhibition of mycelia growth at 250ppm, 500ppm, 1000ppm for *Alternaria solani. Aspergillus terreus*, *Fusarium oxysporum* were also completely inhibited at 1000ppm and 500ppm but minimum inhibition observed at 250ppm; whereas *Curvularia lunata* was also completely inhibited at 1000ppm and 500ppm and 250ppm concentration. In control sets, however no fungal or mycelia inhibition was observed.

Table 1 Effect of *Ganoderma lucidum* (ethanolic extract) on mycelial growth of different fungi

S. no.	Concentration (ppm)	*Alternaria solani* (% Inhibition)	*Aspergillus terreus* (% Inhibition)	*Curvularia lunata* (% Inhibition)	*Fusarium oxysporum* (% Inhibition)
1.	Actidion (Cy) 1000	99.8 ± 0.10	100 ± 0.0	100 ± 0.0	100 ± 0.0
2.	Control (without extract)	0 ± 0	0 ± 0	0 ±0	0±0
3.	250	100 ±0	82±0.626	100±0	93.6±0.379
4.	500	100±0	100±0	100±0	100±0
5.	1000	100±0	100±0	100±0	100±0

Effect of *Ganoderma lucidum* (aqueous extract) on test fungi

In case of *Alternaria solani*, minimum mycelial inhibition was observed at 250ppm, 500ppm and maximum at 1000ppm whereas *Aspergillus terreus* was not completely inhibited at any concentrations. A minor mycelial inhibition percent was observed in case of *A. terreus* but *Curvularia lunata* and *Fusarium oxysporum* were completely inhibited at 1000ppm, 500ppm and 250ppm.

Table 2 Effect of *Ganoderma lucidum* (aqueous extract) on mycelial growth of different fungi

S. no.	Concentration (ppm)	*Alternaria solani* (% Inhibition)	*Aspergillus terreus* (% Inhibition)	*Curvularia lunata* (% Inhibition)	*Fusarium oxysporum* (% Inhibition)
1.	Actidion (Cy) 1000	50.5 ± 0.10	70.6 ± 0.05	70.0 ± 0.06	60.5 ± 0.06
2.	Control (without extract)	0 ± 0	0 ± 0	0 ±0	0±0
3.	250	82±0.622	05±1.030	100±0	100±0
4.	500	82±0.622	04±044	100±0	86±0.533
5.	1000	87±0.536	05±1.030	100±0	100±0

Effect of *Polyporus officinalis* (ethanolic extract) on test fungi

In *Alternaria solani* and *Aspergillus terreus* there was a complete mycelia inhibition observed at 1000ppm and minimum inhibition was observed at 500ppm, 250ppm concentrations respectively. Whereas *Fusarium oxysporum* had maximum mycelia inhibition at 1000ppm and 500ppm followed by 250ppm concentration but *Curvularia lunata* was completely inhibited at 1000ppm, 500ppm and 250ppm concentrations.

Table 3 Effect of *Polyporus officinalis* (ethanolic extract) on mycelial growth of different fungi

S. no.	Concentration (ppm)	*Alternaria solani* (% Inhibition)	*Aspergillus terreus* (% Inhibition)	*Curvularia lunata* (% Inhibition)	*Fusarium oxysporum* (% Inhibition)
1.	Actidion (Cy) 1000	99.8 ± 0.10	100 ± 0.0	100 ± 0.0	100 ± 0.0
2.	Control(without extract)	0 ± 0	0 ± 0	0 ±0	0±0
3.	250	75±0.737	95±0.48	100±0	90±0.32
4.	500	89±1.025	92±0.418	100±0	94±0.362
5.	1000	100±0	100±0	100±0	94±0.36

Effect of *Polyporus officinalis* (aqueous extract) on test fungi

In case of *Fusarium oxysporum*, *Curvularia lunata* and *Alternaria solani*, there were maximum mycelia inhibition observed at 1000ppm conc. while minimum mycelia inhibition was observed at 500ppm, 250ppm concentrations respectively. Whereas a little mycelia inhibition was observed at 1000ppm conc. but there was very little mycelial inhibition observed at 250ppm, 500ppm concentrations respectively.

Table 4 Effect of *Polyporus officinalis* (aqueous extract) on mycelial growth of different fungi

S. no.	Concentration (ppm)	*Alternaria solani* (% Inhibition)	*Aspergillus terreus* (% Inhibition)	*Curvularia lunata* (% Inhibition)	*Fusarium oxysporum* (% Inhibition)
1.	Actidion (Cy) 1000	50.5 ± 0.10	70.6 ± 0.05	70.0 ± 0.06	60.5 ± 0.06
2.	Control (without extract)	0 ± 0	0 ± 0	0 ±0	0±0
3.	250 ppm	81±0.642	1.0±0.0	74±0.766	84.7±0.47
4.	500 ppm	82±0.622	1.5±1.0	78±0.702	88±0.518
5.	1000 ppm	87±0.536	5.7±1.030	82±0.622	89.7±0.58

Effect of *Ganoderma lucidum* (ethanolic and aqueous extracts) on test bacteria

The minimum inhibitory concentration (MIC) was analyzed for aqueous and ethanolic extracts against two bacterial strains *Bacillus subtilis* (strain APR-4, Gram positive) and *Escherichia coli* (EC-1, Gram negative). The antibacterial action of ethanolic extract of *G. lucidum* was observed against *E. coli* having inhibitory zone (0.09 mm) at 1000 ppm concentration and *B. subtilis* had inhibitory zone (0.05mm) at 250 ppm concentration respectively. The aqueous extract of *G. lucidum* had shown zero antibacterial activity/potential against *E. coli* and *B. subtilis* at any test concentrations.

Table 5 Activity of aqueous and ethanolic extracts of *Ganoderma lucidum* against different bacteria

S.no.	Bacterial Type	Bacterial strain	Type of extract	Minimum Inhibitory conc. (MIC)	Inhibition zone (mm)
1.	Gram negative	*Escherichia coli*	Ethanol Extract	1000	0.09± 0.06
2.	Gram positive	*Bacillus subtilis*	Ethanol Extract	250	0.05± 0.06
3.	Gram positive	*E. coli*	Aqueous Extract	-	No inhibitory zone
4.	Gram positive	*B. subtilis*	Aqueous Extract	-	No inhibitory zone

Effect of *Polyporus officinalis* (ethanolic and aqueous extracts) on test bacteria

The ethanolic extract of *P. officinalis* showed a maximum inhibitory zone (10mm) at 1000 ppm concentration for *E. coli* and a remarkable inhibitory zone (0.2 mm) at 250 ppm concentration for *B. subtilis*. While an inhibitory zone (0.3 mm) was observed in *E. coli* at 250 ppm concentration of aqueous extract; no inhibitory zone was observed for *B. subtilis* at any concentration of the aqueous extract of *P. officinalis*.

Table 6 Activity of aqueous and ethanolic extracts of *Polyporus officinalis* against different bacteria

S.no.	Bacterial strain	Type of extract	Minimum Inhibitory conc. (MIC%)	Inhibition zone (mm)
1.	*Escherichia coli*	Aqueous Extract	250	.03 ± 0.006
2.	*Bacillus subtilis*	Aqueous Extract	-	No zone of inhibition
3.	*E. coli*	Ethanol Extract	1000	10± 0.0
4.	*B. subtilis*	Ethanol Extract	250	.02± 0.006

Effect of Ciprofloxacin as a positive control on test bacteria

Ciprofloxacin (0.05%= 500ppm) in ethanol and distilled sterilized water were taken as positive controls and it showed maximum zone of inhibitions (18.4mm) against *E. coli* and (20.2 mm) against *B. subtilis* in ethanolic extract while maximum zone of inhibitions (13.0 mm) against *E. coli* and (12.0 mm) against *B. subtilis* in aqueous extract (see Table -7). But relatively antibacterial activity was low in sample extracts in comparison to standard positive control drug.

Table 7 Activity of aqueous and ethanolic extracts of Ciprofloxacin as a positive control against different bacteria

S.no.	Bacterial strain	Type of extract	Minimum Inhibitory conc. (MIC in ppm)	Inhibition zone (mm)
1.	*E. coli*	Ciprofloxacin (50µg/100µl) (in Ethanol)	500	18.4 ± 0.041
2.	*B. subtilis*	Ciprofloxacin (50µg/100µl) (in Ethanol)	500	20.2 ± 0.06
3.	*E. coli*	Ciprofloxacin (50µg/100µl) (aqueous)	500	13.0 ± 0.04
4.	*B. subtilis*	Ciprofloxacin (50µg/100µl) (aqueous)	500	12.0 ± 0.02

Several previous researches have proved the antimicrobial activity of *Ganoderma lucidum* (basidiocarp and methanolic/ ethanolic extracts) against a wide range of zoo- and phyto-pathogenic fungi (*Botrytis cinerea, Fusarium oxysporum*, *Physalaspora piricola*, etc.) and a few bacteria (*Bacillus subtilis*, etc.) **(Sudhirman and Mujiyati, 1997; Coletto and Mondino, 1991; Kim and Kim, 1999)**. On the other hand, inhibitory action on mycelial growth in *Botrytis cinerea*, *Fusarium oxysporum*, *Mycosphaerella arachidicola* and *Physalospora piricola* was demonstrated by an antifungal polypeptide from fresh fruiting bodies of *Polyporus alveolaris* designated as '*alveolarin'*. Similarly the positive antimicrobial effect was seen in case of *G. lucidum* and *P. officinalis* against the test microbes. So, the present study is exceptional on the aspect that it deals with the antimycobiotic potential of *G. lucidum* against some different phyto-pathogenic fungi as stated above; along with prospecting the potential of an unreported bracket fungus *i.e. Polyporus officinalis* (as a source of anti-fungal and anti-bacterial compounds.

To conclude this, the first report on the screening of the antimicrobial activity against the four fungal pathogens, *viz.* - *Aspergillus terreus, Curvularia lunata, Alternaria solani* and *Fusarium oxysporum* and antibacterial activity against the two bacteria, *viz.* - *Bacillus subtilis* and *Escherichia coli* can serve as prospective potential aspect for much needed novel antibiotics. Further work is needed toward the evaluation of their antimicrobial potential against a wider range of microorganisms, identification of the bioactive principles and elucidation of their mechanism of action.

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STUDY OF BIOSURFACTANT PRODUCING BACTERIA AND PRELIMINARY CHARACTERIZATION OF BIOSURFACTNAT PRODUCED BY *BACILLUS* species ISOLATED FROM PETROLEUM CONTAMINATED SOIL

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ABSTRACT

Chemically synthesized amphiphilic molecules like surfactants are used for variety of purposes, but they are costly and less or non-degradable and may be toxic once released in the environment. Microbially produced biosurfactants are better alternatives of chemical surfactants as they are stable as well as easily biodegradable and also due to structural and functional similarities with chemical surfactants. The aim of this study was to isolate and characterize biosurfactant producing bacteria from oil contaminated sample (garage soil, Anand, Gujarat). Thirty bacterial cultures were isolated on the basis of differences in their colony characters. To confirm and compare the efficiency of biosurfactant production, hemolytic activity and reduction in surface tension was analyzed. Biochemical and morphological tests identified bacterial isolate C5C (Gram positive rods arranged in short chains) as *Bacillus* species. Optimum pH and temperature for biosurfactant production were 5 and 30ºC, respectively. It produced 3.12 g/l of biosurfactant in MSM medium with 1% diesel as carbon source. Biosurfactant produced by C5C contains lipid. No carbohydrates or amino acid component was detected on TLC plate.

Keywords: *Bacillus* species, Biosurfactant, Biochemical characterization, TLC, Mass of Drop

INTRODUCTION

Reduction in surface tension is aided by variety of surfactants. Commercially used surfactants are produced chemically as derivatives from petroleum products **(Ali *et al.*, 2013)**. Such surfactants are used in variety of applications like foaming agent, detergents, emulsifiers, solubilizer, in wetting and spreading etc. because of their amphiphilc nature **(Ali *et al.*, 2013)**. They lower interfacial tension by accumulating at interfaces and micelles formation **(Rufino *et al.*, 2014)**. Major drawback of using the chemical surfactants is their cost and undesirable residues which are difficult to dispose off and degrade leading to environmental pollution **(Batista *et al.*, 2006; Aparna *et al.*, 2012; Marchant and Banath, 2012; Ali *et al.*, 2013)**. Therefore there is continuous search for eco-friendly biosurfactants to replace chemical surfactants.
Biosurfactants are better alternatives because they are easily biodegradable **(Rufino *et al.*, 2014)**. They are structurally and functionally similar to chemical surfactants and synthesized both by bacteria and fungi including yeast **(Satpute *et al.*, 2010; Marchant and Banath, 2012; Ali *et al.*, 2013)**. They are having hydrophobic and hydrophilic parts in their structure. They are produced extra-cellularly or as part of cell structure **(Sneha *et al.*, 2012)**. In recent years, development of biosurfactant technology has taken paramount importance due to their biocompatibility, low toxicity, higher biodegradability and higher stability under extreme conditions like pH and temperature **(Sadat *et al.*, 2013; Kim *et al.*, 2004; Rufino *et al.*, 2014)**. Biosurfactants are so diverse chemically that they can be used in medical field, cosmetic industries, for environmental applications as well as other processes like enhanced oil recovery process. Despite such advantages and applications, biosurfactants are not exploited much because of limited knowledge regarding production condition and extent to which organism produces this substances. Present study was undertaken to screen bacteria isolated from oil spilled region (garage soil) for biosurfactant production and their characterization.

MATERIAL AND METHODS

Sampling area

For isolation of biosurfactant producing bacteria soil samples were collected from different garages around Anand (Gujarat, India). The soil samples from contaminated sites were collected in sterile containers and brought to the laboratory for further studies.

Enrichment and isolation of bacterial cultures

5.0 gm of soil samples were suspended by thorough mixing in 100 ml phosphate buffer saline (PBS). After settling of soil debris 5 ml supernatant was transferred to 50 ml of Bushnell-Haas (BH) Medium [$MgSO_4$ 0.2 g/L, $CaCl_2$ 0.02 g/L, KH_2PO_4 1.0 g/L, K_2HPO_4 1.0 g/L, NH_4NO_3 1.0 g/L, $FeCl_3$ 0.05 g/L] with 1% diesel as sole source of carbon and incubated at 25ºC, 200 rpm for 4 days **(Batista *et al.*, 2006)**. After incubation 1ml of sample was transferred to fresh BH medium (with 1% diesel) and incubated again as stated above. After three cycle of enrichment, loop full of culture was streaked on BH agar medium with 1% diesel and incubated at 37ºC for 1week. Colonies with different morphology were selected and transferred on nutrient agar slant (pH 7.0 ±0.2) and stored at 4ºC.

Hemolytic activity of isolates

Hemolytic activity is considered to be good and easy criteria to screen out biosurfactant producers. For that fresh single colonies of isolated cultures were taken and streaked on blood agar plates. Plates were incubated for 24 to 48 hrs at 37ºC. The plates were visually inspected for zone of clearance (hemolysis) around colonies. Hemolysis was designated as alpha (α), beta (β) and gamma (γ) hemolytic activity and was used as qualitative method for selection of better producer of biosurfactant **(Satpute *et al.*, 2010; Ali *et al.*, 2013; Varjani *et al.*, 2014)**. Five cultures were selected on the basis of the hemolytic activity for further study.

Identification of bacterial culture

The selected bacterial isolates were characterized by morphological/microscopic and biochemical tests such as Gram staining, Methyl Red (MR test), Voges Proskauer (VP test), Citrate utilization test, Nitrate reduction test, H_2S production test, Gelatine hydrolysis test, Glucose and Sucrose fermentation test and catalase test. Cultures were also subjected to Triple Sugar Ion (TSI) test. Results from biochemical analysis were used to find the closest match with known bacterial genus and to assign the bacterial signature according to Bergey's manual **(Nasr *et al.,* 2009; Anandraj and Thivkaran, 2010; Ali *et al.,* 2013)**.

Quantitative measurement of reduction in surface tension

Reduction in surface tension was analyzed from broth medium. For that, bacterial cultures were activated in LB broth for 24 hrs at 37°C. After activation1 ml inoculums with 1.00 $O.D._{600nm}$ cells were transferred to the 100 ml BH medium with 2% diesel as carbon source. Broth was agitated at 200 rpm for one week for growth and biosurfactant production. Surface tension was measured by drop weight method described by **William Burns Tucker (1938)**. It is second most precise and easy method for determination of surface tension. Cells were removed by centrifugation at 5000 rpm for 15 min and supernatant was collected. Cell free supernatant was poured in a burette which was attached to a rubber tube on one side and glass tube of 3 mm diameter at another side. An empty pre-weighed beaker was placed under burette and supernatant was released slowly drop by drop. 50 drops were poured in to the beaker and it was weighed to determine the weight of 50 drops. Mass of one drop was calculated by using following formula **(Ramesh *et al.,* 2011).**

$$Mass\ of\ Drop\ (M) = \frac{(Sample + Beaker\ weight) - (Weight\ of\ empty\ Beaker)}{Number\ of\ drops}$$

Surface tension and surface activity was measured by following formula.

$$Surface\ tension = \frac{Mg \times 10^{-3} \times nM^{-1}}{\pi r}$$

Where, "M" is mass of one drop, "g" is gravity and "r" is radius of glass tube. Value of Surface tension was used to calculate surface activity.

$$\text{Surface activity} = Surface\ tension\ of\ Uninoculated\ medium - Surface\ tension\ of\ Supernatant$$

Optimization of growth medium

pH optimization

The initial pH value of them MSM medium with 1% diesel was adjusted over a range of 2 to 9 and media were sterilized in triplicates. Media were inoculated with 1% v/v inoculum and incubated for seven days at 120 rpm. After incubation period, cell free supernatants (crude biosurfactant) were obtained by centrifugation (8000 rpm for 15 min) and E24 % was determined for samples of each pH according to method suggested by **Chandran and Das (2011)**. For E24% 1ml of cell free supernatant was added in test tube containing 1ml diesel followed by through mixing for 1min by vortex. After 24 hrs emulsification index was calculated as

$$E24\% = \frac{Hight\ of\ Emulsuion\ Formed}{Total\ Hight\ of\ Solution} \times 100$$

Temperature optimization

100 ml of MSM medium with 1% Diesel (pH 7) sterilized at 121 psi for 15 min. After sterilization, 1ml of activated cells with 1.00 $O.D._{600nm}$ were transferred to medium under aseptic condition and incubated at 120 rpm for seven days at different temperatures; 30, 40, 50, 60, 70, 80, 90 and 100°C. Experiment was carried out in triplicates. After incubation E24% was calculated from cell free broth for each flask.

Inoculum development:

For inoculum preparation, single colony of C5C isolate was aseptically transferred to fresh sterile Luria-Bretani broth and incubated at 37°C until its $O.D._{600}$ reaches to 1.00.

