JMBFS

Journal of Microbiology, Biotechnology and Food Sciences

International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

AGROCHEMICALS AFFECT THE ANTIOXIDATIVE DEFENSE POTENTIAL of COTTON PLANTS

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doi: 10.15414/jmbfs.2016.5.6.505-508

ARTICLE INFO

Received 20. 5. 2015
Revised 25. 8. 2015
Accepted 13. 1. 2016
Published 1. 6. 2016

Regular article

OPEN ACCESS

ABSTRACT

Application of insecticides used in cotton fields is often associated with secondary biotic stresses. One of possible reasons of such phenomenon is explained by decreased contents of plants' defense components. As peroxidase (POD) and polyphenoloxidase (PPO) are typical oxidoreductase enzymes scavenging cell oxidative damage, we studied their change levels in cotton leaves in response to the application of three insecticides field experiment. Moreover, the concentration of proline (Pro), methionine (Met) and cysteine (Cys) was studied. The plants were treated with Carbophos, Lannate and Sumi-alfa in early blooming stage at commonly used doses in. Leaf samples were taken on the 10thand 13th days of the treatment. A pyrethroid insecticide Sumi-alfa appeared to negatively impact activities of both POD and PPO ($P \leq 0.05$), contrasting the other two insecticides examined. On the other hand, levels of amino acids with antioxidative properties increased after application of all three insecticides at the end of experiment. Our results show that the oxidative balance of treated plants was interrupted by insecticides (especially Sumi-alfa) with potential impact on vulnerability to secondary stresses. Effects of these insecticides on cotton should be considered and/or studied in more detail for efficient application in agriculture.

Keywords: Cotton plant, Insecticides, Peroxidase, Polyphenoloxidase, Proline, Methionine, Cysteine

INTRODUCTION

Application of insecticides is often reported to be associated with secondary biotic stress. For example increased number of aphids in cotton fields, treated with pyrethroid λ-cyhalothrin against bollworm, has been observed **(Kerns and Gaylor, 1993; Leser, 1994; Ravindhran and Xavier, 1997; Asrorov *et al.* 2014)**. Further, rapid increase in aphid populations after sulprofos and cypermethrin was determined that is unlikely due to direct stimulation of aphid reproduction **(Kerns and Gaylor, 1993)**. Rapid increases in aphid numbers were associated also with applications of the pyrethroid insecticides cypermethrin, and deltamethrin **(Ravindhran and Xavier, 1997)**. Similar observations have been made for different agrochemicals applied on rice **(Wu *et al.* 2001)**. It has been suggested that some agrochemicals might alter the biochemistry of the treated plants **(Leroy *et al.* 2011)** and/or might disrupt the oxidative balance of cells leading to weakening of plant defense. The latter is under normal physiological conditions crucial for optimal functioning of biological processes and in plants is controlled by various antioxidative compounds (e.g. ascorbic acid, glutathione) **(Noori, 2012)** and/or by induction of antioxidative enzymes such as peroxidases, catalases or dismutases **(Zhang** and **Kirkham, 1994)**.

Interruption of oxidative balance results in accumulation of toxic reactive oxygenous species resulting in so called oxidative stress that can seriously injure the tissues. Several pesticides have previously been shown to alter the oxidative balance of cells in mammals and cause cell damages. To these pesticides belong an organochlorine insecticide dieldrin **(Bachowski *et al.* 1998)**, the pyrethroid insecticides Deltametrin **(El-Gohary *et al.* 1999)** and cypermethrin **(Kale *et al.* 1999)**. In plants pyrethroid insecticides were found to decrease catalase and phenylalanine ammonia lyase activities, and total ascorbate and cinnamic acid concentrations in tissues **(Ravindhran and Xavier, 1997; Bashir *et al.* 2007)**. On the other hand, some agrochemical have been suggested as potential plant immunization agents inducing systemic acquired resistance (SAR) in plants, leading to broad-based, long-lasting resistance to a wide range of pathogens **(Enyong Arrey Besong, 2008)**.

The present work was undertaken to study the effect of selected agrochemicals on oxidative balance in cotton plants that might be indicative for explaining the plant vulnerability to secondary stresses. As compounds of antioxidative defense systems we monitored the activity of antioxidative enzymes as well as accumulation of certain amino acids. Of these, peroxidase (POD) was found to have a defense role in eliminating oxidative burst in cotton upon abiotic stress **(Akhunov *et al.* 1999)** and also attack of bacterial blight **(Delannoy *et al.* 2003)**, the phytopathogen *Verticillium dahliae* **(Pshenichnov *et al.* 2011)** and herbivore feeding **(Stout *et al.* 1998)**. POD activity was often found greater in resistant variety than in susceptible **(Akhunov *et al.* 2010)**. On the other hand, polyphenoloxidase (PPO) has been shown to act upon attack of bacterial pathogen *Pseudomonas syringae pv.* **(Li and Steffens, 2002)** and arthropod herbivores **(Felton *et al.* 1989)**, common cutworm *Spodoptera litura* F., cotton bollworm *Helicoverpa armigera* (Hubner) and beet army worm *Spodoptera exigua* (Hubner) **(Thipyapong *et al.* 2007)**. Several previous investigations showed that the activities of these enzymes were affected after application of insecticide imidacloprid at recommended concentration (40 ml/acre) in three Bt cotton hybrids (RCH-134, JKCH-1947, NCEH-6R) **(Kaur *et al.* 2011)** and potato plants **(Chauhan *et al.* 2013)**.

Agrochemicals can also affect nitrogen metabolism **(Slosser *et al.* 2004)** including accumulation of proline (Pro), methionine (Met) and cysteine (Cys) with importance in plant defense. The free Pro has multifunctional role in plant immunity and development, while it is induced by reactive oxygen species signalling **(Ben Rejeb *et al.* 2014)**. Pro is also known to indirectly enhance plant antioxidant defense system **(Hoque *et al.*, 2007; Ozden *et al.* 2009)**. The sulphur containing amino acid Cys plays an important role as an extracellular reducing agent **(Atmaca 2004)**. It is a central metabolite serving as a sulphur donor for the synthesis of Met **(Zagorchev *et al.* 2013)**, which is an efficient scavenger of almost all oxidizing molecules under physiological condition, such as, hydrogen peroxidase, hydroxyl radicals **(Levin *et al.* 1996)**. Under water deficit in cotton plant leaves Met concentration fluctuated widely **(Marur *et al.* 1994)**. Under the effects of cypermethrin Met concentration increased in both organophosphorus insecticide resistant and susceptible plant varieties **(Saleem and Shakoori, 1993)**.

We studied the impact of three agrochemicals organophosphate Carbophos, pyrethroid Sumi-alfa and carbamate Lannate on antioxidative defense components in cotton plants. All three chemicals are being widely used in Uzbekistan for treatment against pests, but their possible (negative) impact on treated plant antioxidative system has not been studied in detail.

MATERIAL AND METHODS

Experimental Design

Field experiments were conducted on cotton variety S 26 (*G. hirsutum*) growing at the pre-bloom stage in the cotton field of the Institute for Plant Protection of the Ministry of Agriculture and Water Resources of Uzbekistan (Tashkent Region, Kibray District, Salar Township). The concentration of each insecticide was prepared according to the consumption norm recommended by producers against cotton pests (Table 1). The solutions were sprayed once in the early morning at 6:00 to 6:30AM. Treatment of plants with three insecticides was a randomized block with 4 replications. Sixteen plants were treated by each insecticide and water was used as control (a total of 4 treatments). The solutions were sprayed once in early morning. A total of 14-16 different developed stages of leaves were taken from the upper, middle, and lower parts of 60-65 plants on the10^{th} and 13^{th} days after treatment that our former results showed the highest changes in the quantity of soluble proteins and sugars were observed on these days **(Asrorov *et al*. 2014)**. They were averaged and lyophilized. During the experiments no effect of insects in the treated fields was observed.

Table 1 Treatment, chemical class and application rates

Treatment	Class	Application rate l/ha
Control		Water
Carbophos, Aerosoyuz, Russia	Organophosphate	0,6
Lannate, Du-Pont, France	Carbamate	0,25
Sumi-alfa, Sumimoto chemical, Japan	Pyrethroid	0,5

Protein Extractions and Analysis

Lyophilized cotton leaves were ground with liquid nitrogen using a mortar and pestle. After grinding, the proteins of control and treated leaves were extracted with Tris-HCl buffer (0.5 M Tris-HCl pH 6.8, 20 mM EDTA, 2 mM PMSF, 1% Triton X-100, and 150 mM DTT) for two hours with stirring. The mixture was filtered and in supernatant proteins were precipitated with cold absolute acetone (1:4 v/v) and centrifuged 30 min (8 000 r/min, at 4^0C). The residue was dissolved in water and freeze-dried. The quantity of soluble proteins was determined according to **Lowry et al. (1951)**. For calibration, albumin bovine (from bovine serum; Sigma A7030) in appropriate amounts was weighed and dissolved in Na_2HPO_4 buffer pH 7 to provide concentrations of 10-100 µg/ml.

POD Assay

The total POD activity of the proteins isolated was determined using benzidine as the chromogenic substrate. Thus, 1.9 ml 0.1 M pH 4.7 acetate buffer (11.5 ml glacial acetic acid + 27.25 g sodium acetate and the volume was adjusted to 1000 ml) was placed in a test tube, and 50 µl of benzidine (40 µg benzidine dissolved in 25 ml 70% ethanol) was added. The solution was stirred thoroughly then 100 µl of protein solution (containing the enzyme) was added. After the addition of 50 µl of hydrogen peroxide and 30 sec passed, the optical density of the solution was measured at 620 nm.

PPO Assay

Total PPO activity of proteins was studied using pyrogallol as a chromogenic substrate. Thus, to 2 ml of phosphate buffer pH 8, having protein solutions (61 g $Na_2HPO_4{\cdot}H_2O$ and 39 g $NaH_2PO_4{\cdot}H_2O$ were dissolved in 200 ml of water) was added 50 µl 0.15 M substrate solution. The intensity of colored solution was determined at 460 nm.

Extraction of amino acids and their modification

Dried samples of treated and control leaves were ground and pooled in liquid nitrogen. 200 mg of each sample was weighed and extracted in 5 ml of water-acetonitrile (9:1) for 15 minutes with homogenizer. The supernatant was isolated after centrifugation for 10 minutes (3000 rpm). Higher molecular compounds were precipitated adding 10% trichloroacetic acid (1:1) for 15 minutes (8000 rpm). 200 µl aliquot was taken and lyophilized. Dry extract was dissolved in 200 µl water-acetonitrile-triethylamine (1:7:1) then the extract was dried. The process was twice repeated. In order to obtain phenylthiocarbamoyl (PTC) derivatives purified samples and standard amino acid were modified with water -acetonitrile-thriethylamine + phenylisotiocyonate (1:7:1:1) for 30 minutes.

HPLC analysis

Quantity analyses of PTC derivatives were conducted in Agilent technologies 1200 (Column: Supelco Discovery HS C18, Cat 56925/-U 7.5 cm x 4.6 mm, 3 µm), with buffer: B – 0.14 M CH_3COONa + 0.05% tetraethyl ammonium, pH 6.4, A – MeCN, gradient % B/min, 0 – 6%, 5 min, 6 – 30%, 30 min, 30 – 60% 5 min, 60 – 100% 5 min. Amino acid derivatives were detected with a flow rate 1.2 ml/min at 269 nm.

RESULTS AND DISCUSSION

The three agrochemicals were once applied on cotton plants for 2 different time periods at doses commonly used in agriculture. Their sound effect on plant metabolism was obvious from data of protein yields isolated from the experimental plants. Sumi-alfa enhanced protein synthesis at both time periods (Fig. 1A). Similar effects were observed for imidacloprid on Bt cotton total protein **(Kaur *et al*. 2011)**. In contrast Carbophos revealed an opposite (negative) effect ($P \leq 0.05$) (Fig. 1 A). Effects of Lannate were significant after 10 days but disappeared at later time (Fig. 1A).

The agrochemicals influenced the activity of antioxidative enzymes investigated. Sumi-alfa significantly inhibited the activity of both POD as well as PPO, after both application times (Fig. 1 B, C). The other two insecticides, in contrast, evoked a multiple increase of PPO activity at both sampling points (Fig. 1 B, C). Their effect on POD, however, was ambiguous since slight decrease ($P \leq 0.05$) after 13 days was only observed for Carbophos and contrasting, time-dependent activity was measured for Lannate (Fig. 1B, C). Previously, imidacloprid insecticide at recommended concentration (40. ml/acre) caused increased POD activity in three Bt cotton hybrids (RCH-134, JKCH-1947, NCEH-6R) **(Kaur *et al*. 2011)**. Moreover, imidacloprid increased the activities of catalase, POD, PPO and the quantity of total protein in potato plants **(Chauhan *et al*. 2013)**. Increased POD activities in imidacloprid applied plants partly coincide with the effects of carbamate lannate and enhanced PPO activity matches with the effects of lannate and carbophos. Decreased POD and PPO activities in Sumi-alfa applied leaves do not correspond with increased levels of POD and PPO activities under treatment of deltamethrin, cypermethrin and fenvalerate either **(Ravindhran and Xavier, 1997)**.

Fluctuating changes were observed with Pro. After treatment with Lannate and Sumi-alfa lower than the control enzyme activities were found in samples taken on 10^{th} day after spray. However, later-on they caused higher enzyme activities than control. Twice higher Pro quantity was calculated after carbamate Lannate. Insignificant slight increases on the 10^{th} day samples and significant increases were studied on 13^{th} day samples after treatment with carbophos ($P \leq 0.05$) (Fig. 1 D). Similar changes were observed with sulphur containing amino acids in all treated samples. All three chemicals significantly increased the quantity of Met and Cys (Fig E, F) ($P \leq 0.05$). The highest increase was observed with samples treated with Carbophos that almost twice higher quantity of Met was gathered (10^{th} day) and mildest effects belonged to Sumi-alfa. ($P \leq 0.05$) (Fig. 1 E). Samples treated with Carbophos on the 10^{th} day contained more Met and Cys than samples taken three days later, whereas, in comparison to control, linear increases were observed with Met and Cys after Lannate and Sumi-alfa treatment. The increased levels of Met in all treated samples match with the effects sulprofos on total essential amino acids **(Kerns and Gaylor, 1993)**.