Production and extraction of biosurfactant:

The production of biosurfactant was carried out in MSM medium with 1% diesel. 1.00 $O.D._{600nm}$ cells were aseptically transferred to production medium with optimized pH. After inoculation medium was incubated for seven days at optimized temperature. Biosurfactant was extracted by solvent extraction method **(Chander *et al.,* 2012)**. In order to precipitate lipids and proteins, 6N HCl was added in supernatant to bring pH 2 and kept overnight at 4°C. White colour precipitates were collected by centrifugation at 10,000 rpm for 20 min. Further extraction was carried out by adding 10 ml chloroform: methanol (2:1 v/v) to the precipitated pellet which was dissolve in 10 ml of distilled water. Phases were allowed to separate. Organic phase was collected in pre-weighted petriplate and kept at room temperature for evaporation. After complete evaporation, petriplate was weighted and produced biosurfactant was calculated as

$$Biosurfactant\ Production = Weight\ of\ petriplate\ after\ drying - Weight\ of\ empty\ petriplate$$

Characterization of biosurfactant by Thin Layer Chromatography [TLC]

To identify the component parts of biosurfactant silica gel plates were used. A spot of crude biosurfactant was placed on silica gel plate. The biosurfactant was separated on plate using following four systems. System 1 Petroleum ether-diethyl ether-acetic acid (80:20:1) for neutral lipid. System 2 Chloroform-methanol-water (65:52:4) for polar lipid. System 3 *n* butanol-acetic acid-water (4:1:1) for amino acid. System 4 ethyl acetate-acetic acid-methanol-water (12:3:3:2) for carbohydrates. After development visualization was carried out for separated components. Lipid was visualized by placing the plates in closed jar saturated with iodine vapour which gave yellow colour spots. Amino acids were visualized by using ninhydrin reagent followed by heating at 90°C for 15 min which gives red or purple colour. Carbohydrate was visualized by detecting red colour spot on spraying with alpha-naphthol-sulphuric acid solution **(Yin *et al.,* 2009)**.

RESULTS AND DISCUSSION

Isolation and screening of bacteria

Thirty bacteria with different colony characteristics were isolated from three soil sample each from various location of a Garage by following three successful cycles of enrichment. Twenty one isolates were found to be Gram positive and rest of nine were Gram negative in gram reaction.

Hemolytic activity and biochemical characterization of bacteria

All isolates were streaked on the blood agar plates. The hemolytic activity was observed in all thirty isolated strain, results showed alpha hemolytic activity by strain SD2-B, SO2-A ,C1C, C6C, C3N, C6N and M-B, beta hemolytic activity by SD1-A, SD1-B, SD1-C, SD1-D, SO1-A, SO1-B, SO2-B, M-A, H-A, H-B, H-C, C2C, C3C, C4C, C5C, C7C, C2N and C4N and hemolytic activity gamma was indicated by SO2-A, H-D, C1N, C5N, C6N and C7N (Table-1). Hemolytic activity is qualitative measurement of biosurfactant ability of cultures **(Satpute *et al.,* 2010)**. Hemolytic zone on blood agar plate have linear relation with the concentration of biosurfactant produced by bacteria **(Youssef *et al.,* 2014)**. Therefore out of thirty bacterial cultures five isolates SD1, SO1-B, SD2-A, SO2-B and C5C were selected for further study on the basis of hemolytic zone on blood agar plates and characterized biochemically (Table-1).

Table 1 Microscopic and biochemical characterization of selected cultures

Test	Cultures				
	C5C	**SD1-A**	**SO1-B**	**SD2-A**	**SO2-B**
Gram Reaction	Positive	**Positive**	Negative	**Positive**	**Negative**
Blood Hemolysis	B	β	β	γ	β
Zone of hemolysis*	++++	+++	+++	+++	+++
Methyl Red test	Negative	**Positive**	Negative	**Positive**	**Positive**
Voges Proskauer Test	**Positive**	**Positive**	**Positive**	Negative	Negative
Citrate Utilization	**Positive**	Negative	Negative	Negative	**Positive**
Catalase test	Negative	**Positive**	**Positive**	**Positive**	**Positive**
Gelatine liquefaction	Negative	Negative	Negative	**Positive**	**Positive**
Nitrate reduction	**Positive**	**Positive**	**Positive**	**Positive**	**Positive**
H_2S Production	Negative	Negative	**Positive**	Negative	**Positive**
Starch Utilization	**Positive**	Negative	Negative	**Positive**	**Positive**
Sucrose fermentation	Negative	**Positive**	**Positive**	**Positive**	**Positive**
Glucose fermentation	**Positive**	**Positive**	**Positive**	**Positive**	**Positive**
TSI**	**A/Ak/-/+**	**A/Ak/-/-**	**Ak/A/+/+**	**Ak/Ak/-/-**	**Ak/A/+/+**

Legends: * (-) no zone ;(+) poor activity; (++) Moderate activity; (+++) Good activity; (++++) Excellent activity, ** - Reaction in slant/Reaction in butt/

H_2Sproduction/Gas production

Results showed that C5C was positive for Voges Proskauer test (VP test), citrate utilization test, nitrate reduction test, starch hydrolysis test and glucose fermentation test (Table-1). These data are quit comparable with results of *Bacillus* **(Aparna *et al.,* 2012)**. Figure 1 showed the morphology of C5C by Gram's staining. SD2-A was able to ferment glucose and sucrose and showed starch hydrolysis. It gave positive result for methyl red test (MR test), but negative for VP test and citrate utilization test. All other positive tests included catalase test, gelatine liquification, nitrate reduction and H_2S production. Triple Sugar Ion slant (TSI) gave alkaline reaction at both slant and butt region with neither H_2S nor gas production (Table-1). Result shows similarities with the results reported for *Pseudomonas* **(Sneha *et al.,* 2012; Sadat *et al.,* 2013)**. SO2-B culture showed similar results as SD2-A except citrate utilization test and TSI reactions. Cultures SD1-A and SO1-B were able to reduce nitrate, ferment glucose and sucrose. They showed positive results for catalase test and VP test. More confirmatory tests are needed to identify the isolate.

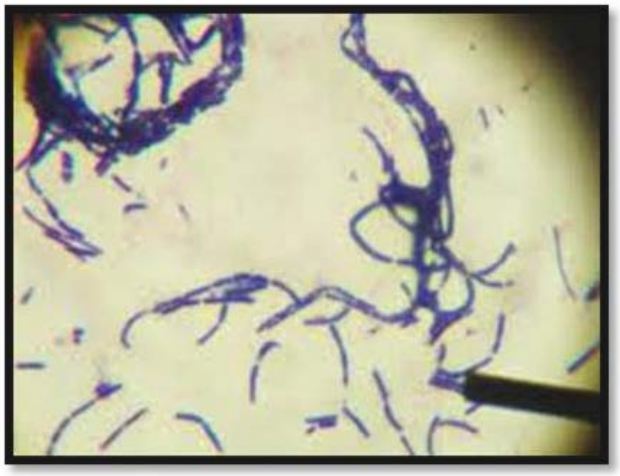

Figure 2 Gram staining of C5C

Quantitative measurement of surface tension of fermented broth

On the basis of hemolytic zone obtained on blood agar plate, cultures C5C, SD1-A, SO1-B, SO2-A and SO2-B which gave excellent or good hemolytic activity were selected for further studies. All five selected cultures were studied further for the reduction of surface tension. Results are shown in Table 2. Culture C5C reduced surface tension of broth by 40.56 mN/m which was lowest compare to other four cultures. **Mathiyzhagan *et al.,* (2011)** reported the surface tension reduction of cell free cultures broth ranging from 64.0 mN/m to 28 mN/m using organisms isolated from oil contaminated soil. *Bacillus subtilis* isolated from Kanchipuram reported to have surface activity 51.38 nm^{-1} **(Ramesh *et al.,* 2011).** Similar results were also observed in the present study.

Table 2 Quantitative measurement of surface tension and surface activity of selected culture broth

Cultures	Surface tension [nm^{-1}]	Surface activity[nm^{-1}]
Control	92.8 x $10^{(-3)}$	00
SD1-A	51.11 x $10^{(-3)}$	41.69
SO1-B	91.24 x $10^{(-3)}$	1.56
SO2-A	78.49 x $10^{(-3)}$	14.31
SO2-B	56.72 x $10^{(-3)}$	36.08
C5C	40.56 x $10^{(-3)}$	51.78

Cultures C5C, SO2-A and SD1-A were found to be good biosurfactant producers according to criteria demonstrated by **Mulligan (2005).** Amongst the isolates selected C5C showed highest surface activity and thus it was further studied for detail study.

Production and extraction of biosurfactant from C5C (*Bacillus* species)

Isolate C5C was used for further studies on biosurfactant production by inoculating it in MSM medium (with 1% diesel as carbon source) at 37^oC at 121 rpm for seven days. Isolate C5C produced 3.12 g/l dry weight of biosurfactant. *Bacillus clausii* 5B produced 2.41 g/l of biosurfactant in minimal medium with 1% w/w glucose as carbon source and ammonium chloride as nitrogen source **(Aparna *et al.,* 2012)**. *Bacillus subtilis* SPB1 produced 2.93 (±0.32) g/l of biosurfactant when media was incorporated with 15g/l of glucose, 6 g/l of urea and 1 g/l of K_2HPO_4 **(Abdel-Mawgoud *et al.,* 2008)**.

pH and Temperature optimization

Biosurfactant produced by C5C gave maximum E24% at pH 5 and 30^oC temperature in MSM medium with 1% diesel (Fig 2 and 3). pH optimization results showed that there was significant increase in emulsification in pH range 2 to 5 and as further increase in pH emulsification reduced. Similarly maximum E24% was observed at 30^oC. After that with E24% is positively decreases. Because of easy evaporation of diesel at higher temperature biosurfactant production might be decrease due to lack of carbon source under batch fermentation operate under shaking flask condition. Surfactin production from *Bacillus subtilis* DSM 3256 was reported to be highest at 6.75 pH and 37.4^oC temperature **(Sen and Swaminathan, 1997)**. Iturine-A was produced maximally by *Bacillus subtilis* at pH 5.9-6.3 and 25^oC temperature **(Mizumoto and Shoda, 2007)**. *Bacillus megaterium* produced maximum glycolipid at pH 8 and 37^oC under solid state fermentation **(Thavasi *et al.,* 2007)**.Similarly optimum condition for surfactin production form *Bacillus subtilis* BS5 was 6.5-6.7 pH and 30^oC as reported by **Abdel-Mawgoud *et al.,* (2008)**. *Penicillum chrysogenum* SNP5 which showed increase in E24% between 2 to 8 pH range, while E24% decreased between pH 8 to 12 **(Gautam *et al.,* 2014).**

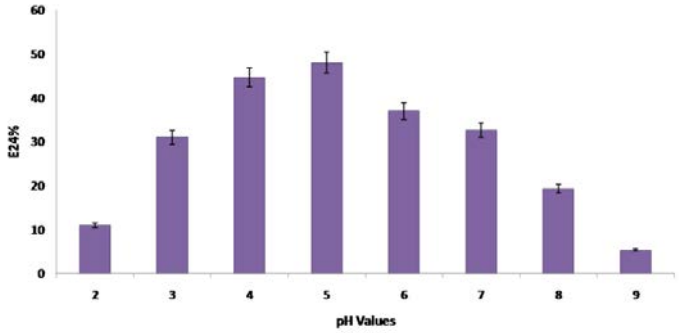

Figure 2 pH optimization

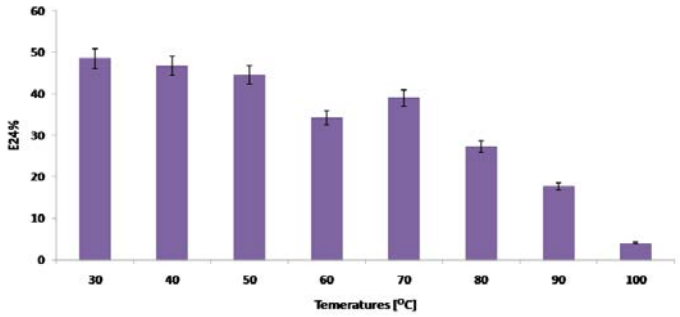

Figure 3 Temperature optimization

Characterization of biosurfactant by TLC

Biosurfactant produced from isolate C5C was analyzed by Thin Layer Chromatography (TLC). As shown in Fig 4, a purple colour spot appearing on TLC (*Rf* value 0.57) suggested presence of polar lipid content in biosurfactant. There was no colour development observed for amino acid or carbohydrate. Similar result was observed by **Aparna *et al.*, (2012)** that *Bacillus clausii* 5B biosurfactant produce red spot on silica gel plate. R_f value of biosurfactant was 0.51. **Nishanthin *et al.*, (2010)** have reported strains BPB7 and BPB13 isolated from petrochemical waste soil produced biosurfactant which is glycolipid in nature. Biosurfactant from *Arthrobacter* strain is reported to be lipopeptides and lipoprotein in nature **(Cipinvte *et al.*, 2011)**.

Figure 4 TLC analysis of biosurfactant

CONCLUSIONS

Thirty cultures were isolated from garage soil near Anand (Gujarat) region. They were characterized morphologically and biochemically. On the basis of screening techniques culture C5C was found to be potential isolate for production of biosurfactant among thirty isolates. It was identified as *Bacillus* sp biochemically as it gave positive response for Voges Proskauer test, citrate utilization test, nitrate test, starch utilization test and glucose fermentation test. Triple sugar ion test showed production of gas with acidic slant and alkaline butt reaction that confirm the *Bacillus* sp. Cultural conditions pH and temperature were also optimized for maximum production of biosurfactant. It produced 3.12 g/l of dry precipitate of biosurfactant in MSM medium (pH 5) with 1% diesel at 30ºC 121 rpm. Further optimization of culture media and condition can be done. Structural characterization, indicate presence of polar lipid content in biosurfactant.

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OPTIMIZATION, PRODUCTION, PARTIAL PURIFICATION AND CHARACTERIZATION OF NEUTRAL AND ALKALINE PROTEASES PRODUCED BY *BACILLUS SUBTILIS*

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ABSTRACT

Different strains of *Bacillus subtilis (Ehrenberg) Cohn,* BIOTECH 1056, 1333, 1573 and 1679, UPCC 1295 and USTCMS 1011 from various culture collections in the country were characterized morphologically, biochemically, and assayed for proteolytic activity. Fermentation parameters like pH, temperature, and incubation time were optimized for the production of proteases that were used as gauges to select the best bacterial isolates for possible commercial application. Among the six isolates studied, USTCMS 1011 gave the highest neutral protease activity of 0.647 U/mg protein at pH 7 and at 37°C for72 hours. The same strain yielded the highest alkaline protease activity of 0.495 U/mg protein at pH 9 and at 30°C for 72 hours. Various beans and fruit extracts or honey were tested as possible protein or carbohydrate sources in the medium. Dextrin produced the highest protease activity with 0.647U/mg protein for neutral protease and 0.495 U/mg protein for alkaline protease as carbohydrate source. *Garbanzos* (chickpea) as protein source, supplemented with yeast extract, gave the highest protease activity at 0.548 U/mg protein for neutral protease and 0.475 U/mg protein for alkaline protease. Both crude proteases were purified by ammonium sulfate precipitation followed by desalting and gel filtration chromatography. Optimal activity of the neutral protease was found at pH 8 and 30°C with an incubation time of 90min while optimal activity of the alkaline protease was at pH 11 and 40°C with only 30min incubation period. Neutral protease showed a single band with molecular weight of 65.4kDa in SDS-PAGE while alkaline protease showed single band with molecular weight of 8kDa.

Keywords: USTCMS 1011, Gel Filtration Chromatography, *Bacillus subtilis*, Neutral Protease, Alkaline Protease

INTRODUCTION

Proteases are essential constituent of all forms of life on earth that function to hydrolyze proteins into smaller fragments. It has been widely used as an important industrial enzyme, occupying nearly 60% of the enzyme sales that are obtained from microorganisms, plants and animals. Proteases have been commercially used for the production of household laundry detergents **(Kumar *et al*, 2002)**, medicine and poultry feeds (Beijing Shifa Technology and Trade Co., Ltd.). Proteases can be isolated from different biological sources. One of the most utilized sources of proteases are the extracellular proteins secreted from the membrane of microorganisms such as *B. subtilis*. These bacterial species are obligate aerobes, mesophiles and spore-forming that are commonly found in the soil. They are stable at pH ranging from 5-11. These bacteria are noted for its ability to degrade plant polysaccharides and pectin. The production of microbial-derived proteases is most advantageous and economically competitive since they are easy to produce commercially at a fast rate and requires less space. Bulk enzymes are preferred over "fine" enzymes, because they are less expensive and can be produced in higher quantity, though it has an inherently lower unit value **(Kumar *et al*, 2002)**. But in terms of relationship between scale values and market volume requirement, proteases command higher enzyme preparation value over pectinase, glucoamylase and amylase. However, environmental and cultural conditions for fermentation are ought to be optimized to obtain higher yield of proteases. Prior to this work, individual independent studies have been carried out on the various strains of *B. subtilis* though no single study has ever been conducted using the four strains of *B.subtilis* namely BIOTECH 1056, BIOTECH 1573, UPCC 1295 and USTCMS 1011 by comparing their activities to that of the previously studied strains, BIOTECH 1333 and BIOTECH 1679 **(Espino *et al.*, 1996)**. Two of the bacterial isolates, BIOTECH 1573 and USTCMS 1011, were both soil isolates from the Philippines. In general, crude proteases can be produced at a less expensive cost and in higher quantity by using cheap sources of proteins and carbohydrates.

MATERIALS AND METHODS

Chemicals and Reagents

Analytical grade reagents were used in all the experiments. The agars and broths used in the study were purchased from HiMedia Laboratories Pvt. Ltd., India. Other chemicals used for the purification method were obtained from Sigma-Aldrich Co., St. Louis, MO, USA and Scharlau Chem-Supply, Gillman, South Australia.

Microorganisms

Different *B. subtilis* isolates namely, *B. subtilis* MNH (BIOTECH 1056), *B. subtilis (Ehrenberg) Cohn* (BIOTECH 1333), *B. subtilis* BIOTECH 1573 (Philippine soil isolate), and *B. subtilis* NRRL B-3749 (BIOTECH 1679) were obtained from the Philippine National Collection of Microorganisms (PNCM) at the National Institute of Molecular Biology and Biotechnology (BIOTECH), University of the Philippines Los Baños, Laguna, Philippines while *B. subtilis* FIRDI 10447 (UPCC 1295) was obtained from the UP Culture Collection, Natural Science Research Institute, University of the Philippines Diliman, Quezon City, Philippines. The *B. subtilis* USTCMS 1011(Philippine soil isolate) was acquired from the UST Collection of Microbial Strains, Research Center for the Natural and Applied Sciences of the University of Santo Tomas, España, Manila, Philippines.

Microorganism's culture condition and inoculum preparation

Inoculum preparation was done according to the culture conditions and method of **El-Safey and Abdul-Raouf (2003)**. The pure culture of *B. subtilis* was maintained in nutrient agar. A 24-hr bacterial culture was maintained in nutrient agar medium at 4°C and subculture was done by streak plate method. Initially, the growth of the bacterial culture and the production of proteases were observed in skimmed milk agar. For every experiment, a 24-hr subculture of microorganism grown in a sterile nutrient agar at 37°C was used. The strain was preserved by inoculating a loopful of the strain into a nutrient broth medium with 70% glycerol and stored at -20°C.