CONCLUSION

Our results show that application of the tested agrochemicals affects cotton plant antioxidative homeostasis that in turn is likely to have impact on defense potential. Among studied three insecticides pyrethroid sumi-alfa was found to negatively affect the activity of both enzymes. On the other hand, application of the tested insecticides evoked the accumulation of amino acids involved in antioxidative (and perhaps other) defense. Nevertheless, impact of these insecticides on defense potential of cotton should be considered and/or studied in more detail for efficient application in agriculture.

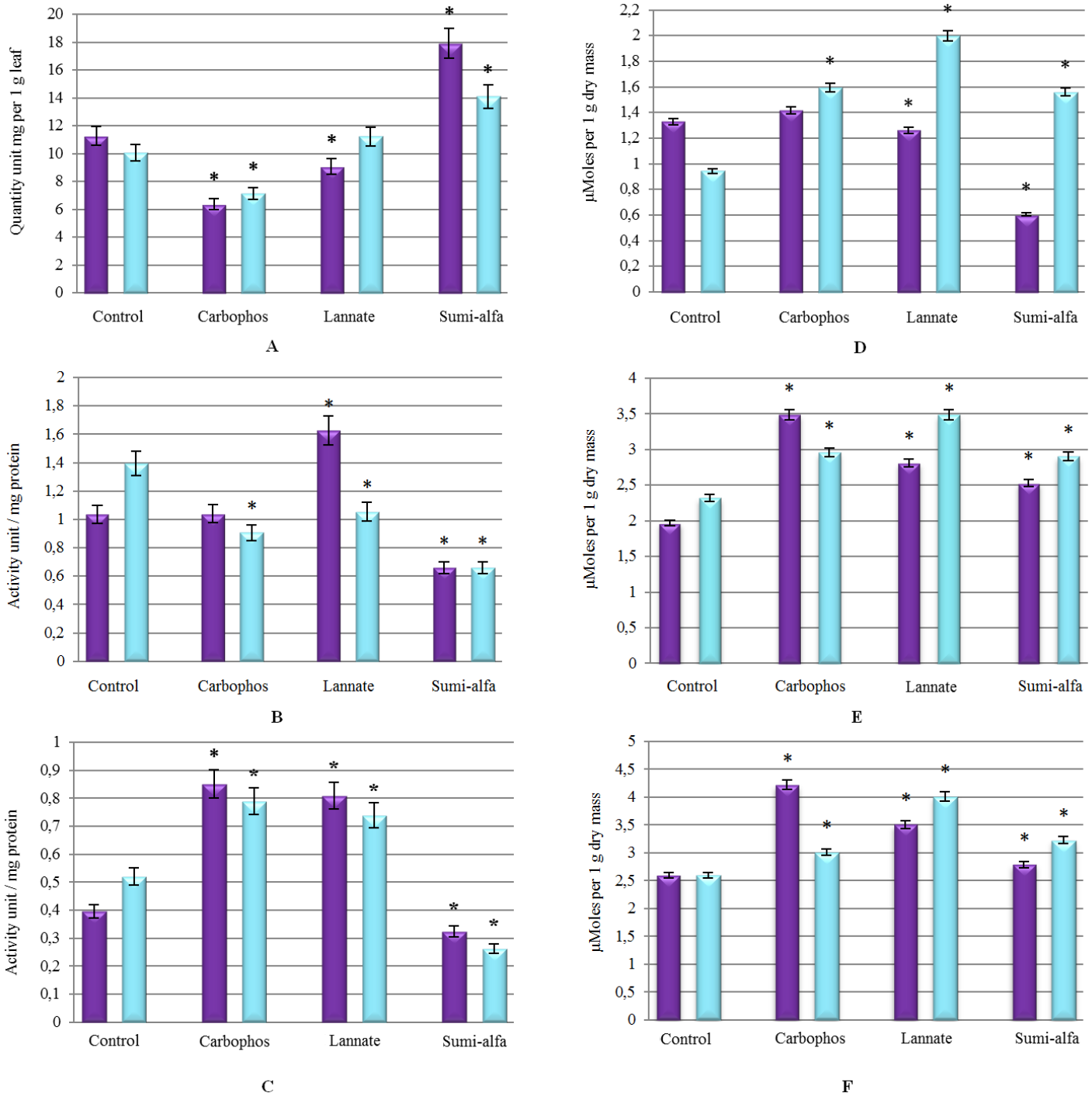

Figure 1 Changes in cotton leaves: **A** – Soluble proteins; **B** – POD activity; **C** – PPO activity; **D** – Pro content; **E** – Met content; **F** – Cys content. Leaves taken for the analysis were statistically different. The SE is less than 2%, data represent ±SEM (m3). Dark colours represent the 10th day and light colors represent the 13th day that samples taken after insecticides spray

Acknowledgments: The Work was supported by CASIA (Central Asia Student International Academic exchange with EU, CASIA II, cohort III) project of European Union Erasmus Mundus Programme. The authors express their sincere thanks to **Dr. Loreta Schwarczova**, programme coordinator in Slovak University of Agriculture in Nitra, **Dr. Mashkura Fakhrutdinova**, coordinator of the programme in National University of Uzbekistan, **Dr. Alim Pulatov**, regional coordinator of the programme (Tashkent Institute of Irrigation and Melioration, Uzbekistan) and **Dr. Ewa Wietsma**, main coordinator of the programme (Wageningen Univerty, Holland) for the financial support with CASIA Programme.
Zdenka Gálová contributed by European Community project: Development of International Cooperation for the Purpose of the Transfer and Implementation of Research and Development in Educational Programs conducted by the Operational Program: Education,
ITMS code: 26110230085

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JMBFS
Journal of Microbiology, Biotechnology and Food Sciences
International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

CLONING, PURIFICATION AND CHARACTERIZATION OF HALOTOLERANT XYLANASE FROM *Geobacillus Thermodenitrificans* C5

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doi: 10.15414/jmbfs.2016.5.6.523-529

ARTICLE INFO

Received 18. 2. 2015
Revised 13. 1. 2016
Accepted 14. 1. 2016
Published 1. 6. 2016

Regular article

OPEN ACCESS

ABSTRACT

High levels of extracellular xylanase activity (994.50 IU/ml) produced by *Geobacillus thermodenitrificans* C5 originated gene was detected when it was expressed in *E. coli* BL21 host. Thermostable xylanase (GthC5Xyl) was purified to homogeneity and showed a molecular mass of approximately 44 kDa according to SDS-PAGE. The specific activity of the purified GthC5Xyl was up to 1243.125IU/mg with a 9.89-fold purification. The activity of GthC5Xyl was stimulated by $CoCl_2$, $MnSO_4$, $CuSO_4$, $MnCl_2$ but was inhibited by $FeSO_4$, Hg, $CaSO_4$. GthC5Xyl showed resistant to SDS, Tween 20, Triton X-100, β- Mercaptoethanol, PMSF, DTT, NEM and DEPC, SDS, and EDTA. A greater affinity for oat spelt xylan was exhibited by GthC5Xyl with maximum enzymatic activity at 60°C and 6.0 pH. The activity portrayed by GthC5Xyl was found to be acellulytic with stability at high temperature (70°C-80°C) and low pH (4.0 to 8.0). Xylobiose and xylopentose were the end products of proficient oat spelt xylanase hydrolysis by GthC5Xyl. High SDS resistance and broader stability of GthC5Xyl proves it to be worthy of impending application in numerous industrial processes especially textile, detergents and animal feed industry.

Keywords: *Geobacillus thermodenitrificans*, thermostable GthC5Xyl enzyme, recombinant xylanase

INTRODUCTION

Hemicellulose in plant cell walls contain xylan as the major component which accounts for one third of the earth's total renewable organic carbon source **(Cheng *et al.*, 2014).** These hetero-polysaccharides are primarily composed of xylose subunits linked by β-1, 4-glycosidic bonds which forms the backbone. This backbone further contains different side groups such as acetyl groups, methyl groups and other sugar molecules **(Collins *et al.*, 2005).** As xylan has a complex and intricate structure, it requires a combination of enzymes for its complete breakdown. These enzymes include endo-1, 4-β-xylanase, β-xylosidase, acetylxylan esterase, arabinose and glucuronidase (Khandeparker and Numan, 2008) out of which the most potent and thus most important one are the endo-1, 4-b-xylanases **(Li *et al.*, 2014).** Xylan is degraded to short xylo-oligosaccharides of various lengths by the action of these enzymes. Xylanases conjoin with β - xylosidases (EC 3.2.1.37) for completely hydrolyzing xylan to xylose monomers. There are various benefits of carrying out xylan hydrolysis at high temperatures by utilizing thermostable enzyme. These advantages include increased reactant and products solubility due to low viscosity, a higher mass transport rate, a decreased hazard of contamination by mesophilic microorganism and half-lives at elevated temperatures leads to better hydrolysis **(Bhalla *et al.*, 2014).** One of the important concern that still persists is the enzyme stability while thermal processing. The changes in the 3D structural confirmation of enzymes at high temperature lead to enzyme inactivation **(Bankeeree *et al.*, 2014).**
There is a frequent isolation and identification of *Geobacillus* from various sources because of its ability to produce thermostable endo-xylanases for xylan hydrolysis. Examples of *Geobacillus* strains producing xylanases with potential industrial applications are novel thermostable endo-xylanase from *Geobacillus sp.* WSUCF1**(Bhalla *et al.*, 2014),** *Geobacillus stearothermophilus* 1A05583 **(Yan Wang *et al.*, 2013)** producing xylanase, *Geobacillus thermodenitrificans* TSAA1 producing Thermostable and Alkalistable Endoxylanase (Verma *et al.*, 2013), novel thermophilic xylanase from *Geobacillus thermodenitrificans* **JK1 (Gerasimova and Kuisiene, 2012),** *G. thermoleovorans* producing highly thermophilic endoxylanase **(Verma and Satyanarayana, 2012),** thermophilic *Geobacillus* sp. 7 1**(Canakci *et al.*, 2012)** producing an alkali-stable endoxylanase.

For application in industry, it is necessary for xylanase to be acellulolytic **(Goswami *et al.*, 2014)** and requiring minimum downstream processing for its production. The aim of this study was to carry out the heterologous expression of *Geobacillus thermodenitrificans* xylanase gene in *E. coli* for increased xylanase production as well as to secrete this expressed enzyme via *E. coli* in the medium to minimize its downstream processing for industrial application. Purification and biochemical characteristic's analysis was further carried out.

MATERIALS AND METHODS

Bacterial Strains, Substrates, Vectors and Chemicals

The chemicals were obtained commercially from Merck A.G. (Darmstadt, Germany), Sigma Chem. Co. (St. Louis, MO, USA), Fluka Chemie A.G. (Buchs, Switzerland), and Acumedia Manufacturers, Inc. (Baltimore, Maryland, USA), Aldrich-Chemie (Steinheim, Germany). The, Wizard Plus SV Minipreps DNA Purification System, Wizard Genomic DNA Purification Kit, dNTP, restriction enzymes and *Taq* DNA Polymerase were obtained from Promega Corp. (Madison, WI, USA). All chemicals were reagent grade and all solutions were made with deionized and double distilled water. *E. coli* JM101: *E. coli* BL21 (DE3): pET28 (a) + were gently provided by Karadeniz Technical University, Molecular Biology Laboratory. The method of Karaoglu was used for culturing of recombinant *E. coli* (Karaoglu *et al.*, 2013).

Screening and Phylogenic Analysis for Xylanase Producing Bacteria

Water and soil samples were collected from the Garam Chashma hot springs of Chitral KPK Pakistan. Enrichment was done using oat spelt xylan (Sigma Chemicals, Germany) as a sole carbon source. Twenty bacterial strains were screened for xylanolytic ability. One prominent isolate was selected and then identified on the basis of cultural, morphological and biochemical properties **(Sneath, 1994)** along with 16S rRNA sequencing (Supplementary Table 1).
NCBI server (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was utilized to recover the partial 16S rRNA via BLAST tool. Similar sequences were then downloaded in FASTA format. Multiple alignment of sequences and calculations of levels of sequence similarity were performed by using ClustalW2 program. Analysis for

closely related organisms was carried out by using obtained phylogenetic tree. Evolutionary history was deduced by Neighbor joining method **(Saitou and Nei, 1987).**

Amplification, Sequencing and Bioinformatics Analysis of Xylanase

Wizard Genomic DNA Purification Kit was used to isolate Genomic DNA. Xylanase gene was amplified by using XyGeoT-F: (5'CTAgCTAgCATgTTgAAAAgATCgCgAAAAg-3') having *Nhe*I and XyGeoT-R: (5'- CCCAAgCTTTCACTTATgATCgATAATAgCCCA-3') having *Hind*III restriction sites in defined PCR conditions. Thermo Cycler (Bio-Rad, Hercules, CA) was used for amplification of xylanase encoding gene. Initial denaturation was at 94°C for 3 minutes followed by 36 cycles (denaturation (94°C for 1 min), annealing (62°C for 40 sec), and extension (72°C for 90 sec) were performed in a PCR vial containing 50 µl of reaction volume, and final extension was done at 72°C for 8 min. The amplified gene was cloned into pGEM®-T Easy vector and positive clones were selected using blue white selection on 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside, Isopropyl β-D-1-thiogalactopyranoside, and ampicillin plate. The white colonies were picked up and confirmed for the insert by double digestion. Two clones having the insert were processed for sequencing.
Nucleotides and their deduced amino acids were analyzed by using BLASTn and BLASTp programmers respectively (http://www.ncbi.nlm.nih.gov/BLAST). CLUSTALW program was used for carrying out multiple sequence alignment of xylanase (http://www.ebi.ac.uk/clustalW) and MEGA 4.0 (with minimum evolution) was used for phylogenetic analysis and dendogram construction of xylanase.