Morphological Characterization of *B. subtilis*

Each of the six strains of *B. subtilis* was subjected to morphological characterization such as Gram staining, spore staining and capsule staining methods. The slides were viewed at High Power and Oil Immersion Objectives and documented.

Biochemical Characterization of *B. subtilis*

Each strain of *B. subtilis* was cultured in different media such as Mac Conkey Agar, Mannitol Salt Agar, Triple Sugar Iron Agar, Simmon Citrate Agar, and Litmus Milk Agar to test for any biochemical reactions. Furthermore, the bacterial strains were also screened for catalase activity.

Media for Proteases Production and Submerged Fermentation

Fermentation was performed with some modifications **(Das and Prasad, 2010)**. The fermentation medium used for protease production consists of the following in 1 L of solution: 60 mL soybean pressed cake extract (SPC extract), 13 g KH_2PO_4, 0.3 g $MgSO_4 \cdot 7H_2O$, $FeSO_4 \cdot 7H_2O$, 8 g dextrin, 0.5 g KCl, 0.2 g $CaCl_2$, 2.0 g yeast extract.

Optimization of Protease Production

A. Effect of pH on Protease Production

The 100 mL media for protease production were prepared in various pH (4.0, 7.0, 9.0 and 10.0) and were inoculated with one loopful of *B. subtilis* and incubated at 37°C using a shaker incubator in 100 rpm for 72 hrs. The protease produced was then measured using casein assay and protein assay **(Das and Prasad, 2010)**.

B. Effect of Incubation Temperature on Protease Production

The media for protease production were incubated at different temperatures (30, 35, 37, 39, 40°C) at pH 6 and were inoculated with one loopful of *B. subtilis* and incubated for 72 hrs. The protease produced was measured using casein assay and protein assay **(Das and Prasad, 2010)**.

C. Effect of Incubation Time on Protease Production

The effect of incubation time was determined by incubating one loopful of bacterial isolates in the medium for protease production at different incubation time (24, 48, 144, 168 hrs) at optimum pH and temperature. The protease produced was measured using casein assay and protein assay **(El-Safey and Abdul-Raouf, 2004)**.

Effect of Modifying Carbohydrate and Protein

Media Composition on Protease Production

This was determined by fermenting a loopful of *B. subtilis* in modified media composition. Soybean pressed cake extract and dextrin were incorporated to the medium as standard nitrogen and carbon sources, respectively. Other sources such as *garbanzos* (chickpea) extract, black bean extract, and mung bean extract were used as new possible nitrogen sources, at 0.6% concentration and were incorporated in the media for protease production (nutrient medium stated above) aside from soy bean pressed cake extract. Whereas, mango extract, banana extract and honey, tested as alternative carbon sources, at 0.8% concentration were incorporated in the media as new carbon sources for protease production other than dextrin.

Enzyme Production

Different strains of *B. subtilis* were fermented using the optimized fermentation parameters and media composition for protease production. Optimum pH was used by adjusting the initial pH of the media using Na_2CO_3. The propagation medium was sterilized at 121°C for 15 min. The medium (10ml in 250 mL Erlenmeyer flask) was inoculated with a loopful of *B. subtilis* and incubated at optimized temperature and incubation time in a rotary shaker (Thermoshaker Max Q4000, Thermo Scientific, USA) operated at 200 rpm for 72 hrs.

Product recovery

After incubation, the medium was centrifuged at 3,000 to 13,000 rpm (KUBOTA KS-5000P, Kubota, Japan) at 4°C for 15 min to separate the microbial cells. The supernatant was collected because it contains the extracellular enzyme that was secreted by the bacteria. The collected solution was stored at 4°C until further use.

Purification of Proteases

A. Ammonium Sulfate Precipitation

The bacterial supernatant was fractionated by using ammonium sulfate with 80% of saturation and was salted out using the desired buffer for the enzyme. All steps were carried out at 4oC. The proteins were re-suspended at 0.1M Tris-HCl buffer at pH 7.8 for neutral proteases and 0.1 M glycine-NaOH at pH 10-11 for alkaline proteases **(Adinarayana *et al*, 2003)**.

B. Dialysis

Dry membranes (Sigma D9652-100FT Dialysis tubing cellulose membrane, Sigma-Aldrich) were used for the dialysis of the collected protease, the dialyzing bag was boiled using distilled water with EDTA. The dialyzing buffer used was the same as the buffer used in desalting the enzymes. Both types of enzymes were purified using this technique. The enzyme was recovered from the membrane and stored at 4°C until further use.

C. Gel-filtration Chromatography

The protein obtained after precipitation with 80% ammonium sulfate was dissolved in the desired buffer, dialyzed and loaded into a column of Sephadex G-75 (Sigma-Aldrich) equilibrated with 0.1 M Tris-HCl buffer at pH 7.0-8.0 for neutral protease and 0.1 M glycine-NaOH buffer at pH 10.0-11.0 for alkaline protease. The chromatography was performed at a flow rate of 60 mL/hour using the same buffer. Five milliliters of eluate were collected per fractions. Collected proteases have high caseinase activity and demonstrated high peaks at 280 nm.

Characterization of Purified Enzymes

A. Effect of pH on enzyme activity and stability

The activity of purified enzyme was measured at different pH values (2.0-11.0). The pH was adjusted using phosphate-citrate buffer (pH 2.0-7.0), Tris-HCl buffer (pH 8.0), and glycine-NaOH buffer (pH 9.0-11.0). The mixture was incubated at 40°C for 30 min and the activity of the enzyme was measured. The stability of the enzyme was determined using different buffers (pH 2.0-11.0) and was incubated for 48 hrs. The activity of the enzyme at each exposure in different pH and time were measured using casein assay and protein assay.

B. Effect of temperature on enzyme activity and stability

The activity and stability of the purified enzyme were both determined by incubating the mixture at different temperatures (20, 30, 40, 50 and 60°C) for 30 min and 1 hour, respectively. The activity of the enzyme at the different temperatures and their stability were measured using casein assay and protein assay **(Adinarayana *et al*, 2003) (Das and Prasad, 2010)**.

C. Effect of Incubation Period

The purified protease was incubated at 40°C at their respective pH and incubated for of 30, 60, 90, 120, 150 and 180 min. The enzyme activity was determined using casein assay and protein assay.

Homogeneity determination using SDS-PAGE

Fractions with high activity in casein assay from gel chromatography were run in SDS-PAGE. Two (2) microliter of sample were mixed with 2.5µL Bolt® LDS Sample Buffer (4x) (Novex, USA), 1µL Bolt® Reducing Agent (10x) for reduced samples and none for non-reduced samples and brought the final volume to 10µL using ultra-pure water. The samples were incubated for 10 minutes at 70°C. Ten microliter of samples and standard were loaded in a pre-cast gel (Bolt® Mini Gels, Novex, USA). The pre-cast gel was placed in Bolt® Mini Gel Tank (Novex, USA) and added with 400mL of 1X MES Running buffer (Novex, USA). The running condition set for the gel was 80V and run for 60 minutes. After running, the gels were stained with Coomassie Blue R-25 (Sigma-Aldrich, USA) and destained using methanol and acetic acid. Protein standard used was SeeBlue® Plus2 Prestained Standard (Novex, USA).

Tyrosine Standard Curve

Stock solution of tyrosine was prepared at 100 µg/ml and aliquots of which were prepared (10-100 µg) then was read at 280 nm. The standard curve was composed of tyrosine concentrations versus the absorbance at 280 nm **(Kumar *et al*, 2002)**.

Casein Assay

A 2.5 mL casein solution of 0.6 % casein in 0.01M Tris-HCl (pH 7.2) or 0.01M glycine-NaOH (pH 10) was incubated with 0.5 mL of diluted enzyme solution for 30 min at 40ºC. The reaction was stopped by adding 10% trichloroacetic acid (TCA) solution. After 30 min, the mixture was centrifuged at 10,000 rpm for 5 min. The enzyme activity was then calculated from the standard curve of L-tyrosine. in the Absorbance of each concentration of tyrosine (10-100 µg/mL) read at 275 or 280 nm for the preparation of the standard graph. Enzymatic activity which was measured as one unit, means that one amount of enzymatic activity reacts with one micromole of substrate per minute of reaction. Enzyme activity was dependent on the temperature, particular pH, substrate concentration and the active site of enzyme **(Espino *et al*, 1996; Kumar *et al*, 2002)**.

Protein Assay

The protein concentration was measured using Lowry method using bovine serum albumin (BSA) as the standard. The concentration of protein during purification studies was measured at 280 nm.

Statistical Analysis

The incubation parameters and other conditions were statistically analyzed by one-way analysis of variance (ANOVA) using MS Excel determine the effects of different variables. The $p<0.05$ value was considered to be significant.

RESULTS

Six strains of *B. subtilis* were collected from different culture collections in the Philippines and screened for proteolytic activity. The different strains of bacteria were partially identified to confirm the identity of the bacteria from the culture collections and then preceded to identification of best strain that produced proteases through optimization of fermentation parameters like temperature and pH independently. Some of the strains are known to produce proteases and some of the strains acquired from different culture collections are studied to produce proteases (BIOTECH 1573 and BIOTECH 1679) while some (USTCMS 1011, UPCC 1295, BIOTECH 1056, BIOTECH 133) are not yet screened and studied to produced proteases.

Partial Identification of Six Strains of *B.subtilis*

The results of morphological and biochemical characterization confirmed the identity of the *B. subtilis* strains that were used in the study. The results showed that they were all gram positive, spore forming and encapsulated bacteria that were lactose and glucose fermentor and can produce catalase. The six strains of *B. subtilis* showed a negative result in Mannitol Salt Agar (MSA) since the agar is specific for *Staphylococcus aureus,* negative in Simmon Citrate Agar (SCA) and MacConkey Agar (MCA) since both media are selective for *Escherichia coli.*

Fermentation Parameters

Tyrosine standard curve was used to determine the proteolytic activity of the enzyme based on its reactivity towards the substrate and was used to determine enzyme activity in each fractions of purification. The protein concentration was determined based on the absorbance acquired and was used to compute the specific activity of the enzyme. (The linear equation obtained from the standard curve was $y=0.002x+0.0197$ with a linearity (R^2) value of 0.9929. The equation was significant on the whole research since it was used to determine the enzymatic activity throughout production, purification, and optimization.

The pH of the media and the incubation temperature were optimized for the production of neutral and alkaline proteases with highest activity. The six different strains of *B. subtilis* were incubated at different temperatures and different pH of the media. Results are shown in Figure 1 and 2.

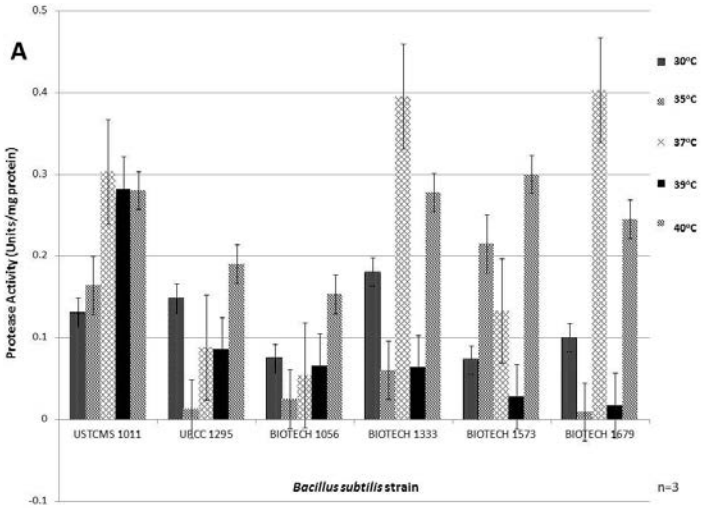

Figure 1A Neutral protease activities (U/mg protein) of six strains of *Bacillus subtilis* at different incubation temperature using media for protease production according to **Das and Prasad, 2010** and **Espino *et al*, 1992** at pH 6.

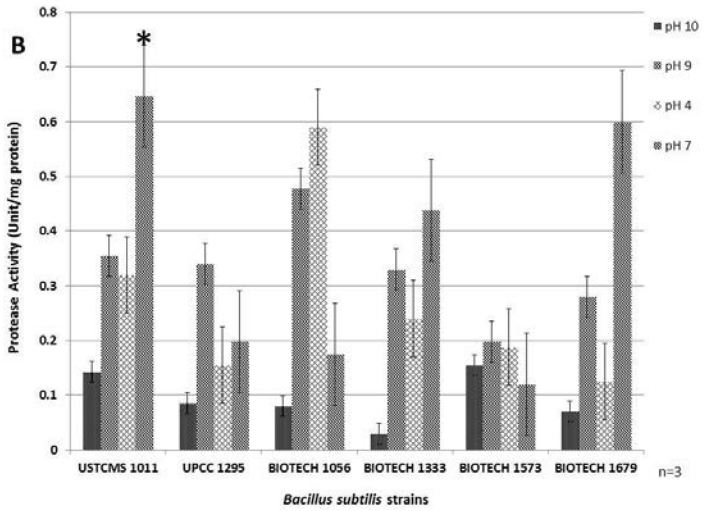

Figure 1B Neutral protease activities (U/mg protein) of six strains of *Bacillus subtilis* at different pH medium using media for protease production according to **Das and Prasad, 2010** and **Espino *et al*, 1992** at 37ºC.

Evaluating the two graphs (Fig. 1A and 1B), it showed that the strain USTCMS 1011 produced the highest neutral protease activity (0.6466±0.05 U/mg) based on the fermentation parameters at pH 7.0. and at 37ºC and thus was used in the protease production experiment.

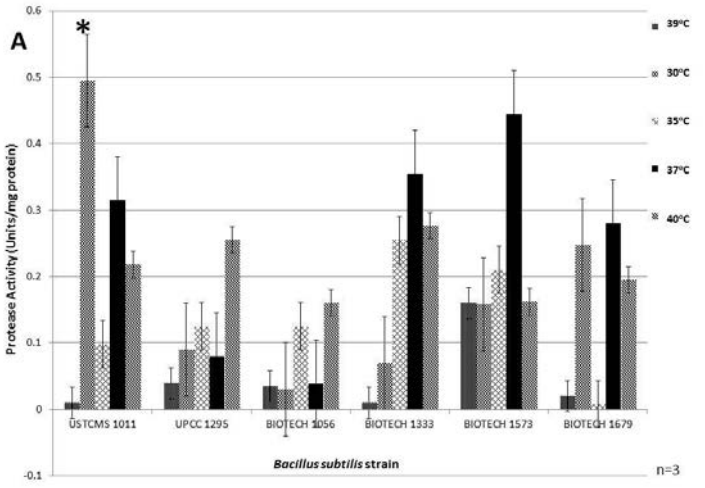

Figure 2A Alkaline Protease activities (U/mg protein) of six strains of *Bacillus subtilis* at different incubation temperature using media for protease production according to **Das and Prasad, 2010** and **Espino *et al*, 1992** at pH 9.

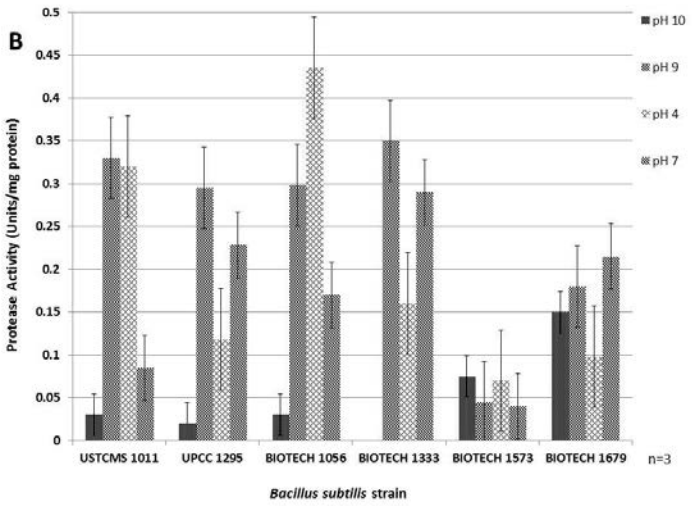

Figure 2B Alkaline Protease activities (U/mg protein) of swix strains of *Bacillus subtilis* at different pH medium using media for protease production according to **Das and Prasad, 2010** and **Espino *et al*, 1992** at 32ºC.

Figure 2 illustrates the alkaline protease activity at different pH of media and different incubation temperature. USTCMS 1011 had the highest alkaline protease activity based on the two graphs that yielded 0.4954±0.032 U/mg. Since USTCMS 1011 gave a significantly highest alkaline protease activity ($p<0.05$), it was used for the production of the alkaline protease at pH 9 and at 30ºC.

Media Composition

Additional fermentation parameters were optimized in this study such as the media compositions wherein different protein and carbohydrate sources were used. The protein and carbohydrate sources that gave the highest proteolytic activity was used for the production of laboratory scale enzymes. The concentration of the protein and carbohydrate sources used was 0.6% (w/v) and 0.8% (w/v), respectively. Results are shown in Figure 3.

Black beans, *garbanzos* and mung beans,incubated with or without yeast, were used as protein sources.Statistical analysis showed significant differences among different protein sources. Optimum proteolytic activity was found when garbanzos with yeast was used, where highest protein activity for both alkaline and neutral protease at 0.4754±0.028 U/mg protein and 0.5476±0.041 U/mg, respectively were recorded. Results are shown in Fig. 3A.

Incubation time of 24, 48, 72, 144, and 168 hours were used in the experiment (data not shown). At 72 hours, both the neutral and alkaline proteases significantly yielded the highest proteolytic activity ($p<0.05$). Neutral protease gave 0.6466±0.05 U/mg protein and alkaline protease gave 0.4955±0.032 U/mg protein. Results are shown in Fig. 3C.

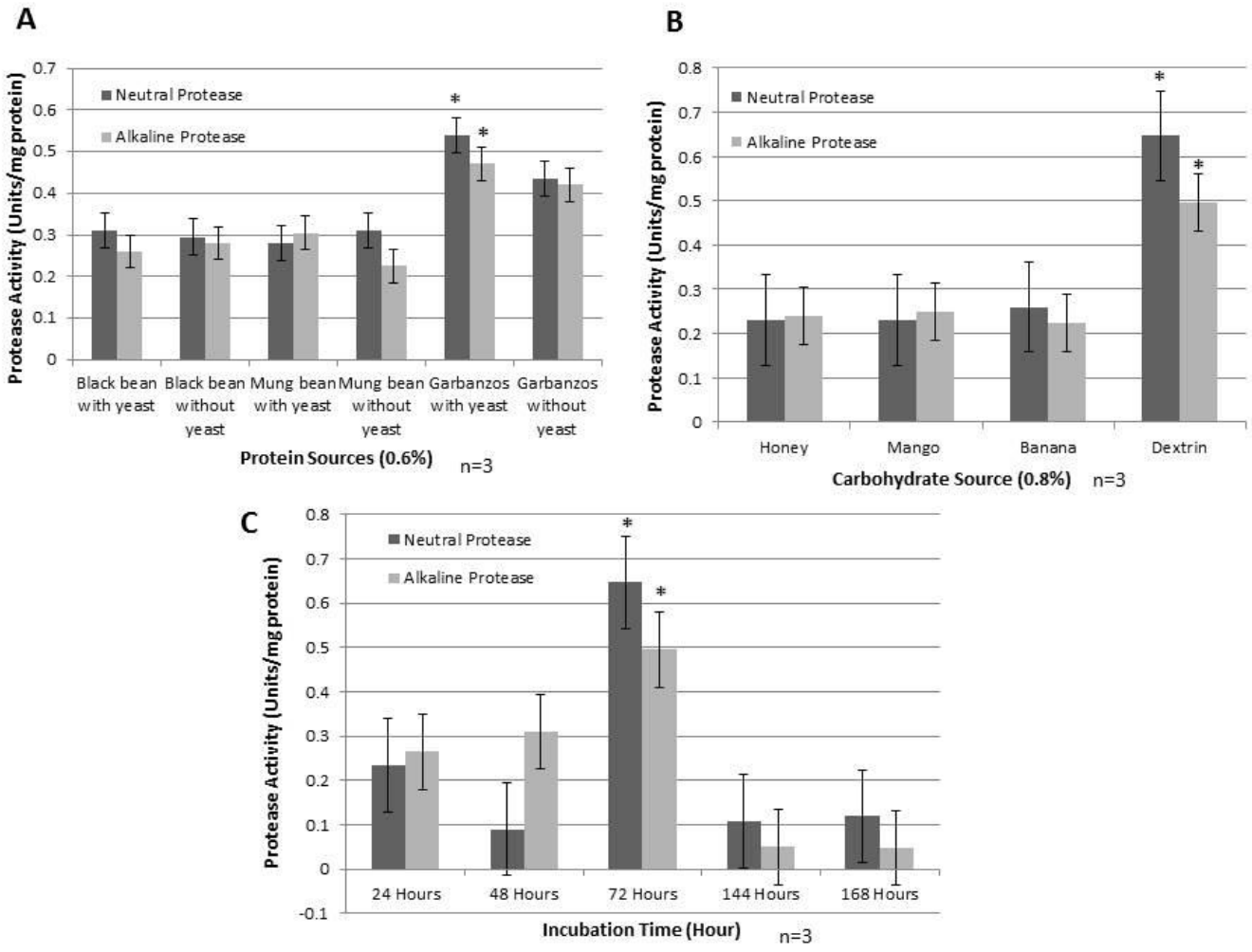

Figure 3 (A) Neutral and Alkaline protease activity (U/mg protein) of *B. subtilis* USTCMS 1011 at different protein sources with a concentration of 0.6% incorporated in the media for protease production according to **Das and Prasad, 2010** and **Espino *et al*, 1992** at pH 7, 37ºC and pH 9, 30ºC respectively. (B) Neutral and Alkaline protease activity (U/mg protein) of USTCMS 1011 at different carbohydrate sources with a concentration of 0.8% incorporated in the media for protease production according to **Das and Prasad, 2010** and **Espino *et al*, 1992** at pH 7, 37ºC and pH 9, 30ºC respectively. (C) Effect of different incubation time (hours) on USTCMS 1011 production of neutral and alkaline protease using the media for protease production according to **Das and Prasad, 2010** and **Espino et al, 1992** at pH 7, 37ºC and pH 9, 30ºC respectively.

Purification by Gel Filtration

Neutral and alkaline proteases were produced from *B. subtilis* USTCMS1011 by using the optimized fermentation parameters and media composition. The protease was partially purified by salting-in in a solution of ammonium sulfate (% w/v) and desalting the enzyme by dialysis. The dialyzed product was subjected to gel filtration for further purification. The absorbance of each fraction that were collected were read at 280nm to detect the presence of proteins. High peak at 280nm indicated the presence of the enzyme on the different fractions. The fractions were also assayed using casein assay to constantly

monitor the presence and the activity of the enzyme in each fractions. Results are shown in Figure 4.

The test for protease acivity of neutral protease was done at 280nm . This test was used to confirm the presence of neutral protease on the different fractions (shown in Fig. 4A). The highest protease activity at 18.411 units/mL in the reaction mixture was observed in fraction 5. The fraction with the highest proteolytic activity was collected and used for optimization of partially purified enzyme.Similarly, the test for alkaline protease activity was done at 280nm . This test was used to confirm the presence of alkaline protease on the different fractions (shown in Fig. 4B). The highest protease activity at 11.064 units/mL was observed in fraction 5. Figure 4B also showed the high peak and high protease activity in fractions 5 and 6. The presented data was in enzyme activity (units/mL) since protease activity was being determined on the gel purification and not the specific activity (units/mg protein) of the enzyme in relation to its substrate.

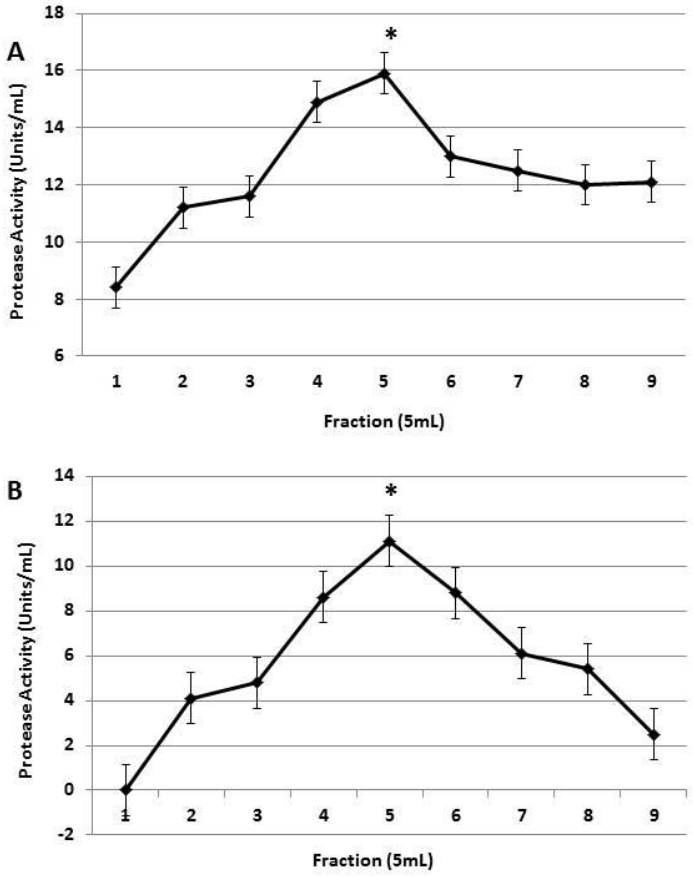

Figure 4 (A) Total neutral protease activity (U/mL) after gel filtration chromatography of 5 mL per fraction. (B) Total alkaline protease activity (U/mL) after gerl filtration chromatography of 5mL per fraction.

Purification of Crude Enzyme

The total activity and total protein were determined using casein assay and protein assay (Lowry method). After the determinations, the total protein, specific activity, percentage yield, and the purification fold of the enzyme were calculated. Results are shown in Table 1 and 2.

The purification folds for gel-filtration chromatography were 31.719 and 1.330 for neutral and alkaline proteases respectively.

The trend on the total protein, total activity, specific activity, percentage yield, and purification fold based on the different purification steps. As the total protein, total activiy and percentage yield decreases the specific activity and purification fold increases.

Optimization of Environmental Factors Effecting the Partially Purifed Enzyme

Environmental factors like the pH of the buffer used in the casein assay, pH stability of the enzyme, temperature used in the assay, thermal stability of the enzyme, and the incubation time used in the assay were optimized (Fig. 5, Fig. 6, and Fig. 7). These factors were optimized together with the partially purified enzyme. High activity in the different environmental factors were applied and used as basis for further study.

The neutral and alkaline proteases were subjected to different temperatures during the assay. The optimum temperature for neutral protease was at 30°C and 40°C for alkaline protease. Both enzymes were subjected to different temperatures prior to use in the assay (**Pagare *et al*(2009)**; **El-Safey and Abdul-Raouf (2004)**; **Kumar *et al*(2002)**). Both of the enzymes are stable in five different temperatures. Neutral protease showed the highest activity at 30 minutes and decreased its activity as the incubation time increased. While the alkaline protease had its highest activity at 90 minutes and decreased its activity as the incubation time increased. Both enzymes showed that the time of incubation had an effect on the proteolytic activity of the enzymes.

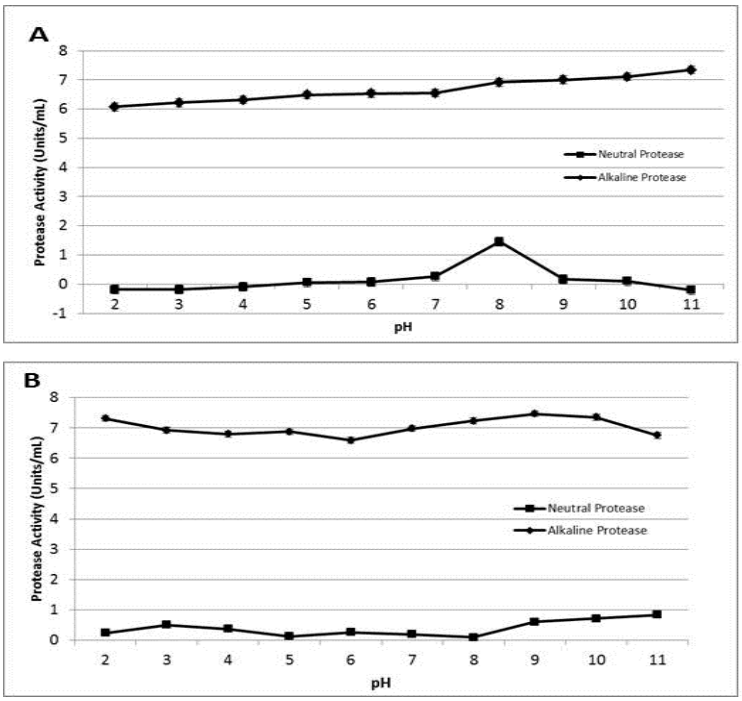

Figure 5 (A) Optimum pH environment for partially purified neutral and alkaline proteases reaction. (B) pH stability of partially purified neutral and alkaline proteases incubated for 48 hours.

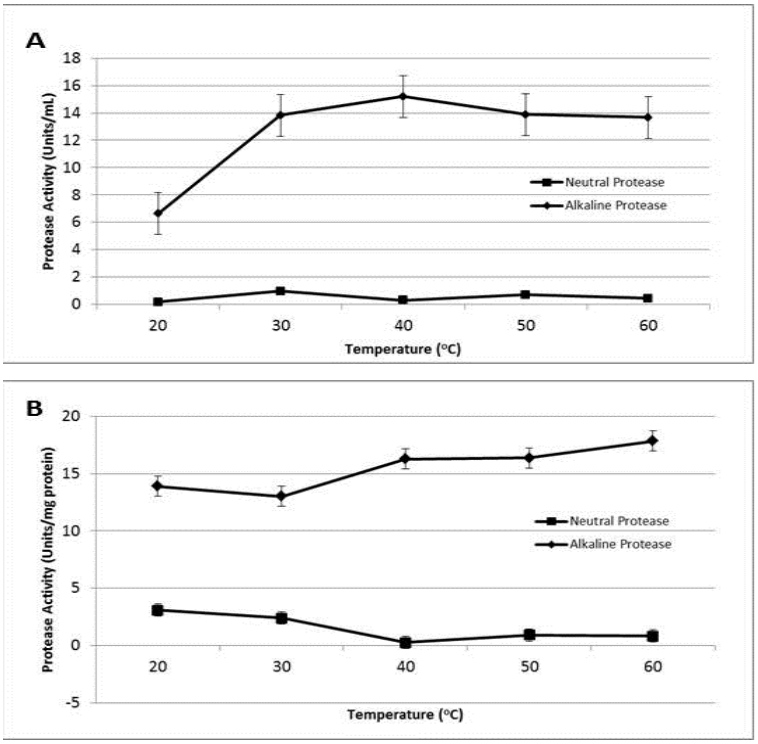

Figure 6 (A) Optimum thermal environment for partially purified neutral and alkaline proteases reaction. (B) Thermal stability of partially purified neutral and alkaline proteases incubated for 1 hour.

Table 1 Neutral Protease Purification Table

Method	Total Protein (mg/mL)	Total Activity (Units/mL)	Specific Activity (Units/mg protein)	% Yield	Purification fold
Crue Enzyme	17.84	2.40	0.13	100	1.00
Ammonium Sulfate Precipitation	2.28	2.23	0.98	93.28	7.32
Desalting by Dialysis	1.12	1.56	1.39	64.95	10.37
Gel-Filtration Chromatography Sephadex G-75	0.10	0.42	4.26	17.43	31.72

Table 2 Alkaline Protease Purification Table

Method	Total Protein (mg/mL)	Total Activity (Units/mL)	Specific Activity (Units/mg protein)	% Yield	Purification fold
Crue Enzyme	8.90	4.76	0.53	100	1.00
Ammonium Sulfate Precipitation	4.31	2.35	0.54	49.79	1.03
Desalting by Dialysis	2.69	1.56	0.58	32.96	1.09
Gel-Filtration Chromatography Sephadex G-75	1.36	0.96	0.70	20.30	1.33

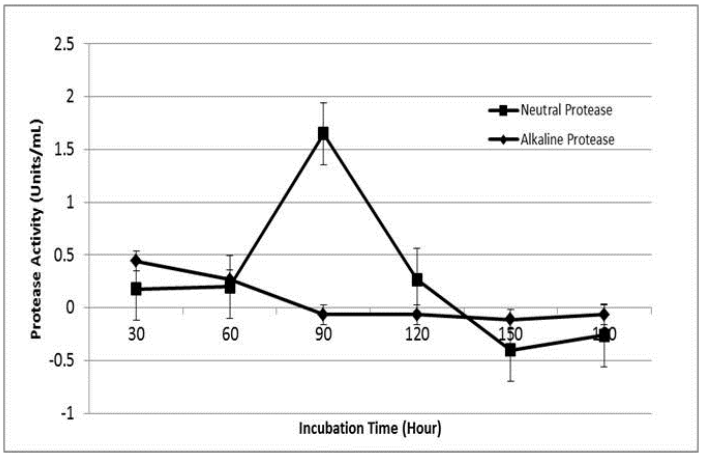

Figure 7 Optimum incubation time of reaction for both partially purified neutral protease and alkaline proteases.

Homogeneity determination using SDS-PAGE

Fractions collected from the gel chromatography with high proteolytic activity were analyzed by using SDS-PAGE to determine the presence of proteins and its molecular weight. Protein standard used in this analysis were Insulin B Chain (3kDa), Aprotinin (6kDa), Lysozyme (14kDa), Myoglobin Red (17kDa), Carbonic Anhydrase (28kDa), Alcohol Dehydrogenase (38kDa), Glutamic Dehydrogenase (49kDa), BSA (62kDa), Phosphorylase (98kDa), Myosin (188kDa). Molecular weight was determined by linear semilogarithmic plot of the molecualar weights of standard and its Rf values. Based on the Rf value of the of the purified neutral protease, the calculated molecular weight of the protein band was 65.4kDa which coincide in the band of BSA and Phosphorylase (Fig. 8A). While based on the Rf values of the purified alkaline protease, the calculated molecular weight of the band was 8kDa.

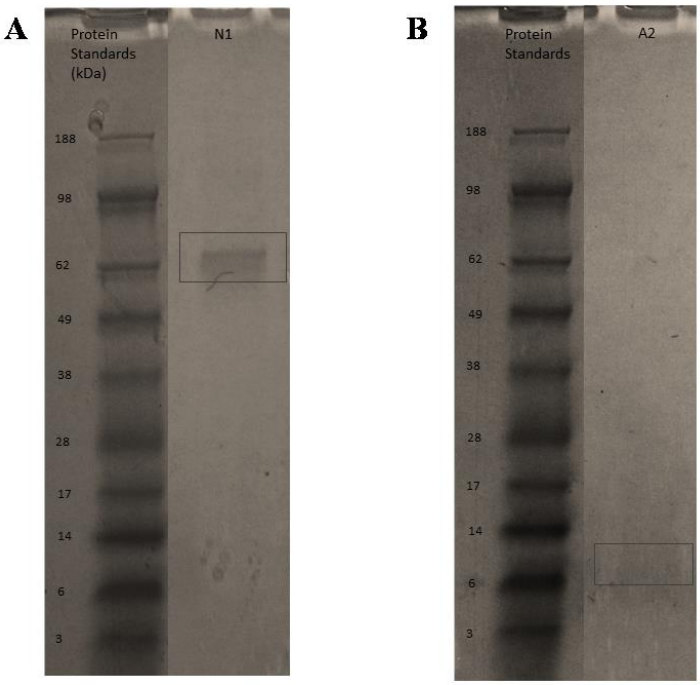

Figure 8 (A) SDS-PAGE molecular weight determination of partially purified neutral proteases. (B) SDS-PAGE molecular weight determination of partially purified alkaline proteases.

DISCUSSIONS

Bacillus subtilis is known to produce many kind of enzymes, namely, RNAse, alkaline phosphatase, levansucrase, amylase and several proteases **(El-Safey and Abdul-Raouf, 2004)**. Neutral and alkaline proteases were the two enzymes from *B.subtilis* that were of interest in this study. Data presented from the optimization of fermentation medium up to the characterization of the enzyme itself showed that *B.subtilis* was producing extracellular proteases as part of its defense mechanism and as part of its metabolism. In this study, six strains of *B.subtilis* were screened for the production of proteases, optimized the fermentation medium for high production of proteases. Since USTCMS 1011 showed high proteolytic activity in the optimization of pH and temperature of the media of both neutral and alkaline protease (Figs1 and 2), it was used for further experiments and for the production of two proteases. **El-Safey and Abdul-Raouf (2004)**, noted in their study that optimum protease production by *B. subtilis* may range from 30°C to 45°C. The pH of media noted in the study of **El-Safey and Abdul-Raouf (2004)** with the optimum protease production was at pH 7 and at the pH range of 6.0 to 8.0 and for alkaline stable protease at pH 11. The results of

this study conformed that optimum fermentation condition for protease production was at thermal range of 30ºC to 45ºC and has a buffer pH of 6.0 to 8.0 for neutral condition and 9.0 to 11.0 for alkaline condition.

Garbanzos with yeast extract significantly showed the highest proteolytic activity among the other protein source ($p<0.05$). *Garbanzos* is known to have a value of 8.86 g of protein per 100 g, while mung bean and black bean have 7.02 and 1.08 g respectively and yeast extract added in the media is also an established protein source.

The abovementioned protein source were used because of its lower unit value and is more economical than using pure yeast extract as protein source. Results showed that *garbanzos* was a good substitute to soybean which was use in research of protease production of **Espino *et al.* (1996)**.; soy bean has been considered to have high protein content than other legumes and other animal products and contains all essential amino acids and all essential fats while *garbanzos* also contain high protein, essential fats, and nutrients; and since garbanzos has a lower economic value than soy bean, it was used in protease production experiment as a substitute to soybean with a little amount of yeast extract for additional protein source in the media rather than using pure yeast extract. Ammonium sulfate was also noted to be good source of nitrogen for *B.subtilis* as per **El-Safey and Abdul-Raouf (2004)**.