Construction and Expression of the Recombinant Vector pET_GthC5Xyl

Complete xylanase gene was amplified and purified by gel extraction and ligation was done into pGEM®-T Easy Vector. Heat shock method **(Belduz *et al.*, 1997)** was used to transformed the ligated product in *E. coli* JM101 competent cells.
Recombinant vector pET_GthC5Xyl having *Nhe*I and *Hind*III restriction sites compatible to pET28a (+) vector was constructed. The xylanase gene cloned pGEM®-T Easy Vector was isolated and digested with respective restriction enzymes. Xylanase gene product was purified by gel extraction and ligated into already purified and digested pET28a (+) vector using T4 DNA ligase overnight at 16°C. Heat shock method **(Belduz *et al.*, 1997)** was used to transform the ligated product in *E. coli* JM101 competent cells. The positive clones were established by double digestion of the recombinant plasmid with specific restriction enzymes. Two plasmids having xylanase gene was sequenced using the Taq DyeDeoxy Terminator Cycle Sequencing Kit according to the manufacturer's instructions, and analyzed with an Applied Biosystems (Macrogen, Korea) Model 370A automatic sequencer. The recombinant vector pET_GthC5Xyl was transformed into *E. coli* BL21 (DE3) cells. Four clones were grown in LB broth overnight and 1% (v/v) of this was used as inoculum to cultivate *E. coli* BL21 (DE3) cells. 1 mM IPTG was used for induction of the recombinant xylanase at OD_{600nm} of 0.5–0.6. For higher expression of xylanase the induced cell were cultivated for 20 h.

Purification of the Recombinant GthC5Xyl

After higher expression of recombinant xylanase the culture was centrifuged for 15 minutes at 10,000 rpm and suspended in 50 mM sodium phosphate buffer. 60% ammonium sulphate was used for precipitation of enzyme and then DEAE affinity chromatography was used to purify the enzyme. The obtained 60% precipitates were loaded on a column (1.5 × 50 cm) of DEAE-Sepharose pre-equilibrated with 10 mM sodium phosphate buffer pH 6.0. The column was washed with 1000 ml of the same buffer at flow rate of 0.5 ml/min. After washing, column was eluted with linear gradient of (0.55 M) NaCl in sodium phosphate buffer. The active fractions were combined and concentrated by ultrafiltration (Sartorious, 30000 MWCO filters).
GthC5Xyl enzyme purity was checked by reverse phase C-18 column (4.6 x 250 mm; E. Merck, Germany) of High Performance Liquid Chromatography (HPLC System 600 Waters, Waters Corporation, Massachusetts, USA).The solvent system acetonitrile-water (70:30) at a flow rate of 0.5 ml/min was employed for the separation of sample components. Absorbance was read at 280 nm using a highly sensitive photo-diode array (PDA) detector (996 Waters).

SDS-PAGE, Zymogram Analysis and Protein Identification

The fractions containing xylanase activity were analyzed by SDS-PAGE and zymography as described by Liao *et al.* **(Karaoglu *et al.*, 2013**; **Liao *et al.*, 2012).** SDS-PAGE was performed using an 11% (w/v) polyacrylamide gel with a 5% stacking gel with the Mini-Protean II system (Bio-Rad, Hercules, CA) according to Laemmli (1970). The protein bands were visualized by staining with Coomassie Brilliant Blue R-250 (Bio-Rad, Hercules, USA). For the zymogram analysis, briefly, after performing SDS-PAGE the zymogram gel was soaked for 30 minutes in 2.5% (v/v) Triton X-100 to remove the SDS and re-nature the proteins in the gel, which was then washed thoroughly in 50 mM Phosphate buffer (pH 6) and incubated at 60 °C for 30 min in 1% xylan. The gel was flooded in 0.1% (w/v) Congo red solution for 15 min and destained with l M NaCl until hydrolysis zones appeared against a red background. The reaction was then stopped by dipping the gel into 5% acetic acid solution. To classify the protein sequence, a homology search was performed using Mascot (http://www.matrixscience.com). The partial amino acid sequence was used to identify analogous proteins through a BLAST search of the NCBI protein sequence database. Amino acid homology alignment of the predicted GthC5Xyl with other highly homologous known xylanases was carried out.

Activity Assay, Determination of Protein Concentration and Kinetics of Xylanase

Dinitrosalicylic acid (DNS) method **(Miller, 1959)** was used for studying the activity of purified GthC5Xyl via measuring the reducing sugar release from oat spelt xylan. All xylanase assays was performed with 100 mM sodium phosphate buffer pH. 6.0 (unless otherwise specified). The xylanase assay was carried out by incubating suitable diluted enzyme with 1% xylan in 100 mM sodium phosphate buffer for 20 minutes at 60ºC. 3,5-dinitrosalicylic acid reagent (DNSA) was used for the estimation of liberated sugars. One unit of xylanase is defined as the amount of enzyme that liberates 1 µmol of reducing sugar under the assay conditions using oat spelt xylan as the substrate. Bradford method (1976) was used for determining protein concentration. In this procedure, Bovine serum albumin was used as standard **(Bradford, 1976).** Kinetic parameters V_{max} (µmol/min/mg) and K_m were determined by Michaelis-Menten plots of specific activities at multiple xylan concentrations varying between 0.5 mg/ml to 30 mg/ml.

Determination of pH and Temperature Effects on Activity and Stability of GthC5Xyl

The effect of temperature on activity of GthC5Xyl was determined spectrophotometrically using xylan as substrate. By using the method previously described enzyme activity was assayed over a range of temperatures from 40°C to 100°C. Results were expressed as relative activity (%) obtained at optimum temperature. GthC5Xyl temperature stability was determined by incubating enzyme at 40, 50, 60, 70, 80, 90 and 100°C for 200 min and then measuring the residual activity.
pH optimum was determined at 540_{nm} and 60°C by using buffer solutions of different pH. Results were expressed as relative activity (%) obtained at optimum pH. pH stability of enzyme was determined by incubating enzyme at each pH value for 200 min at 60°C and then measuring the residual activity.

Activator and Inhibitor Effects of Metal Ions on GthC5Xyl Activity

For the determination of activation and inhibition effect of various metal ions, the enzyme was incubated for 20 min with 1, 5 and 10 mM of bivalent metal ions such as, Zn^{2+}, Mn^{2+}, Mg^{2+}, Cd^{2+}, Ca^{2+}, Hg^{2+}, Ni^{2+}, Fe^{2+} and Cu^{2+} at optimum reaction conditions. GthC5Xyl activity was defined 100% without metal ions and residual activity (%) was determined spectrophotometrically (Table 2).