The different sources of carbohydrate used were dextrin, honey, banana and mango peelings. Statistical analysis showed significant differences among different carbohydrate sources. Optimum proteolytic activity was found when dextrin was used ($p<0.05$) as carbohydrate source in both alkaline and neutral proteases activity of 0.4954±0.032 and 0.6466±0.05 U/mg protein, respectively. Results are shown in Fig. 3B. Based on the studies conducted, dextrin served as the most prominent carbohydrate source because of its structure which consists of branching unit of glucose and easily for the bacteria to degrade and turn it into glucose subunits, which is the primary source of carbon for bacteria **(Espino *et al*, 1996)**. In addition, honey, banana and mango peelings were found to have anti-bacterial properties thereby attaining lower proteolytic activities. **El-Safey and Abdul-Raouf (2004)** also noted in his study that lactose and sucrose were also very good sources of carbon for the bacteria while glucose supressed protease production.

Incubation time of 72 hours was used for the production of protease in both alkaline and neutral protease together with other fermentation parameters because the results showed high production of protease in those conditions and that incubation time was also the optimum incubation time for protease production as performed by **Espino *et al* (1996)**, **Das and Prasad (2010)** and **Kumar *et al* (2002)**.

The pH of the culture strongly affects many enzymatic processes and transport of compounds across the cell membrane **Das and Prasad (2010)**. The optimum pH for partially purified neutral proteases were obtained as pH 8 and at pH 11 for alkalaine proteases. Neutral protease had no activity on acidic and basic pH while the alkaline protease showed an increase in the activity as the pH used in the assay increases. Both proteases were observed to be stable at pH 2-11. **El-Safey and Abdul-Raouf (2004)**, **Adinarayana *et al* (2003)** and **Ahmed *et al* (2011)** noted in their research that neutral protease has an optimal activity at pH 7.0 and was stable at pH ranging from 2.0 to 9.0 while the alkaline protease has an optimum activity at pH 11 and was stable at pH ranging from 5.0 to 12.0. Optimum temperature for partially purified protease for it to be fully active was observed in this study at 30ºC and 40ºC; but according to **El-Safey and Abdul-Raouf (2004)**, **Ahmed (2011)** their purified protease were active at 35ºC to 37ºC, 40ºC and was stable at 25ºC to 42ºC. In that note, results obtained in this study conform that purified proteases are active at buffer pH of 7.0 to 8.0 for neutral proteases and pH 9.0 to 11.0 for alkaline proteases and that they are stable at pH 2.0 to 12.0. Also, purified proteases are active at thermal incubation of 30ºC to 40ºC and are active at 25ºC to 42ºC.

Based on literatures, alkaline serine protease has a lower molecular weight than neutral protease. **Adinaraya *et al.* (2003)**, characterized their isolated alkaline protease with a molecular weight of 15kDa to 19kDa. Neutral protease in the other hand has a molecular weight of 44kDa and 50kDa **(Younis *et al*, 2009)**.

CONCLUSION

Among the six tested *B. subtilis*, strain USTCMS 1011 had the highest proteolytic activity for neutral and alkaline protease. The optimum conditions for neutral protease were pH 7 and 37ºC while for alkaline protease, pH 9 and 30ºC. High proteolytic activity was observed both in neutral and alkaline proteases using *garbanzos* with yeast extract as the protein source and dextrin as the carbohydrate source. Furthermore, partially purified proteases have an optimum pH of 8 at 30ºC for 90 min that is also stable at pH 2-11 and at 30-40ºC for neutral proteases. On the oher hand, alkaline protease had an optimum pH of 11 at 40ºC for an incubation period of 30 minutes and was also stable at pH 2-11and at 30-40ºC. Molecular weight of enzyme produced was noted to be at 64.5kDa for neutral proteases and 8kDa for alkaline proteases.

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GROWTH AND BIOGENIC AMINE POTENTIAL OF *Lactococcus lactis* subsp. *lactis*, isolated FROM TURMERIC (*Curcuma longa* Linn.), WITH PROBIOTIC CHARACTERISTICS IN SKIM MILK

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ABSTRACT

The growth and biogenic amines, (BA), (histamine and tyramine) potential of *Lactococcus lactis* subsp. *lactis* with potential probiotic characteristics isolated from turmeric (*Curcuma longa* Linn.) was studied in skim milk. Probiotic characteristics were determined in terms of acid and bile tolerance, hydrophobicity and antimicrobial activity. Fermentation of skim milk was followed for 48 hrs at 37 ºC determining pH, cell counts and histamine and tyramine with qualitative screening for proteolytic activity. Isolate showed good acid and bile salt tolerance (>90 %) with acceptable adhesion and antimicrobial activity. The maximum viable cell count (8.35 log cfu mL^{-1}) was observed after 48 hrs of incubation in skim milk with corresponding low pH of 4.06. Histamine or tyramine was not detected throughout 48 h of incubation as determined by HPLC. These properties suggest *Lactococcus lactis* subsp. *lactis* isolated from fresh turmeric is a safe potential probiotic in terms of BA production in milk.

Keywords: Biogenic amines, Histamine, Tyramine, Probiotic, *Lactococcus lactis*

INTRODUCTION

There is an increasing trend today in food industry to use probiotic starter cultures to promote the health and well being of the consumers. Probiotics are live microbial feed supplements which beneficially affects the host by improving the indigenous micro flora (**Fuller, 1989**). Lactic acid bacteria (LAB) play a major role as probiotic or functional starter organisms in food and beverage industry and are considered to have a safe history of application and consumption in fermented products (**Wood *et al.*, 1995; Caplice *et al.*, 1999; Leroy *et al.*, 2004**). *Lactobacillus* and *Lactococcus* species are mainly used as probiotics in fermented milk products. Since *Lactococcus* strains are widely used as starter cultures, several works showed their application as probiotics in fermented dairy products (**Grahn *et al.*, 1994; Kimoto *et al.*, 1999; Pianpumepong *et al.*, 2012; Enan *et al.*, 2013**).

Strains with probiotic features were isolated from natural habitats or from fermented products (**Oberman *et al.*, 1998**). The recent trend shows the isolation of wild-type of strains from traditional products to be used as starter or probiotic cultures in food fermentation (**Beukes *et al.*, 2001**). LAB isolated from various kinds of fermented plant products show potential use as starter cultures with improved quality of the end products (**Mourad *et al.*, 2004; Prachyakij *et al.*, 2008**). However there is no reported evidence of potential probiotic bacteria isolated from turmeric rhizomes and use in milk fermentation. New or potential probiotic strains should be evaluated not only for their beneficial health effects, but also for their harmful metabolites. Biogenic amines (BA) are considered as undesirable metabolic product of probiotic or functional starter organisms with potential heath risk to consumers (**Holzapfel *et al.*, 1995; Leroy *et al.*, 2004; Ammor *et al.*, 2007**).

Biogenic amines, histamine and tyramine are the most extensively studied amines in milk and milk products due to their toxicological effects. Histamine has been reported as the causative agent of histamine intoxication, while tyramine has been reported to affect the hypertensive crisis in the individuals being administrated monoamine oxidase (MAO) inhibitor drugs (**Anderson *et al.*, 1993; Silla Santos 1996; Zaman *et al.*, 2009**). The high amount of histamine and tyramine can be formed during storage or processing of fermented foods by the activity of bacterial decarboxylase enzyme (**Halász *et al.*, 1994**). Accumulation of biogenic amines in fermented milk or cheese can be affected by the availability of free amino acids and the presence of decarboxylase positive microorganisms (**Fernández-Garcia *et al.*, 2000**). Microorganisms possessing decarboxylase activity can be non-starter lactic acid bacteria and other spontaneous microflora (**Roig-Sagués *et al.*, 2002**) and starter microorganism (**Fernández-Garcia *et al.*, 2000**).

Several species of lactic acid bacteria have been identified as biogenic amines formers in milk. Several species of *Lactobacillus bulgaricus*, *L. casei* and *L. acidophilus* are reported as histamine formers (**Edwards *et al.*, 1981; Stratton *et al.*, 1991; Petridis *et al.*, 1996**). According to **Chander *et al.*, (1989)** *Lactococcus lactis* was found to produce histamine, tyramine and tryptamine. As reported by **González de Llano *et al.*, (1998)**; **Halász *et al.*, (1994)**; and **Straub *et al.*, (1995)** amine forming ability should be one of the concerns in selecting starter cultures. Thus testing for BA formation by commonly used or intended to use starter or probiotic LAB strains is essential. However sufficient progress has not yet been made in the optimal strains selection in terms of BA formation and information relevant to the BA formation by probiotic *Lactococcus lactis* subsp. *lactis* in milk system is still scarce.

Therefore, the aim of this study was to study the growth characteristics and biogenic amine potential of non dairy based *Lactococcus lactis* subsp *lactis* isolated from fresh turmeric rhizomes during the fermentation of skim milk.

MATERIAL AND METHODS

Isolation and Identification of Lactic Acid Bacteria (LAB)

Fresh turmeric rhizomes were purchased from Thakun, Suraththani Province, Thailand. Lactic acid bacteria were isolated and identified and characterized as previously reported (**Adnan *et al.*, 2007; Pianpumepong *et al.*, 2010**). In brief, 10 g of chopped turmeric rhizomes was mixed with 90 mL of sterile distilled water and extracted for 2 min using stomacher. Serial dilutions in sterile NaCl (0.85 %) were prepared with the 1 mL of extracted suspension. Diluted suspension (0.1 mL) was spread on MRS agar (Himedia, Mumbai, India) plates containing 0.5 % (w/v) calcium carbonate solution and incubated anaerobically at 37 ºC for 24-48 hrs. Acid producing bacteria were recognized by the appearance of clear zone around the colonies. The purity of the colonies was examined on MRS agar plates containing 0.06 g L^{-1} bromocresol purple as an indicator. The

medium colour changed from purple to yellow as a result of pH reduction indicating the production of a lot of lactic acid during the log phase of bacterial growth. Isolates were tested for catalase production by placing a drop of hydrogen peroxide solution (3 % (v/v) in sterile distilled water) on bacterial cells. Immediate formation of bubbles indicated the presence of catalase in the cells. Following the catalase test isolates were further analyzed and identified by using API kits (bioMérieux, Inc., Durham, NC, USA).

Probiotic Characterization

Probiotic characteristics of the strain *Lactococcus lactis* subsp. *lactis* were investigated as acid and bile salt tolerance, hydrophobicity, and antimicrobial activity. Acid tolerance was tested as reported by **Pianpumepong *et al.*, (2010).** Cell suspension was added to MRS broth maintained at pH 3.0 and a control at pH 6.4 and incubated for 2 h at 37 ºC. At the end of 2 h incubation viable cell count was determined by growing them in MRS agar for 48 hrs at 37 ºC. Acid tolerance was measured as percentage of cell survival calculated by comparing the initial bacterial cell count to the count after 2 h incubation at pH 3.0. Bile salt tolerance was observed as reported by **Pianpumepong *et al.*, (2010).** Bacterial cell count was compared to the cell count in MRS broth with added bile salt and without bile salt after 24 h of incubation. Antibacterial activity was tested against *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella* spp. and hydrophobicity and antimicrobial activity were calculated as reported by **Pianpumepong *et al.*, (2010).**
Lactococcus lactis subsp. *lactis* with potential probiotic characteristics was further studied for their growth characteristics and biogenic amine potential in skim milk.

Preparation of Inocula

The strain *Lactococcus. lactis* subsp. *lactis* was maintained along with other isolated bacterial strains at the culture stocks of Biotechnology Laboratory of the Asian Institute of Technology, Pathumthani, Thailand. Prior to the inoculation the strain was subcultured ten times in MRS broth containing 0.1 % histidine (Serva, Heidelberg, Germany), 0.1 % tyrosine di-sodium salt (Biochemica, Hessen, Germany) and 0.005 % pyridoxal-5-phosphate (Sigma Aldrich, Buchs, Switzerland) according to the previously described method (**Bover-Cid *et al.*, 1999**) in order to enhance amino acid decarboxylase activity.

Preparation of Milk Samples

Powdered skim milk (Himedia, Mumbai, India) was reconstituted with distilled water (10 %), sterilized at 121 ºC for 5 min according to the manufacturer's specification and analyzed for microorganisms, pH and biogenic amines histamine and tyramine prior to the inoculation. The sub cultured strain was aseptically added to the milk at the rate of 1 % (v:v) and incubated at 37 ºC for 48 hrs. Samples were taken at 6 hrs interval within first 24 hrs and then after 24 hrs interval and analyzed for pH, titratable acidity, colony forming units and biogenic amines, histamine and tyramine. Two replicates were done for the analysis.

Determination of Viable Cell Count

Samples of the sterilized reconstituted skim milk were analyzed for mesophilic bacteria (APHA, 1992). Mesophilic counts were obtained for 10^0 and 10^{-1} dilutions on plate count and MRS agar after 48 hrs of incubation at 37 ºC. Milk samples were serially diluted by 10 fold in 0.5% sterile peptone water. Serial dilutions (sample volume of 0.1 mL) were plated on MRS agar (Himedia, Mumbai, India) and inverted plates were incubated at 37 ºC for 48 hrs. Petridishes with 30- 300 separate colonies with white, smooth appearance were selected for the enumeration and number of colony forming units was recorded per mL of sample.

Determination of pH

The fermented milk samples with L. *lactic* subsp. *lactis* were tested for pH. The pH was measured using an electronic pH meter (Model Jenway 3310, Stone, Staffordshire ST15, OSA, UK). The pH meter was calibrated using standard buffer solutions (Merck, Darmstadt, Germany) of pH 4.0 and 7.0 prior to the analysis.

Screening for Proteolytic Activity

Proteolytic activity of the selected strain was qualitatively screened in MRS agar plates supplemented with 10% skim milk. Medium plates were bored with sterile cork borer. Strains (500 µl) were inoculated into the holes and incubated at 37 ºC for 4 days. Proteolytic activity was recognized by the clear halo around the colonies.

Determination of Biogenic Amines, Histamine and Tyramine

Biogenic amines in milk samples were extracted according to the previously described method of **Santos *et al*., (2003).** The filtered supernatants were stored at -20 ºC until BA analysis. The amines were separated and quantified by HPLC, following the procedure optimized in this laboratory (**Priyadarshani *et al.*, 2011**) with a little modification, using similar equipment and chromatographic conditions. The pre-column derivatization of acid extract was done similarly to the conditions described by **Priyadarshani *et al.*, (2011)**, but with addition of 200 µl of NaOH instead of 20 µl. The identification of amines was performed by comparison of retention times of amines in samples to standard solutions spiked to milk. Quantification of histamine and tyramine was done by using external calibration lines prepared with recovery data obtained by spiking known amounts (0.5 – 200 mgL^{-1}) of standard histamine and tyramine to the milk samples followed by extraction and HPLC analysis. The linear regression equations between recovered BA and peak areas are y= 460333+69183x and y=303311+46289x for histamine and tyramine respectively with corresponding correlation coefficient of 0.9929 and 9948. All the samples and replicates were injected at least in duplicate to the HPLC column. The quantity of each amine was expressed in mgL^{-1} milk.

Statistical Analysis

Statistically significant differences were evaluated by one-way analysis of variance with Fisher's LSD test at a 95 % significance level using Minitab (version 14) statistical software.

RESULTS

Screening for Lactic Acid Bacteria (LAB)

The MRS medium was used to isolate the lactic acid bacteria from fresh turmeric rhizome. The LAB was selected from the colonies on MRS plus bromocresol purple agar. The colour of the media changed to yellow indicating the acid production. The isolates were found Gram positive and catalase negative (data not shown). The isolates were further specifically identified at species level by API 50 CHL media. A strain of *Lactococcus lactis* subsp. *lactis* was used for further tests.

Probiotic Characterization

Probiotic characterization was determined by acid and bile salt tolerance, hydrophobicity, and antimicrobial activity. The LAB strain, *Lactococcus lactis* subsp. *lactis* isolated from fresh turmeric rhizomes showed important probiotic characteristics as shown in the Table 1.

Table 1 Probiotic characteristics of *L. lactis* subsp. *lactis* isolated from fresh turmeric rhizomes

Probiotic characteristic	Experimental results
Acid tolerance (% Survival rate)*	95.00 ±1.13
Bile tolerance (% Survival)*	90.56 ±2.83
Hydrophobicity (%)**	58.87
Antimicrobial activity against indicator bacteria	
Escherichia coli	+++
Salmonella spp.	+++
Staphylococcus aureus	+++

* Values are expressed as mean ± standard deviation
** The cell surface hydophobicity (%) was calculated according to the **Gusils *et.al.* 2002** using H (%) = $((A_o - A)/ A_o) * 100$, where *Ao* = Optical density of cell suspension; *A* = Optical density of lower aqueous phase.
+++, inhibition zone of more than 10 mm

Characteristics of Sterilized Reconstituted Skim Milk

Microbial counts on sterile skim milk are shown in Table 2. The initial pH of the milk samples were varied in between 6.6 to 6.8. The variation could be expected due to the variation of pH in the distilled water used to prepare milk samples and different lots of powdered milk used in the same brand. No histamine or tyramine was detected in the sterilized reconstituted skim milk samples.

Table 2 Colony forming units of mesophilic bacteria in sterilized skim milk samples

Milk Sample	Colony forming Units per mL			
	Total Plate Count Agar		MRS agar	
	10^0	10^{-1}	10^0	10^{-1}
1	0.5	0	0	0
2	0	0	0	0
3	0	0	0	0

Values are mean value for duplicate analysis

Growth Characteristics of *Lactococcus lactis* subsp. *lactis* in Skim Milk

The growth study showed that the probiotic *Lactococcus lactis* subsp. *lactis* grow well in skim milk. Growth of the strain is shown in Figure 1A. The initial viable cell count was 7.15 log cfu mL^{-1}. The strain attained maximum viable cell number of 8.35 log cfu mL^{-1} after 48 hrs of incubation. The pH of the skim milk inoculated with *L. lactis* subsp. *lactis* decreased from 6.8 initially to 4.06 after 48 hrs of incubation (Figure 1B).

The analysis of proteolytic activity on skim milk agar plates revealed that tested strain of *L. lactis* subsp. *lactis* did not show clear proteinase activity as clear hallo around the colonies after 4 days of incubation was not observed (Figure 2).

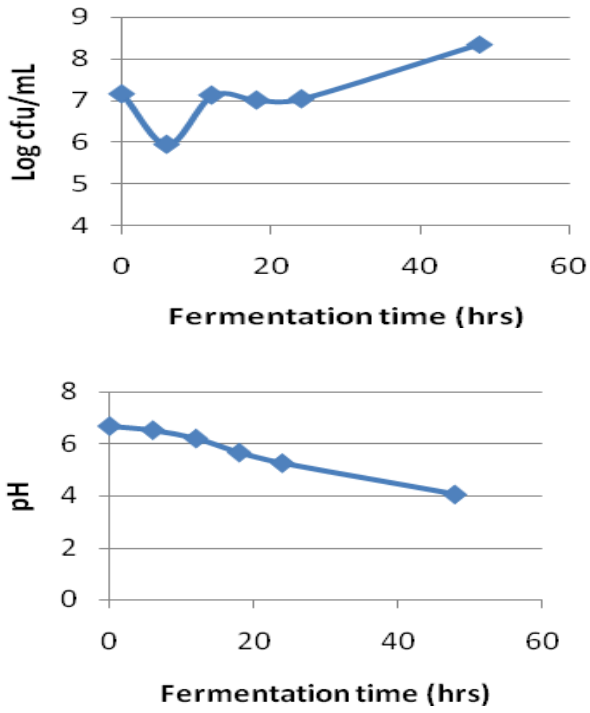

Figure 1 (A-B): A: Total colony forming units Log CFU mL^{-1}; B: pH of reconstituted and sterilized skim milk fermented with *Lactococcus lactis* subsp. *Lactis,* isolated from turmeric rhizomes. Results are the average of the duplicate treatments

Biogenic Amine (Histamine and Tyramine) Formation by *L. lactis* subsp. *lactis* in Skim Milk

Quantification was done by exploration of the standard calibration curves prepared from peak areas obtained by recovered amines spiked to skim milk. Calibration lines for both histamine and tyramine were constructed separately by plotting peak height vs. amount of amine. Linear regression analysis was performed for obtained data. The results of regression analysis were shown in the Table 3. The correlation coefficient for both histamine and tyramine were found to be 0.99. Therefore it is revealed that there is a linear relationship between amount of amine and detector response. This further indicated that the method applied for derivatization and HPLC analysis in this study was satisfactory. Potential to produce histamine and tyramine in milk during fermentation was not observed for the probiotic *Lactococcus lactis* subsp. *lactis* (Figure 3) as none of the amines were detected.

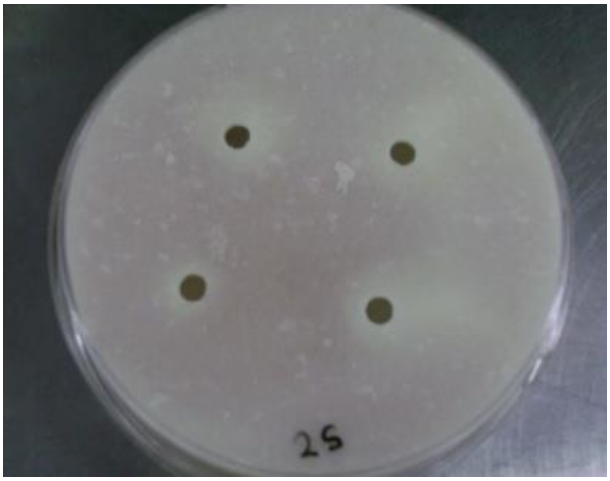

Figure 2 Proteolytic activity of strain *Lactococcus lactis subsp. Lactis* isolated from fresh turmeric rhizomes after 4 days incubation at 37 °C on skim milk

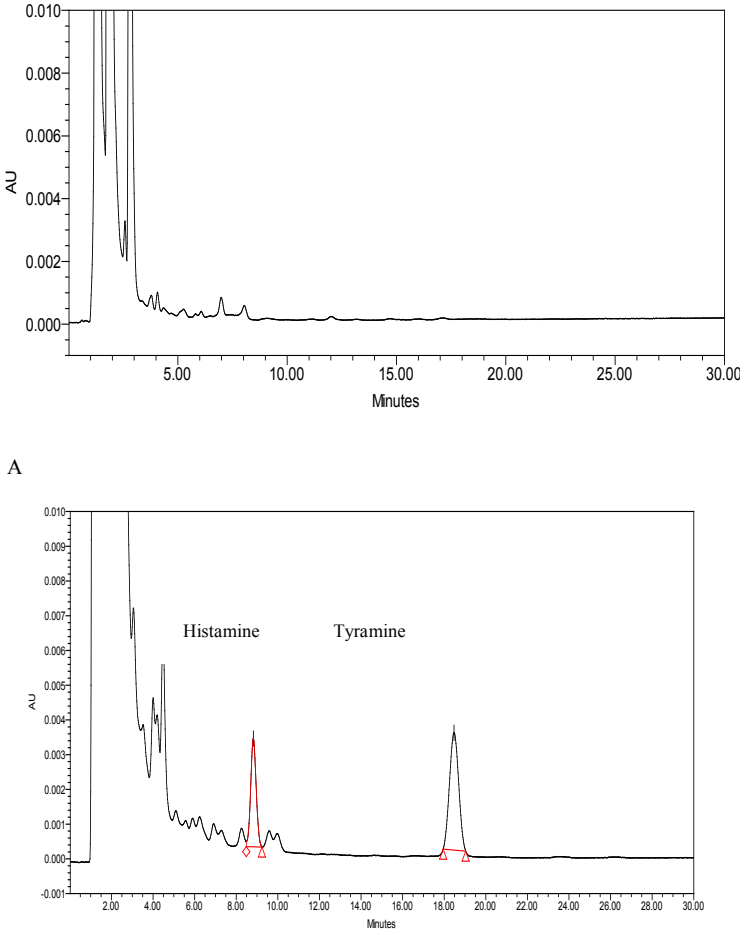

Figure 3 Representative HPLC chromatograms of skim milk A: inoculated with *Lactococcus lactis* subsp. *lactis* isolated from fresh turmeric rhizomes after 48 hrs of incubation at 37 °C. B: spiked with authentic biogenic amines, histamine and tyramine 5 mgL^{-1}

DISCUSSION

A non-dairy based *Lactococcus lactis* subsp. *lactis* was isolated from fresh turmeric rhizomes using MRS medium. Isolate was found Gram-positive and catalase negative. The strain was specifically identified by API 50 CHL media at species level. The probiotic isolate was selected based on acid tolerance, bile salt tolerance, hydrophobicity and antimicrobial activity. The ability to resist low pH is an important selection criterion for probiotic microorganisms as acidic gastric juice in stomach destroys most of the ingested microorganisms. As reported by **Pianpumepong *et al.*, (2010)**, *L. lactis* subsp. *lactis* was able to tolerate 2 h of acid exposure at pH 3 and with good survival rate at 0.3 % bile salt. The ability of LAB to survive the passage through acidic media is variable and strain dependant. The survival rate of approximately 85 % is considered to be very

significant for the probiotic field (**Fernádez *et al*., 2003; Pennacchia *et al*., 2004**). The isolated strain showed >90 % of survival rate at both low pH and bile salt.

Adhesion to intestinal surfaces is an important property of probiotic bacteria. The hydrophobicity test was used to demonstrate adhesion capacity of the culture to the intestinal epithelium of the host. Several studies revealed the possibility of *Lactococcus* strains to be present in the human or animal gastrointestinal tract (**Gruzza *et al*., 1992; Klijn *et al*., 1995**). According to **Kimoto *et al*., (1999)** highest adhesion rate is observed with *Lactococcus lactis* subsp. *lactis* NIAI527. The turmeric derived *L. lactis* subsp. *lactis* studied in present study showed >50 % adhesive capacity.

The isolate was found to produce strong inhibition zones (zone of inhibition of more than 10 mm) against three indicator organisms. The high antagonistic activity was demonstrated against *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella* spp. which are considered to be disease causing agents in the lower digestive tract in human. Similar to this study **Enan *et al*., (2013)** shows the antibacterial activity of *Lactococcus lactis* subsp. *lactis* Z_{11} isolated from Zabady (Arabian yoghurt) against *Listeria monocytogenes*, *Bacillus cereus* and *Staphylococcus aureus.*

The growth and survival of probiotic bacteria in milk and milk products are affected by various factors. Some of these are: acid and hydrogen peroxide formation by yoghurt starter; availability and content of oxygen in milk, package, and temperature. (**Bolduca *et al*., 2006; Shah, 2000a**). Moreover their growth and survival was found to be affected by milk composition (chemical and microbiological), amount of milk solids and nutrient availability (**Shah, 2000b**). Among probiotics *Lactococci* have numerous nutritional requirements for growth, especially nitrogen sources (**Law *et al*., 1976**) and amino acid requirement reported to be strain dependent (**Chopin, 1993**). They utilize peptides or proteins as nitrogen sources through proteolytic enzyme activity (**Leitão *et al*., 2000**). The content of these compounds are low in raw milk. Hence presence of proteolytic system is an important aspect for probiotic lactic acid bacteria which are used in food fermentation (**Kojic *et al*. 1991**). The pH reduction during incubation enhances the proteolysis to liberate short peptides and free amino acids. Presence of proteinase enzymes then enhances the growth in milk. The present study revealed the proteinase negative (Prt^-) characteristics as milk protein degradation was not observed on the skim milk agar plates. Sharp reduction in pH during first 24 h of incubation was not observed in *L. lactis* subsp. *lactis* in milk when compared to other strains studied (data not shown). Similarly to this study **Durlu-Ozkaya, (2001)** reports slow acidifying activity of *L. lactis* subsp. l*actis* strains in milk at 30 ^oC. In this study, highest viable count of 8.35 log cfu mL- with concomitant low pH of the medium was observed at the 48 h of incubation.

Many factors are needed to be fulfilled in order to accumulate BA in milk. The factors of concern are: availability of precursor amino acids; presence of decarboxylase positive microorganisms and availability of proper conditions for growth and decarboxylation (**ten Brink *et al*., 1990; Russo *et al*., 2010**). Various factors are related to such conditions in milk such as milk treatments, use of starter cultures and enzymes, the duration and the temperature of fermentation, the level of proteolysis, the pH, the NaCl concentration, the presence of oxygen, the activity of water and relative humidity, the bacterial density and water activity and synergetic effects (**Gardini *et al.,* 2001**). The main producers of BA in milk and milk products are LAB including *Enterococcus*, *Lactobacillus*, *Leuconostoc*, *Lactococcus* and *Streptococcus* (**Bonetta *et al*., 2008; Calles-Enríquez *et al*., 2010; Linares *et.al*., 2012**). However, biogenic amine production by the *Lactococcus* strains has not been much documented.

de Llano *et al*., (1998) has reported high level of tyramine production by wild dairy *L. lactis* subsp. *lactis* in decarboxylase broth supplemented with amino acid precursors. But no tyramine production was detected in cultures of these strains grown in milk. However, evolution of tyramine production is observed in skim milk supplemented with tyrosine after 18 h of incubation. In contrast, **Durlu-Ozkaya *et al*., (2001)** report decarboxylase negative strains of *L. lactis* subsp. *lactis* isolated from Beyaz cheese made from raw ewes' milk. In the present study no histamine or tyramine production was detected in milk cultured with L. *lactis* subsp. *lactis* strain isolated from turmeric throughout the 48 h of incubation time.

CONCLUSION

This study showed that *Lactococcus lactis* subsp. *lactis* isolated from fresh turmeric rhizomes carries potential probiotic characteristics and does not produce histamine and tyramine in fermented milk. This suggests *Lactococcus lactis* subsp. *lactis* isolated from turmeric as a potential non dairy based functional starter. However results of BA formation by the same strain in laboratory media will not imply the similar behavior in complex food matrixes. This makes the situation more complex and implies that tests on the probiotics for amine production in the food matrix into which it is planned to be applied should be done for positive BA producers. BA production capability should be an important criterion for choice of probiotic and starter cultures and only those strains not producing BA should be used as probiotics or functional starter cultures.

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WASTE BIODEGRADATION AND UTILIZATION BY *PSEUDOMONAS* SPECIES

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ABSTRACT

Waste utilization is the global problem of modern society. Waste products display negative influence on environment and human health for a long time, consequently different methods have been developed for waste disposal. They include application of physical, chemical and biological degradation. Special interest is induced by using microbial cultures such as *Pseudomonas*. The article considers *Pseudomonas* genus as the effective key to the solution of relevant problem lifting adverse effects of toxic by-products. The studies revealed that pseudomonades due to metabolic and physiological diversity are able to degrade a wide range of compounds hazardous to living organisms. Indeed, during biodegradation bacteria produce secondary metabolites that can be used in medicine, industry, agriculture and bioremediation. Waste serves in this case as a cheap source of carbon and nitrogen. The genus possesses vast potential in both tackling contamination problems and production of valuable compounds for numerous applications.

Keywords: *Pseudomonas*; waste; biodegradation; utilization; biosynthesis

INTRODUCTION

Waste management is a worldwide challenge urging effective solution strategy. Waste can cause deleterious impact on human health and condition of environment. About 85% of various wastes are produced in USA, Europe and Oceania. These materials are discharged in amount 1.6 kg per capita in USA, 1.5 kg in Europe, 0.8 kg in Oceania and 0.4 kg in Asia **(Williams, 2005)**. There are several approaches to solve the problem, including recycling and utilization. Recycling can occur by different ways via physical, chemical and biological degradation.

On the other hand, genus *Pseudomonas* is known for its ability to utilize a large variety of compounds and produce a wide range of secondary metabolites and biopolymers owing to metabolic and physiological diversity. Using industrial, agricultural and household wastes as media for biodegradation or production of various compounds is becoming more attractive. Several studies indicated favorable application prospects of this genus.

METABOLIC PROCESSES IN *PSEUDOMONAS*

Catabolic pathways of *Pseudomonas*

Pseudomonas species include bacteria inhabiting various ecological niches. Pseudomonades are able to live in both simple and complex media utilizing many compounds as substrates owing to the nutritional versatility. The variety is provided by diverse metabolic pathways observed in these bacteria. These biochemical routes allow to degrade a wide spectrum of substances, including toxic ones to other prokaryotes and to higher organisms. The breakdown of various compounds makes *Pseudomonas* most appropriate in biodegradation of waste, especially persistent components.

Petroleum and its fractions consist of several classes of compounds (mainly hydrocarbons): saturates, aromatics, other organic chemicals containing nitrogen, oxygen, sulfur, and traces of metals. The saturates include normal alkanes, branched alkanes, and cycloalkanes called paraffins, isoparaffins, and naphthenes, respectively. Normal and branched alkanes have the general formula C_nH_{2n+2}, with n usually ranging from 1 to 40. Cycloalkanes are distinguished by one or more carbon rings to which hydrogen atoms are attached according to the formula C_nH_{2n} while aromatic compounds contain one or more aromatic rings. These are major constituents of petroleum **(National Research Council, 1985; Robbins and Hsu, 2000)**. Hydrocarbon components may turn out to be carcinogens or organic pollutants, hence bioremediation is considered as effective solution of the problem.

Alkane utilization via oxidation to l-alkanols (terminal oxidation) has been described for *Pseudomonas*. Enzymatic system involved in oxidation consists of three components: a particulate hydroxylase (EC 1.14.15.3) and two soluble proteins, rubredoxin and rubredoxin reductase (EC 1.18.1.1), mediating electron transfer between NADH and the hydroxylase. The product of the alkane hydroxylase reaction further acts as substrate for alcohol- (EC 1.1.1.1) and aldehyde dehydrogenases (EC 1.2.1.5) converting l-alkanol to fatty acids. They are degraded by β-oxidation to acetyl-CoA, which enters the central metabolism, or poly-(3-hydroxyalkanoate) generated under nitrogen or phosphorous limitation. Alternative pathway for alkane utilization (subterminal oxidation) produces secondary alcohol that can be converted into ketone. The latter is oxidized to ester which in turn is hydrolyzed by esterase (EC 3.1.2.2) to an alcohol and fatty acid **(Ramos, 2004)**.

Branched alkanes are more recalcitrant to degradation than linear because of methyl branching obstructing access of enzymes. However, some investigations in this direction were carried out. Mutants of *P. citronellolis* were able to grow on 2,6-dimethyloctane via a combination of alkane and citronellol degradative pathways **(Fall *et al.*, 1979)**. 2-methylhexane maybe be decomposed to 5-methylhexanoic and iso-valeric acid accompanied by the second pathway via 2-methylhexanoic acid **(Thijsse and Van der Linden, 1961)**.

Degradation of cyclic alkanes also has been studied. The formation of valeric acid, formic acid, cyclohexanol and hexanedioic acid from cyclohexane by *Pseudomonas* was reported **(Imelik, 1948)**. Other researchers showed that hydroxylase system of microbial cells transformed cycloalkanes into cycloalkanols as the reaction products **(van Beilen *et al.*, 1994; van Ravenswaay Claasen and Van der Linden, 1971)**. Cyclohexanol was further degraded via cyclohexanone and ε-caprolactone to adipic acid **(Anderson *et al.*, 1980)**.

Pseudomonas strains are able to degrade a number of aromatic compounds from various sources, including petroleum and dyes. So far 11 central catabolic pathways with many different peripheral pathways have been revealed. By-paths split substrates into a few intermediates (usually dihydroxybenzenes or dihydroxyaromatic acids), which are then ring-cleaved and converted to tricarboxylic acid cycle intermediates through the corresponding central pathways **(Timmis, 2010)**. There are many studies related to aromatic catabolic pathways of *Pseudomonas*, so we'll concentrate on degradation of caffeine and nicotine.

Several biochemical routes of nicotine utilization by microorganisms were detected: pyridine, pyrrolidine and demethylation pathways. The attack of

nicotine molecule at the pyrrolidine ring is typical for *Pseudomonas* genus **(Brandsch, 2006)**. However, this pathway varies in different strains. Nicotine degradation by *P. putida* S16 starts via oxidation with the formation of methylmyosmine, which, upon addition of water, spontaneously leads to ring opening and formation of pseudooxynicotine. Subsequent steps are the formation of 3-succinoylpyridine (SP) after removal of methylamine and hydroxylation of the pyridine ring at position 6 with generation of 6-hydroxy-3-succinoylpyridine (HSP) followed by an oxidative decarboxylation to 2,5-dihydroxypyridine (DHP) and succinate. DHP is further degraded into lactic and 3-hydroxybutyric acids **(Brandsch, 2006; Wang *et al*., 2007)**. Formation of HSP from nicotine by *Pseudomonas* sp. HZN6 is controlled by the similar mechanism, however, pseudooxynicotine at first is transformed into 3-succinoylsemialdehyde-pyridine and then into SP **(Qiu *et al*., 2012)**. *Pseudomonas* sp. CS possesses both pyrrolidine and demethylation pathways. The strain is able to provide direct demethylation to form 3-(3,4-dihydro-2*H*-pyrrol-5-yl) pyridine, and hydroxylation at position 2 of pyrrolidine ring to yield 1-methyl-5-(3-pyridyl) pyrrolidine-2-ol, later converted to cotinine **(Wang *et al*., 2012)**. *Pseudomonas* sp. Nic22 generates a wide spectrum of intermediates, including hazardous compounds such as myosmine (3-(4,5-dihydro-3H-pyrrol-2- yl)pyridine), 2,3'-dipyridyl and cotinine, further subject to complete degradation **(Chen *et al*., 2008)**. *P. geniculata* N1 can decompose nicotine with formation of 6-hydroxynicotine, 6-hydroxy-*N*-methylmyosmine, 6-hydroxy-pseudooxynicotine, 2,6-dihydroxypseudooxynicotine, myosmine and cotinine **(Liu *et al*., 2014)**. Another nicotine degradation pathway was established for *P. plecoglossicida* TND35. The strain provided for the oxidation of the pyrrolidine ring to *N*-methylmyosmine. This intermediate was further hydroxylated at 2nd position of pyrrolidine ring to form cotinine analogue, 2,3-dihydro-1-methyl-5-(pyridin-3-yl)-1*H*-pyrrol-2-ol. The metabolite can be demethylated and oxidized to another cotinine analogue, 5-(pyridin-3-yl)-1*H*-pyrrol-2(3*H*)-one, or oxidized and released methylamine, that leads to the pyrrolidine ring opening to form an end product 4-hydroxy-1-(3-pyridyl)-1-butanone. Additionally, the strain is able to disrupt bond between pyridine and pyrrolidine ring of the nicotine molecule, forming 3,5-bis(1-methylpyrrolidin-2-yl) **(Raman *et al*., 2014)**. Thus the transformation of nicotine by *Pseudomonas* can proceed along different metabolic pathways with formation of a wide range of compounds, hence new variations of nicotine degradation can be expected.