Analysis of Hydrolysis Products and Shelf life Determination of Xylanase

For analysis of xylanase hydrolysis product purified GthC5Xyl was mixed with 100 mM sodium phosphate buffer (pH 6.0) containing 1% (w/v) xylan and incubated for 10 h at 60°C. For the removal of insoluble materials samples were centrifuged for 12 min at 3000 g. TLC pates were then spotted with 3 µl aliquots. Chromatography by ascending method was then performed on silica gel 60 F_{254}TLC plates (Merck) with n-butanol, acetic acid and water (2:1:1) containing solvent system. Plates were then sprayed with 5% (v/v) sulfuric acid in ethanol and then heated at 120°C for about 10 min for the sugar detection. Shelf life determination was done by keeping GthC5Xyl in refrigerator at 4°C as well as at room temperature. Samples were then removed at different intervals and residual activity was determined for 16 weeks.

RESULTS

Isolation and Identification of Bacteria

Bacterial strains, which formed clear halos around their colonies on xylan agar plates, were picked up for further studies. The isolate was confirmed as *Geobacillus thermodenitrificans* C5 with partial 16S rRNA sequencing having a length of 1419 bp nucleotide. The sequence was deposited in GenBank (Accession No. KP203956). The Fig. 1 shows phylogenetic relation of isolate. It shows a very close relation with *Geobacillus thermodenitrificans* subsp.*calidus* F84b (EU477773) and *Geobacillus thermodenitrificans* subsp. *thermodenitrificans* NG80-2 (CP000557) (Fig. 1) having 99% similarity.

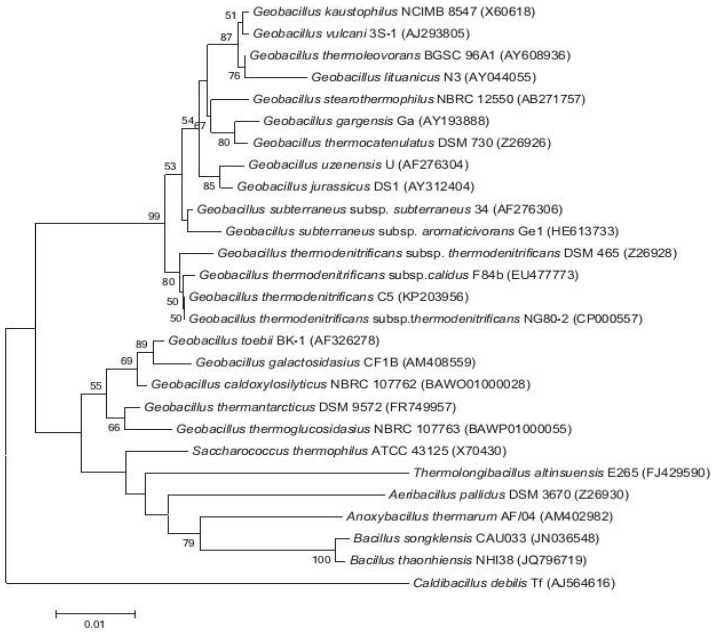

Figure 1 Phylogenetic tree of *Geobacillus thermodenitrificans* C5. The evolutionary history was inferred using the Neighbor-Joining method. Evolutionary analyses were conducted in MEGA 5.0.

Cloning and Sequence Analysis of Xylanase

Xylanase encoding gene having 1230 bp was amplified by using *G. thermodenitrificans* DNA as template with translational initiation codon ATG and termination codon TGA. Confirmation of xylanase gene cloning in pET28 (+) vector was done by recombinant vector double digestion with *Nhe*I and *Hind*III restriction enzymes. A successful transformation and expression of the construct pET_GthC5Xyl was carried out in *E. coli* BL21 (DE3) cells. Xylanase expression was induced with 1 mM IPTG at 37°C and higher production of recombinant xylanase was achieved.

Bioinformatics Analysis

Deduced amino acid sequence of xylanase from *Geobacillus thermodenitrificans* C5 having showed no presence of cysteine amino acid residues but an excess of (Asp+Glu) negatively charged residues. Aliphatic index of 37 was demonstrated by the *in silico* analysis. A wide resemblance with various GH10 family endoxylanases and high homology with other *Bacillus* sp and *Geobacillus* sp was depicted by amino acid sequence analysis with BLASTp (Fig. 2). Available crystal structure of xylanase (PDB ID, 1HIZ chain A) from *G. thermodinitrificans* was utilized for proposing GthC5Xyl secondary and tertiary structures. A total of 11 α helices along with 5 sharp turns and 13 β sheets were found in secondary structure. Important catalytic residues Glu was found within the conserved region present inside a "bowl" shaped structure of GH10 xylanase via 3D structure obtained from PyMol PDB viewer. Phylogenetic relationship of GthC5Xyl of *G. thermodenitrificans* C5 with other xylanases available at NCBI database showed maximum identity with xylanase of *Geobacillus* sp. TC-W7 (GQ857066) and *G. thermodenitrificans* strain JK1 (JN209933).

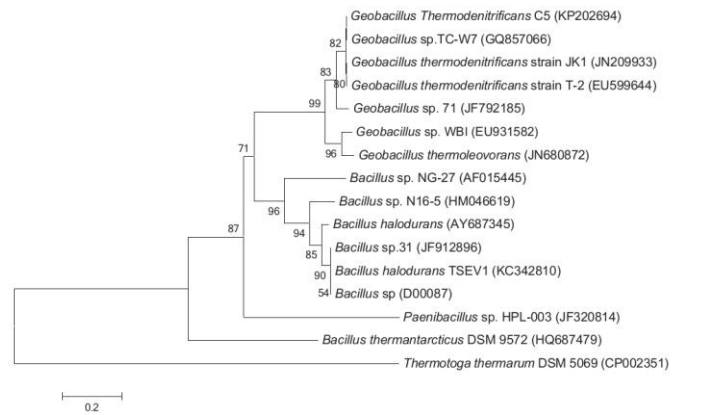

Figure 2 Phylogenetic relationship of *xyl* of *G. thermodenitrificans* C5 with other xylanases available at NCBI database. Neighbor-joining tree showed maximum identity with xylanase of *Geobacillus* sp. TC-W7 (GQ857066) and *G. thermodenitrificans* strain JK1 (JN209933)

Purification of the GthC5Xyl

Elution of recombinant GthC5Xyl was done by DEAE affinity chromatography column with 0.55 M of NaCl (Supplementary Fig. 2). The protein eluted was visualized as a single band on 15% SDS- PAGE which indicated its homogeneous nature (Fig. 3). GthC5Xyl's molecular weight was found out to be approximately 44 kDa. This was confirmed by denaturing protein markers. SDS-PAGE and reverse phase HPLC on C-18 column were used to check the purity of purified protein. A single peak was revealed at retention time of 2.5 min by HPLC chromatogram confirming the purity of preparation (Fig 4) with control of *Bacillus subtilis* (Supplementary Fig. 3). Purified xylanase yield was 176.28% with a specific activity of 1243.12 IU/mg and an overall purification fold of 9.89 (Table 1).

Table 1 Summary of GthC5Xyl purification steps

Purification step	Total Protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification Yield
Cell extract	4.49	564.1286	125.6411	100	1
Precipitate	3.03	714.4617	235.7959	126.6487	1.876742
DEAE-Sepharose	0.8	994.5004	1243.125	176.2897	9.894258

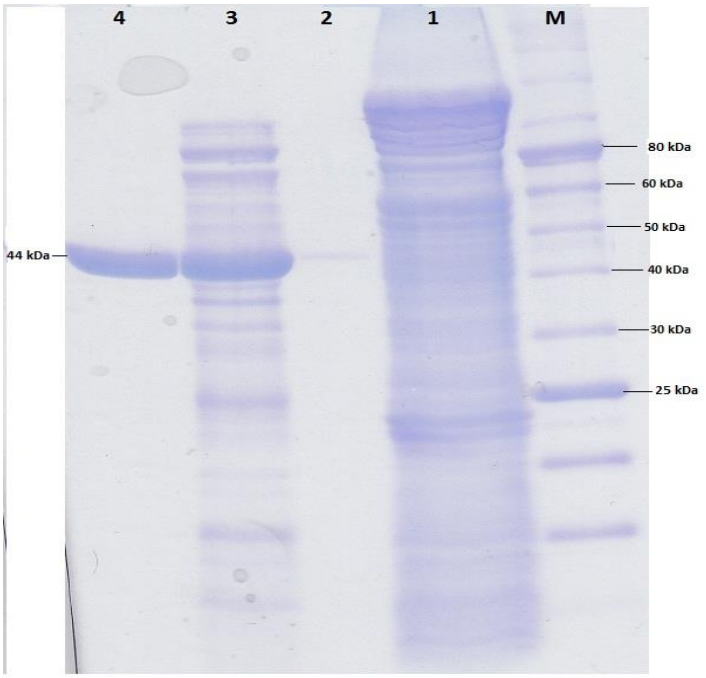

Figure 3 SDS-PAGE showing purified recombinant *Xyl* enzyme obtained from ion-exchange column chromatography by DEAE Sepharose. M, SDS-PAGE molecular mass standards 10–250 kDa New England Biolab; 1, culture supernatant; 2, Induced supernatant; 3, ammonium sulfate precipitation; 4, Purified GthC5Xyl

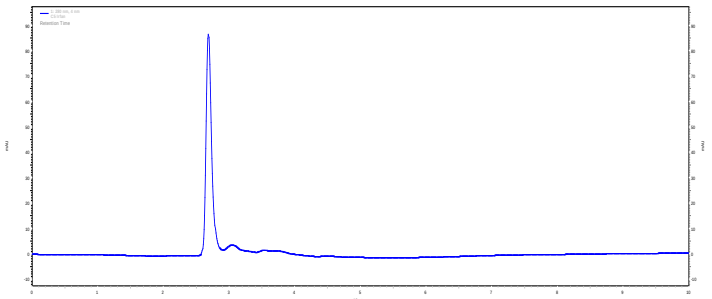

Figure 4 HPLC profile of the purified xylanase from *Geobacillus thermodenitrificans* C5 using a reverse phase C-18 column (4.6 x 250 mm) HPLC chromatogram of the purified enzyme shows a single peak at a retention time of 2.5 min confirming that it was a pure preparation.

Biochemical Characterization of Recombinant Xylanase

The purified GthC5Xyl exhibits activity over a broad range of temperature (40–90°C) and pH (3.0–9.0) with optimum temperature at 60°C (Fig. 5) while optimum pH was 6.0 (Fig. 6). The recombinant GthC5Xyl retained more than 80 % activity after exposure to 60°C for 200 min, and retained more than 70%

activity after exposure to 70°C and 50°C for 200 min (Fig. 7). The enzyme retained more than 80% activity after 3 h at various pH values (6.0, 7.0 and 8.0) (Fig. 8).

In order to verify the effect of substances on GthC5Xyl activity, the purified GthC5Xyl was incubated in the presence of several metallic ions, and detergents such as, sodium dodecyl sulfate (SDS), tetrasodium ethylenediaminetetraacetate (EDTA), dithiothreitol (DTT), phenylmethylsulfonyl fluoride (PMSF), and β-mercaptoethanol, at 1 mM, 5 mM and 10 mM concentrations (Table 2). In general, the GthC5Xyl activity was enhanced with increased concentration of the substances used. Hg^{2+} and $FeSO_4$ were strong inhibitors of the GthC5Xyl while $CaSO_4$ partially inhibit GthC5Xyl at higher concentration. The GthC5Xyl has been inhibited by $AlCl_3$, $ZnSO_4$, $MgCl_2$ and MgSO4 at 10 mM concentration (Table 3). CsCl inhibited the GthC5Xyl at 5.0 mM concentration only. $CoCl_2$, AgNO3, $MnSO_4$, $CaCl_2$, KCl, $CuCl_2$, $MnCl_2$ and $CuSO_4$ does not show inhibitory effect on GthC5Xyl.

The GthC5Xyl is quite stable in the presence of the detergents tested but half of its activity is inhibited in the presence of 1% CTAB concentration and more than 80% activity is retained even at 1% concentration of Tween 20. Triton X-100 and Tween 40 inhibit enzyme at higher concentration only.

The effect of NaCl on activity of GthC5Xyl revealed that the activity was increased with increasing concentrations till 0.8 M. At 1 M of NaCl, relative activity was 101% which sharply decreased to 71 % at 1.5 M of NaCl (Table 3).

Table 2 The effects of various metal ions, detergents and inhibitors on GthC5Xyl

Control	**100%**		
	1 mM 0.1% (v/v)	5 mM 0.5% (v/v)	10 mM 1% (v/v)
Metal Ions			
$AgNO_3$	91.24	126.81	111.35
CsCl	105.16	85.05	89.69
$MnSO_4$	135.43	157.75	107.48
$AlCl_3$	107.71	110.57	81.96
$CoCl_2$	125.27	120.63	116.76
$ZnSO_4$	93.83	91.24	81.96
$MgSO_4 \cdot 7H_2O$	107.48	98.20	71.13
$CuSO_4$	115	105	92
$FeSO_4$	88	74	66
$MnCl_2$	139	124	125
LiCl	101	97	93
$CaCl_2$	105	101	91
$MgCl_2$	115	98	77
$CaSO_4$	94	88	81
Hg	57	30	
KCl	106	92	91
$CuCl_2$	105	130	101
Inhibitors			
EDTA	104	90	79
TSC	103	103	102
β- Mercaptoethanol	96	97	97
NEM	102	100	74
DEPC	104	102	79
DTT	106	107	113
PMSF	100	86	76
NBS	100	73	54
Detergents			
Tween 20	99	96	84
Tween 40	90	81	63
CTAB	84	46	43
SDS	96	91	85
Triton X-100	98	87	73