Bacteria utilize caffeine via two catabolic pathways: *N*-demethylation and C-8 oxidation. The N-demethylation pathway is the most common, detected in over 80% of reported isolates, including *Pseudomonas*. During degradation each of the three methyl groups of caffeine (1,3,7-trimethylxanthine) was removed in series, molecular oxygen was incorporated and formaldehyde and water molecule were produced. At the first step theobromine (3,7-dimethylxanthine) is mainly formed with small amounts of paraxanthine (1,7-dimethylxanthine) for some strains. Later theobromine or paraxanthine are *N*-demethylated to form 7-methylxanthine converted into xanthine. Then uric acid produced from xanthine enters normal purine catabolic pathway **(Summers *et al*., 2015)**. Additionally, *P. putida* CBB5 was reported to degrade theophylline (1,3-dimethylxanthine), 3-methylxanthine and 1-methylxanthine (products of other variant of *N*-demethylation pathway) to xanthine. The strain also oxidized these compounds to 1,3-dimethyluric, 1- and 3-methyluric acids – terminal metabolites of CBB5 **(Yu *et al*., 2009)**.

Plastics are mainly represented by organic polymers of high molecular weight. They are generally not biodegradable and arouse pollution problem. Plastics are utilized in the course of several stages: biodeterioration, depolymerisation, assimilation and mineralization. During biodeterioration bacteria split materials into tiny fractions. Depolymerisation is cleavage of polymeric molecules into oligomers, dimers and monomers. Assimilation is integration of intermediates into microbial metabolism and finally mineralization releases simple molecules, like CO_2, N_2, CH_4, H_2O and different salts in the environment. The biodegradation process can stop at each stage **(Lucas *et al*., 2008)**. The investigation concerning plastic decay is under way and some examples are mentioned below in other chapters. However, there is a lot of work for researchers, especially concerning utilization of resistant polymers. Some examples of plastic degradation are mentioned below.

On the other hand, degradation of natural polymers such as chitin is a more studied topic. Chitin is widespread in nature as a structural element in many organisms, e.g., fungi, crustaceans, insects or algae. This compound consists of (1→4)-β-linked N-acetyl-D-glucosamine. The single sugar units are rotated 180° to each other, with the disaccharide N,N'-diacetylchitobiose as the structural subunit. Chitin is classified into three different crystalline forms: α-, β-, and γ, differing in the orientation of micro-fibrils. Chitin usually is associated with other adjacent structural polymers such as proteins or glucans, which often contribute more than 50% of the total chitin-containing tissue. The polymer can be degraded via two pathways: the (1→4)-β-glycoside bond is exposed to hydrolysis or the compound is deacetylated to chitosan or possibly even cellulose-like forms after additional deamination. Further intermediates can be reintegrated into cell material or mineralized and removed from the system **(Beier and Bertilsson, 2014)**. Research showed that *Pseudomonas* is able to convert chitin into chitosan **(Ghorbell-Bellaaj *et al*., 2012)**.

Thus genus *Pseudomonas* possesses various metabolic pathways. They engage numerous compounds as sources of valuable nutrients (e.g. essential amino acids from keratin) **(Chaturvedi and Verma, 2014)** and even degrade toxic chemicals, like free cyanide and thiocyanate by hydrolytic mechanism, resulting in accumulation of ammonium in the medium **(Mekuto *et al*., 2016)**. Metabolic diversity provides vast opportunities for *Pseudomonas* application in waste disposal.

Biosynthesis of biosurfactants and polyhydroxyalkanoates

Pseudomonas is able either to degrade waste and its components or utilize it for biosynthesis of valuable products. The genus produces a wide range of substances, however, many studies related to waste disposal deal with biosurfactants (mainly rhamnolipids) and polyhydroxyalkanoates (PHAs). The corresponding biosynthetic pathways were thoroughly investigated for *Pseudomonas* genus.

Biosurfactants are the surface-active agents capable of reducing surface and interfacial tension between liquids, solids and gases and they find use in cosmetic, food, petroleum industries, medicine and bioremediation. These compounds are non-toxic and biodegradable in contrast to chemical analogs **(Chamy and Rosenkranz, 2013)**. Biosurfactants are usually glycolipids, composed of carbohydrates in combination with long-chain aliphatic acids or hydroxyaliphatic acids. Formation of mono and di-rhamnolipids is mediated by rhamnosyltransferase I and II (EC 2.4.1.-), respectively. Two quorum sensing systems (QSS) are involved in regulatory mechanisms of rhamnolipid synthesis. In the first QSS genes *rhlA* and *rhlB* encoding rhamnosyltransferase I are positively regulated by rhlR (transcriptional activator). The latter and autoinducer are encoded by *rhlR* and *rhlI*, respectively. Two signal molecules, N-butanoyl-L-homoserine (PAI-2) and hexanoyl-L-homoserine lactone are produced by *rhlI*. Transcriptional activator binds to autoinducer PAI-2 and this complex causes activation of *rhlA* and *rhlB*. The second QSS containing two genes (*lasR* and *lasI*) positively regulates the transcription of *rhlR* system. Rhamnosyltransferase I catalyses the synthesis of mono-rhamnolipid from deoxythymidine-diphospho-L-rhamnose (dTDP-L-rhamnose) and β-hydroxydecanoyl-β-hydroxydecanoate. Di-rhamnolipid is produced from mono-rhamnolipid and dTDP-L-rhamnose **(Satpute *et al*., 2010)**.

PHAs, like biosurfactants are characterized by low toxicity and biodegradability and can be used in the packaging, pharmaceutical, food industries, agriculture and medicine. PHAs are composed of medium-chain length (*R*)-3-hydroxyfatty acids (6–14 carbon atoms). There are several metabolic pathways of biosynthesis depending on the carbon source. If cells utilize fatty acids, the latter are converted to (*R*)-3-hydroxyacyl-CoA via β-oxidation pathway. Then PHA synthase (EC 2.3.1.B3) catalyzes the enantioselective polymerization of (*R*)-3-hydroxyacyl-CoA to PHA. If the carbon source is oxidized to acetyl-CoA, the intermediates of fatty acid biosynthesis are formed *de novo* **(Ramos, 2004; Rehm, 2008)**.

APPLICATIONS OF *PSEUDOMONAS* IN WASTE BIODEGRADATION

Plastics are the common compounds replacing traditional materials in diverse fields. Most plastic products are resistant to biodegradation and can display toxic properties threatening the environment and human health. However, the same synthetic polymers may be degraded by bacteria including pseudomonades. An example is polyvinyl alcohol (PVA) that is widely used in adhesive, paper-coating and textile industries and detected in wastewater **(Chen *et al*., 2007, Chiellini *et al*., 2006)**. Experiments revealed that *P. alcaligenes* can reduce the pollution caused by PVA-based materials. However, elevated rate of degradation was achieved when PVA concentration was low, pH was alkaline, the inoculum size was large and minimum flow rate was maintained. Nevertheless, the strain demonstrated effective degradation of PVA **(Bharathiraja *et al*., 2013)**. Decomposing potential of different *Pseudomonas* strains was compared in experiments with low density polyethylene (LDPE) used widely as packaging material. After 120 days of incubation, the extent of weight reduction was 20% for *P. aeruginosa* (PAO1), 11% for *P. aeruginosa* (ATCC), 9% for *P. putida*, 11.3% for *P. syringae* strains, 0.3% for the negative control. Maximum fall in tensile strength and carbonyl index was reached by PAO1. The degradation products comprise long chain fatty acids, esters, hydrocarbons, oxygenated chemical compounds predominantly containing aldehydes, ketones, ester and ether groups, unsaturated fatty acids, etc. **(Kyaw *et al*., 2012)**. Other reports are focused on biodegradation of resistant polymers. Some bacterial strains are able to decompose these compounds. *P. aeruginosa* strain HE858284 was confirmed to partially degrade the recalcitrant nylon-6 polymer to 6-aminohexanoic acid, ε-caprolactam and some unidentified oligomers **(Sanuth *et al*., 2015)**.

Biodegradation of caffeine is also a possible application for *Pseudomonas*. Caffeine is used in food industry and medicine, however, its production yields annually nearly two million tons of residues in the form of pulp, husk, spent-grounds and residual water. These wastes are rich in carbohydrates, proteins and minerals, but also contain toxic compounds such as tannins, polyphenols, and caffeine that can be toxic in elevated concentrations to saprophytic organisms involved in biotransformation processes in the environment. Nevertheless, several microorganisms, including *Pseudomonas* strains are able to utilize caffeine. *Pseudomonas* sp. NCIM 5235 can completely degrade 1.2 g/L and 5 g/L

of caffeine in 15 and 48 h, respectively. The isolate also broke down 59.9 and 21.5% of caffeine in 96 h when initial concentration of caffeine in the medium was 7.5 and 10 g/L, respectively. Decomposition of caffeine occurred via demethylation. By 24 h both 3,7-dimethylxanthine at concentration 0.28 g/L and 7-methylxanthine at concentration 0.37 g/L were formed when initial concentration of caffeine was 5 g/L. By 48 h the level of 3,7-dimethylxanthine decreased to concentration 0.15 g/L and the amount of 7-methylxanthine increased to concentration 0.78 g/L, with formation of xanthine at concentration 0.32 g/L. By 84 h all metabolites were completely utilized **(Dash and Gummadi, 2010)**.

Wastes containing high concentrations of water-soluble nicotine have been classified as toxic and hazardous for human health and the environment. Biodegradation is a viable method for nicotine removal. Microorganisms able to degrade nicotine include *Pseudomonas* species. For example, *Pseudomonas* sp. Nic22 is the isolate with higher capacity for degradation than other studied 17 strains. Compared to the pure nicotine, this strain could decompose the substance in tobacco extract with less intermediates detected **(Chen *et al*., 2008)**. *Pseudomonas* sp. ZUTSKD was able to grow and degrade nicotine at substrate concentrations 2–5.8 g/L. At 30°C and pH 7.0, with additional 15 g/L $Na_2HPO_4{\times}6H_2O$ and 6 g/L KH_2PO_4 in 5% TWE (tobacco waste extract), *Pseudomonas* sp. ZUTSKD could utilize 97% of nicotine (1.6 g/L) in 12 h. The strain degraded nicotine completely when the reducing sugar concentration in TWE was lower than 8 g/L. Yeast extract and phosphate additions improved nicotine degradation in 5% TWE **(Zhong *et al*., 2010)**. However, biodegradation of the tobacco nicotine is carried out by diverse biochemical pathways. Some metabolic intermediates may be harmful for health or environment, so it is essential to further study the conversion products before transferring the biotechnology to industry.

Toner used in copiers and printers releases significant quantities of waste. Toner powders consist of very small thermoplastic particles, usually styrene-acrylate copolymer. Black toners contain carbon black or iron oxides as pigments, color toners additionally include various organic pigments. Such additives as wax, silica and minor mineral amounts also can be components of the powder. It seems natural therefore, that these wastes are considered to be toxic to human health and the environment. However, certain bacteria, including *Pseudomonas* species can solve this problem. It was stated that some strains inoculated in minimal salt media were able to grow successfully in the presence of toner powder. Among various tested combinations, mix of 2g powder and 10g soil provided for the best bacterial growth **(Sepperumal *et al*., 2014)**.

1-Propanethiol (PT) is an important mercaptan primarily resulting from anaerobic decomposition of proteins and it appears to be severely toxic to humans. *P. putida* S-1 isolated from activated sludge is able to degrade this compound. Addition of PT in the preincubation medium could enhance PT degradation. Cells pre-grown in media containing mercaptan degraded 28 mg/L of PT within 10 h in contrast to cells pre-grown in other media (the least time 15 h). *P. putida* S-1 cultivated in stirred tank reactor removed 86% of PT with inlet concentration of approximately 100 mg/m^3 after 12 h. Disposal efficiency increased to almost 100% under the same conditions by 24 h. Degradation exceeded 98% at concentration 200 mg/m^3 and decreased at concentration 400 mg/m^3, recovering with time. Additionally, gaseous isopropanol at 200 mg/m^3 level could be effectively removed in the reactor when mixed with the stream of PT, reaching elimination degree of 88%. Dipropyl disulfide, 3-hexanone, 2-hexanone, 3-hexanol, 2-hexanol, and propanethiol S-oxide were identified as the major intermediates and CO_2, SO_4^{2-}, and biomass were the final products of PT degradation **(Chen *et al*., 2016)**.

Carbazole is carcinogenic and mutagenic heterocyclic compound found in the waste of various pharmaceutical, petrochemical and dye industries. Several bacteria including *Pseudomonas* have been reported to utilize carbazole. *Pseudomonas* sp. GBS.5 cells immobilized in polyvinyl alcohol-alginate attained the maximum carbazole degradation rate (52.62 ppm/h) in contrast to cells immobilized in calcium alginate beads (39.06 ppm/h) **(Bhatnagar *et al*., 2016)**.

Another experiment proved that phenol can be used as the sole carbon and energy source for *Pseudomonas* spp. ETL 2412. Phenol in water and effluents is the major organic chemical associated with various applications. However, this compound is hazardous to human health. The bacterium decomposed phenol to 76.43±1.23 mg/L concentration from an initial value 200 mg/L. Phenol removal efficiency changed with cultural conditions. The optimal parameters for phenol decay were found at pH 7.5 (82.63%), temperature 30°C (78.69%) and 0.25% supplemented glucose level (98.28%) **(Shah *et al*., 2014)**.

Mineral oil released into the environment is able to affect health of many living organisms. Moreover, oil contamination causes detrimental effect on soil properties such as fertility, permeability, water-holding and binding capacity. Biodegradation is a promising method to eliminate oil spills by microorganisms capable to utilize toxic contaminants as a source of carbon and energy. One experiment revealed that isolates of *P. fluorescens* degraded 68.5% of waste lubricating oil in a week. However, *Acinetobacter* sp. was more effective than *Pseudomonas* (71.9% of the oil after 7 days) **(Umar *et al*., 2013)**. The other experiment showed ability of *Pseudomonas* sp. to degrade motor oil. 70.61% of oil pollution was gone following 6 days of bacterial inoculation. However, oil is composed only of hydrocarbons, while the bacteria require extra nutrients for fast degradation rate. Addition of glycerol increases decomposition extent to 85.61%. Inorganic salts promote bioconversion of 93.18% oil. In case of the surfactant 81.82% oil cleavage was recorded on the final day of incubation. Combination of glycerol and inorganic salts resulted in degradation of 96.97% oil. It was found to be the most effective treatment intensifying oil break-down **(Sathiya-Moorthi *et al*., 2008)**. *P. aeruginosa* DQ8 was able to grow with diesel oil and crude oil as the sole carbon and energy sources, digesting the wide range of their components. 83±1.0% of total petroleum hydrocarbons (TPHs) of diesel oil were degraded and most of the fractions, including C12–C25 n-alkanes were consumed by the strain. 79.3±0.85% of crude oil TPHs were transformed, and more than 60% of those were decomposed in the first 5 d. The strain efficiently utilized various oil fractions. The alkane fraction was distinguished by the fastest degradation rate. The others, such as the aromatic hydrocarbon, nonhydrocarbon and asphaltene fractions were split by 52.3±2.1%, 66.3±5.3% and 46.6±3.4%, respectively **(Zhang *et al*., 2011)**.

The other studies traced ability of some bacteria, including *Pseudomonas* to use chitin or keratin as carbon sources that enhance crude oil removal. Bacterial cultures were grown on media with oil, or oil supplemented with chicken-feathers or shrimp wastes. Keratinolytic bacteria were better enzyme producers than the chitinolytic ones. The presence of organic, mainly keratinous wastes promoted oil hydrocarbon disposal to 90%, detoxifying thereby doses of the pollutant over 58.300 mg/L in a relatively short period. Oil degradation in the presence of chicken-feathers was 3.8 times more intense than with shrimp wastes, and almost twice as active in comparison with oil-only cultures **(Cervantes-González *et al*., 2008)**.

Pseudomonas may cope with cyanide contamination. Significant sources of cyanide pollution are anthropogenic activities such as cyanidation process in the mining industry. It is a highly reactive chemical forming metal-complexes harmful to the environment. Cyanide is mostly removed from industrial effluents by alkaline chlorination, hydrogen peroxide or ozonation. However, these methods produce hazardous end-products. Some bacteria, including *Pseudomonas* are able to decompose cyanide and thiocyanate. They degraded 250 and 450 mg/L CN^-, achieving disposal efficiency of 80 and 32% within 150 h, respectively. Additionally, the SCN^- degradation degree was 78 and 98% for non- and cyanide-spiked cultures **(Mekuto *et al*., 2016)**.

Dye contamination is a critical ecological problem. There are over 100000 commercially marketed dyes, with more than $7{\times}10^7$ tons produced annually worldwide **(Rafi et al., 1990)**. Dyes are widely used in textile, food industries, cosmetics and printing. Huge amounts of dye-saturated textile fabrication effluents pose grave environmental challenges. 10–15% of pigments are wasted in the course of dyeing process **(Zollinger, 1987)**. Dye contamination characterized by toxicity, carcinogenicity and mutagenicity raises dramatic public health concern. Additionally, dyes obstruct light penetration and oxygen transfer in water bodies and may persist in the environment for an extended period of time. Use of physical and chemical methods in contrast to biological treatment of dye-contaminated wastewater is expensive and sometimes produces hazardous by-substances multiplying pollution problems. On the other hand, some microorganisms are capable to cause decolorizing effect. The results showed that in fed-batch process, *P. oleovorans* transformed 50 mg of dye in 192 h. However, when decolorization was performed in the anoxic reactor, bacterial culture fully degraded 25 mg of dye during 24 hours **(Silveira *et al*., 2011)**. *P. aeruginosa* ETL-1 was shown to disrupt three triarylmethane dyes within 24 h. Under shaking conditions, percentages of dye decolorization were 90%, 75%, and 66% for basic violet 14, basic violet 3, and acid blue 90, respectively, whereas poor decolorization (< 30%) was recorded for all three dyes under static conditions. *Triticum aestivum* and *Lens esculenta* were used in dye phytotoxicity studies. The length of shoots, roots, and seedlings was more significantly affected by the tested dyes rather than by their degradation metabolites, indicating less toxic nature of the latter **(Shah *et al*., 2013a)**. Decolorization rates can be changed by varying cultural conditions. The optimum pH and temperature values for depigmentation usually are 7–9 and about 37°C, respectively. Elevated concentrations of dyes and oxygen, addition of heavy metals decelerate bleaching process. Such compounds as glucose, peptone, yeast extract, or starch promote decolorization to a certain extent. Yet increased concentrations of these compounds may cause the interfering action because the bacteria will utilize them instead of the dyes **(Shah *et al*., 2013a,b,c)**. The purified laccase enzyme (EC 1.10.3.2) derived from *P. fluorescens* was shown to decompose congo red with maximum effect upon 96 h (38.09%) **(Vandana and Peter, 2014)**.

USE OF WASTE SUBSTRATES IN PRODUCTION OF VARIOUS COMPOUNDS BY *PSEUDOMONAS*

Wastes in biosurfactant production

Pseudomonas strains can be used in utilization of waste vegetable oils. The food industry and households produce and consume frying oils in large quantities. The experiments revealed bacterial ability to utilize these substrates. Most of the tested *Pseudomonas* cultures showed satisfactory growth on basal medium with 2% or 4% used olive or sunflower oil. Additionally, used olive oil induced biosurfactant production **(Haba *et al*., 2000)**. Residues from corn, babassu,

cottonseed, palm oil refinery and soybean soapstock waste as the best substrate generating 11.7 g/L of rhamnolipids, can be used for biosurfactant production **(Nitschke *et al*., 2005)**. Enhanced degradation of oil-rich soybean wastes (acid oil, deodorizer distillate, soapstock) and increased biosurfactant production in comparison with soybean oil was demonstrated by *P. aeruginosa* MR01. Among these wastes soapstock provided for maximum specific growth rate, higher extent of biodegradation and biosurfactant purity grade **(Partovi *et al*., 2013)**. Similar experiments were carried out with orange peelings, carrot peel waste, lime peelings, coconut oil cake, and banana waste. The orange peel was found to be the best substrate for generation of 9.18 g/L of rhamnolipid biosurfactant reducing surface tension down to 31.3 mN/m **(George & Jayachandran, 2009)**.It was established that biosurfactants can be produced by *Pseudomonas* utilizing distillery and whey wastes **(Babu *et al*., 1996)**, waste motor lubricant oil and peanut oil cake **(Thavasi *et al*., 2011)**, waste frying rice bran oil **(Venkatesh and Vedaraman, 2012)**, waste frying coconut oil **(George & Jayachandran, 2013)**, corn steep liquor and molasses **(Gudiña *et al*., 2013)**, distillery waste with other industrial wastes viz. curd whey waste, fruit processing waste and sugar refining effluent **(Dubey *et al*., 2012)**, waste rapeseed oil **(Możejko and Ciesielski, 2014)**, saponified waste palm oil **(Możejko and Ciesielski, 2013)**. All these wastes show great potential as cheap and high-energy sources in production of valuable compounds by *Pseudomonas* genus.

Various non-traditional oils such as jatropha oil, karanja oil and neem oil may be applied as renewable and low cost substrates for rhamnolipid production. These oils cannot be used for edible purposes either due to a strong disagreeable odor or toxic components. Such exotic oils contain various minor constituents that may affect the metabolic activity of microbes and hence rhamnolipid synthesis. Neem oil contains a number of triterpenoids, mainly azadirachtin (0.4–0.9%) which acts as an insecticide and thioketone as aromatic. Karanja oil contains flavonoids such as karanjin (2.2–4.5%) and pongamol (0.4–0.9%) possessing insect-repelling properties. Jatropha oil contains curcin, lectins and most toxic phorbol esters (0.6%) distinguished by carcinogenic activity. However, experiments revealed that jatropha and karanja oils can be used in biosurfactant production **(Pratap *et al*., 2011)**. Phorbol esters are also components of seed cake generated as by-product in the course of biodiesel process based on *Jatropha* seeds. Phorbol esters are known to display toxic properties, so that seed cake cannot be consumed as cattle feed, unlike other oilseeds. These compounds can cause environmental problems and aren't destroyed even by heating at 160°C for 30 min. Nevertheless, *P. aeruginosa* PseA completely degraded phorbol esters in nine days under optimal conditions **(Joshi *et al*., 2011)**.

Wastes in production of polyhydroxyalkanoates

Such media as glycerol, cassava wastewater (CW), waste cooking oil and CW with waste frying oils can be used as cheap carbon substrates in production of both rhamnolipids and PHAs. The results show that composition of rhamnolipids and PHAs varies with both the carbon source and the applied strain. The best combination is use of strain *P. aeruginosa* L2-1 with mix of CW and waste frying oil as the carbon source **(Costa *et al*., 2009)**. Sugar refinery waste (cane molasses) with urea served as the potent nitrogen source used in production of PHA, like poly-3-hydroxybutyrate (PHB). Under optimal conditions maximum biomass level and PHB production - 7.60 g/L and 5.32 g/L, respectively, were achieved after 54 h of fermentation. Total sugar concentration decreased to 17.8 g/L at the end of production phase in comparison with initial concentration of 40 g/L **(Tripathi *et al*., 2012)**. The experiments showed that glycerol as the by-product of biodiesel manufacturing from kitchen chimney dump lard proved the better source for PHA synthesis than commercial glycerol, sugarcane molasses and glucose. Such glycerol application contributes into cost reduction of biodiesel technology by engaging side product as the main ingredient of nutrient medium **(Phukon *et al*., 2014)**. *Pseudomonas* sp. strain DR2 synthesized PHA from glucose, citrate, glycerol, palmitate, corn oil, and waste vegetable oil. 37.34% and 23.52% PHA levels were attained using corn oil and waste vegetable oil, respectively. Composition of these compounds depended on different substrates. PHA from corn oil consists of three major 3-hydroxyalkanoates: octanoic (C8:0, 37.75% of the total 3-hydroxyalkanoate content), decanoic (C10:0, 36.74%), and dodecanoic (C12:0, 11.36%). The proportion of 3-hydroxyalkanoates in the waste PHA derived from vegetable oil is hexanoic (5.86%), octanoic (45.67%), decanoic (34.88%), tetradecanoic (8.35%), and hexadecanoic (5.24%). It appears that inexpensive vegetable oil and waste can serve as alternative substrates for PHA production **(Song *et al*., 2008)**.

Some reports pointed out that PHAs may be obtained using plastics, which will resolve both problem of waste disposal and synthesis of the end product. Polystyrene can be converted into the desired compound in two steps. The first step involves the pyrolysis of polystyrene to styrene oil at the temperature 520°C. The process results in the generation of oil composed of styrene (82.8%) and low levels of α-methylstyrene, toluene, styrene dimer, and traces of other aromatic compounds. The second step includes bacterial transformation of the styrene oil to PHA by *P. putida* CA-3. Cells grown in shake flask culture consume 1 g of styrene oil to yield 62.5 mg of PHA and 250 mg of bacterial biomass. A low level of PHA accumulation was observed in the first 10 h of growth, followed by a dramatic rise between 16 and 24 h, and a steep fall thereafter. After 48 h of fermentation 1.6 g of PHA and 2.8 g of bacterial biomass was derived from 16 g styrene oil by *P. putida* CA-3 in a 7.5 liter stirred tank reactor **(Ward *et al*., 2006)**. Change of nitrogen concentration exerts some effect on cell dry weight and PHA production. At supply rate 1 mg N/l/h 1.1-fold rise of PHA ratio in dry biomass was recorded. The maximum amount of PHA (4.2 g) produced from styrene by *P. putida* CA-3 in the bioreactor occurred at nitrogen feed rate 1.5 mg/l/h. The rates 1.75 and 2 mg N/l/h resulted in critical decrease of PHA intracellular level **(Goff *et al*., 2007)**. Polyethylene terephthalate also can serve as the substrate for PHA production. Conversion of polyethylene terephthalate similar to polystyrene proceeds in two stages: pyrolysis to styrene oil followed by *Pseudomonas* production of polymers. *P. putida* strains accumulated PHA with maximal productivity of approximately 8.4 mg PHA/l/h for 12 h before the synthesis rate fell sharply. *P. frederiksbergensis* accumulated PHA at the highestl rate of 4.4 mg PHA/l/h but there was no slow down in the production rate over time **(Kenny *et al*., 2008)**. *P. aeruginosa* PAO-1 synthesized biomass with almost 25% PHA content when supplied with the polyethylene pyrolysis wax in the presence of rhamnolipids. The change of nitrogen source from ammonium chloride to ammonium nitrate resulted in faster bacterial growth and the earlier onset of PHA accumulation **(Guzik *et al*., 2014)**.

Wastes in production of enzymes

Up to 30–40% of the production costs of industrial enzymes are accounted for by the growth substrates. The use of cost-effective growth medium for the synthesis of proteases can significantly improve economics of the process. For example, *P. aeruginosa* MN7 was shown to grow on the shrimp waste powder as the sole source of carbon, nitrogen, and salts. The strain was found to produce protease (elastase) (EC 3.4.24.26), with maximal synthesis of the enzyme at substrate concentration 60 g/L **(Jellouli *et al*., 2008)**. Wheat bran can be used as the feedstock for production of alkaline protease by *P. aeruginosa*. The higher levels of protease activity could be achieved using the following cultural conditions: wheat bran concentration (5%, w/v); pH (9.0); NaCl (12.5%); temperature (45°C); inoculum size (5 ml); incubation period (48 h) and agitation rate (100 rpm). Under these conditions, *P. aeruginosa* produced alkaline protease (EC 3.4.24.40) showing activity 582.25±9.2 U/ml **(Meena *et al*., 2013)**. Animal fleshing, a solid waste generated by tanning industry, also can be used as the only source of nitrogen and carbon for synthesis of alkaline proteases. The enzyme produced by *P. aeruginosa* reached maximal activity (1160-1175 U/mL) after 20 hours of incubation. That protease preferentially degraded non-fibrillar proteins, like albumin and globulin. However, increasing amino acid content in the exocellular medium suppressed protease activity upon further fermentation **(Kumar *et al*., 2008)**.

Microbial strains are essential in control of castor oil wastes. Two different *P. aeruginosa* strains are able to degrade castor oil with rates 5.7 and 3.8 g/L a day. Additionally, these strains produce lipase (triacylglycerol acylhydrolase, EC 3.1.1.3) with maximum activity of 1121 and 470.82 U/ml at 37°C and activity of 358.72 and 147.18 U/ml at 75°C. The activities were evaluated by the rate of *p*-nitrophenol production. Thus, *P. aeruginosa* strains can be used both in castor oil waste control and production of lipases **(Amara and Salem, 2009; Sharon *et al*., 1998)**. Palm fatty acid distillates, crude soybean acid oil, and wastewater from soybean oil refining process added at 2% concentration to the basal medium were also tested as nutrient sources for lipase production. The results demonstrated that the maximum lipase synthesis was attained using wastewater from palm oil refining process (70.29±0.09 U/mg), followed by wastewater from soybean oil refining process (62.59±0.38 U/mg) and crude soybean acid oil (62.48±0.43 U/mg) **(Supakdamrongkul *et al*., 2014)**.

Bacterial consortia, including some *Pseudomonas* strains (*P. fluorescence, P. putida*) might be engaged in degradation of banana waste (leaves and pseudostem) by solid state fermentation for cellulase (EC 3.2.1.4) production. The consortia exhibited high levels of enzyme production and pattern of biosynthesis with maximum specific activities lying between 15 and 25 days **(Dabhi *et al*., 2014)**.

Wastes in production of pigments

Another application for wastes is production of pigments, like melanin that mostly acts as antioxidant. Melanins find use in agriculture, medicine, cosmetic and pharmaceutical industries. It was found that vegetable waste from cabbage leftovers mixed with marine broth in 70:30 ratio is suitable for production of melanin (2.79±0.2 mg/mL) but the yields were lower compared with pure marine broth (5.35±0.4 mg/mL). Pigments from the different nutrient sources have shown different elemental composition. However, functional groups appeared to be the same **(Tarangini and Mishra, 2013)**.

In order to find cheap medium for pigment production by *P. fluorescens* seven different combinations of agricultural wastes were tested. Combination IV (rice straw + rice husk + wheat husk + cow dung + coconut water) provided for both increased bacterial growth and high rate of green pigment (pyocyanin) production. Pyocyanin finds application in organic light emitting devices, biosensors as a redox compound, microbial fuel cells (MFC) as electron shuttle, medicine as antitumor agent **(El-Fouly *et al*., 2015)**. Combination VI (rice straw

+ tapioca waste + cow dung + maida) was also found to be appropriate for growth but the production of pigments was relatively inferior to combination IV. The other variants turned out to be not adequate **(Poorni *et al*., 2011)**.

Other waste applications

Tobacco wastes have potential application in the biosynthesis of valuable chemicals, especially pyridines, like SP. SP (γ-oxo-3-pyridinebutanoic acid) is a simple molecular pyridine compound that can be transformed into hypotensive agents of mammals (ω-heteroaroyl-(propionyl)-l-prolines). *P. putida* S16dspm is able to utilize nicotine and form the target metabolites. Under optimal conditions of fed-batch fermentation the engineered strain produced SP at concentrations 9.8 g/L and 8.9 g/L and conversion yields of 83.8% and 89.9% during 45 h from aqueous nicotine solution and crude suspension of the tobacco waste, respectively **(Wang *et al*., 2015)**. Another compound that can be produced from tobacco wastes is HSP. This metabolite is a potential building block in the synthesis of drugs, insecticides and other compounds possessing biological activities, such as the analgesic molecule epibatidine. Under optimal conditions, recombinant strain *P. putida* P-HSP generated HSP at concentrations 6.8 g/L and 16.3 g/L with productivity 0.25 g/L/h and 0.71 g/L/h and the yields 36% and 75% from tobacco waste and nicotine, respectively. The HSP concentration derived from crude tobacco waste extract was lower than from pure nicotine, probably, because the former carries complex components inhibiting enzyme activity. Nevertheless, in long-term perspective tobacco wastes may be regarded as substrates for production of valuable compounds **(Yu *et al*., 2014)**.

Shrimp shell waste may serve as a source of chitin in production of chitosan used as fruit juice clarifier. The strain *P. aeruginosa* A2 was applied for chitin extraction. Demineralization advanced rapidly during the first three days of incubation period. At the same time, a swift pH drop took place. From the fourth day, these two parameters remained constant. Demineralization maximum (92%) was achieved when pH of the culture equaled 4.4. However, protease activity reached its highest level (1230 U/mL) after 1 day of incubation, and 90% protein removal was recorded. Chitosan converted from chitin was found to be effective in the clarification of different fruit juices at low concentration (0.025% for orange and apple juices and 0.1% for lemon juice), and it doesn't affect biochemical parameters of the juices. Presumably, it may serve as an ideal flocculation clarifier for fruit juices. Additionally, the antioxidant activity of shrimp waste hydrolysates produced during fermentation was tested. The results indicated that hydrolysates exhibited important radical-scavenging activities. The antioxidant activity increased and reached peak value after 3 days (90%) **(Ghorbell-Bellaaj *et al*., 2012)**. Similar experiment with crab shell waste samples allowed to study the effect of temperature, concentrations of shell waste and glucose, shell size on demineralization (DM) and deproteinization (DP) in the process of chitin extraction by *P. aeruginosa* F722. The optimal temperature for DM was found to be 30°C. The efficiency of DM was 92%, while the efficiency of DP was 63%. DM and DP efficiencies decreased with the increase of the solid to liquid ratio. The best results were registered for 5% CS waste. 90.5% DM was achieved by 7 days of incubation with 10% glucose and 10% inoculum of *P. aeruginosa*. The DM rate was gradually increased for both large (particle size 20–35 mm) and small (3–10 mm) shell samples during the cultivation. At day 7, the DM rates were 91.5% and 84.0% for the large and small size samples, respectively. The DP rates were 62% for the large size samples and 58.5% for the small ones **(Oh *et al*., 2007)**.

About 30% of annual world cheese whey volume remains underutilized, ending up as waste or animal feed. The studied *P. taetrolens* strain is able to derive lactobionic acid from whey. Lactobionic acid displays antioxidant, biodegradable, biocompatible and chelating properties, and therefore it may fit into various applications. Membrane-bound system catalyzed lactose oxidation to lactone intermediate (lactobiono-δ-lactone), with carbonyl group subsequently hydrolyzed by lactonase into lactobionic acid. The strain produced 42.4 g/L lactobionic acid after 32 h fermentation started with a 30% inoculum volume, evidencing the role of cheese whey as an alternative substrate for production of this compound **(Alonso *et al*., 2011)**.

Pseudomonas bacteria may be applied in biodegradation and conversion of excess waste glycerol. Glycerol is a colorless compound containing three hydroxyl groups in its structure. It is the by-product of fat processing, ethanolic fermentation of glucose and biodiesel industry released in growing amounts. The waste glycerol can be utilized as a carbon source for microbial growth and converted into various products, like 1,2-propanediol, 1,3-propanediol, succinic acid, ethanol, xylitol, propionate. Chemical synthesis of 1,3-propanediol demands high energy input and expensive catalysts while microbial metabolic route requires ambient temperature and atmospheric pressure **(Deckwer, 1995; Hao *et al*., 2008)**. It is possible to transform glycerol into 1,3-propanediol using *Pseudomonas* strains, with the best results achieved in 72 h process at 50 g/L glycerol concentration. In turn, 1,3-propanediol can be used in industrial production of unsaturated polyester resins showing fire and electrical resistance **(Karve *et al*., 2014)**.

Wastes may be applied in fabrication of biofuel. Biofuel is usually produced by microbial fermentation of the sugars. World pulp and paper industry puts out about 300-350 million tons of paper and board of various types, so that paper wastes make a splendid source of biofuel, if two major plant components, starch and cellulose, are converted to sugars readily utilized by microorganisms. The studies indicated that the waste paper substrate (basically consisting of starch after chemical hydrolysis) was converted into simple fermentable sugar by *P. aeruginosa*. The product yield of the microbial hydrolysis of waste paper substrate was 45%. It was enough for further production of ethanol by *Saccharomyces cerevisiae* **(Prema *et al*., 2015)**.

Waste can be used as substrate for MFC. MFC converts energy available in a biological substrate directly into electricity. Cells function on different carbohydrates, including complex substrates in wastewaters and alleviate thereby environmental contamination problem. However, the tests showed lower coulombic efficiencies (the ratio of the output of charge to the input of charge) of complex wastewater substrates with the highest value of 36% as compared to easily degradable synthetic wastewaters with the peak level of 98% **(Fornero *et al*., 2010)**. Nevertheless, some studies in this direction were carried out **(Majumder *et al*., 2014)**.

CONCLUSION

Ability of *Pseudomonas* to produce and utilize a wide range of compounds makes this genus extremely attractive in various applications, including waste disposal. Investigations revealed *Pseudomonas* potential in degradation of agricultural, industrial and household wastes. It's especially important with regard to compounds hazardous for the environment and humans. Annually new studies concerning waste utilization emerge and the data show high efficiency of this bacterial genus in resolving contamination problems.

Moreover, metabolic versatility of *Pseudomonas* allows to gain valuable biological products, primarily biosurfactants and PHAs suitable for various industrial processes and often distinguished by superior properties than chemical analogs in terms of lower toxicity and biodegradability. As a result it's possible to manufacture a broad spectrum of bioactive agents using waste materials as the sole source of carbon and nitrogen.

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