Table 3 Effect of NaCl on purified xylanase GthC5Xyl

Salt Concentration	**Xylanase activity %**
0	100±0.08
0.2 M	101 ±0.07
0.4 M	104±0.10
0.6 M	105±0.20
0.8 M	106±0.15
1 M	101±0.22
1.2 M	98±0.30
1.5 M	71±0.24

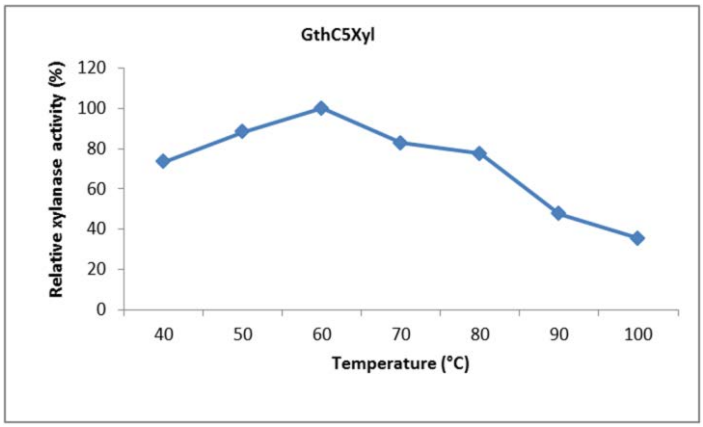

Figure 5 The effect of temperature on the activity of purified xylanase.

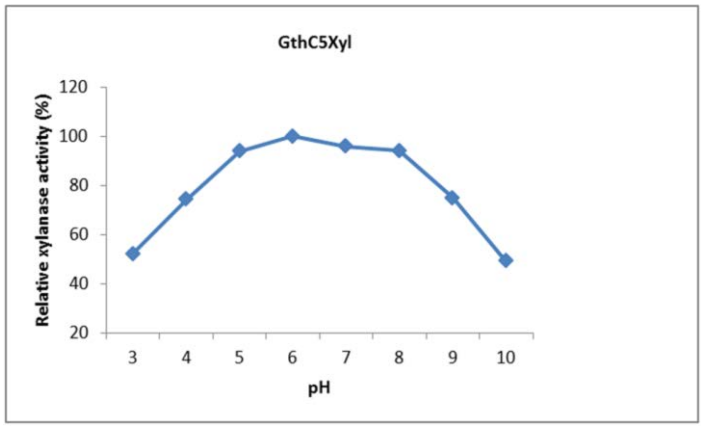

Figure 6 The effect of pH on the activity of purified xylanase.

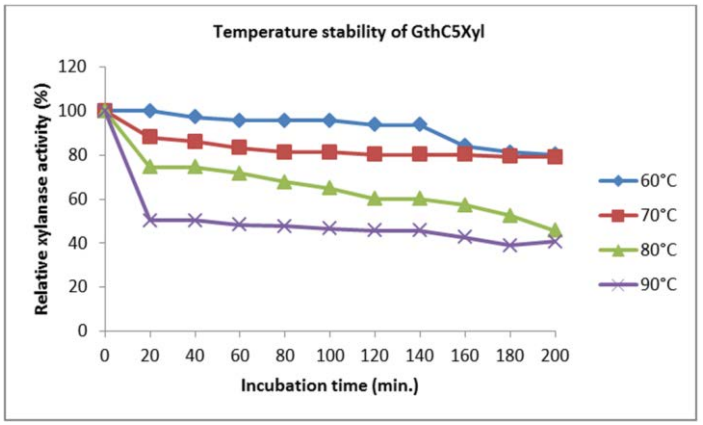

Figure 7 The effect of temperature on the stability of purified xylanase.

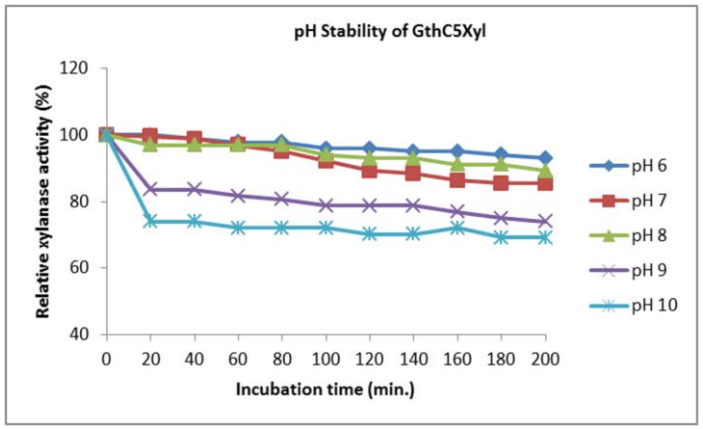

Figure 8 The effect of pH on the stability of purified xylanase

Substrate Specificity, Kinetic Parameters, Mode of Hydrolysis and Determination of Shelf Life of Xylanase

Recombinant GthC5Xyl enzyme activity was evaluated at 60°C and pH 6.0 for 20 min with various substrates for the determination of enzyme specificity (Supplementary Table 2). Specificity of the recombinant enzyme was towards polymeric xylan source and attacked no other substrate such as insoluble xylan, carboxymethyl cellulose, Avicel, filter paper, pNP-β- Xylopyranoside, pNP-α- L-arabinofuranoside, pNP-α- glucopyranoside, pNP- β- galactopyranoside, pNP-α-D- Xylopyranoside and pNP- acetate.

The K_m and V_{max} of GthC5Xyl (for oat spelt xylan) were 3.9084 mgml−1 and 1839.86 μmolmg−1min−1, respectively (Supplementary Fig. 1). TLC of the oat spelt xylan hydrolyzed product was done for the analysis of purified GthC5Xyl mode of action (Fig. 9). A range of xylooligosacharides were released by the action of GthC5Xyl on xylan. Xylopentose and xylobiose were the main products released.

No activity was lost by purified GthC5Xyl when stored for 12 weeks at 4°C but after that decline was observed. After 16 weeks, 90% of initial activity was retained by the enzyme which is an important fact for its industrial application. Conversely enzyme remained completely stable for five weeks at room temperature but showed 80% and 70% residual activity after storage for 10 and 12 weeks, respectively.

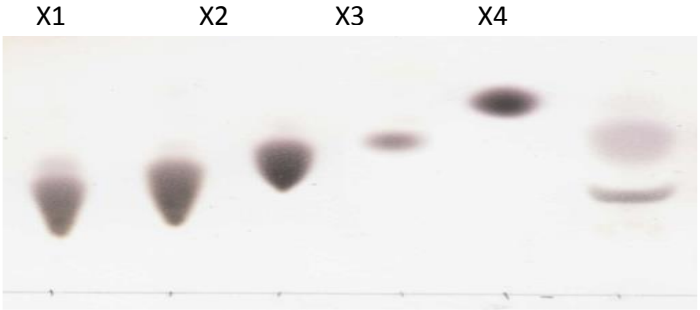

Figure 9 TLC analysis for hydrolysis products released from oat spelt xylan by xylanase from *Geobacillus thermodenitrificans* C5, 1: GthC5Xyl; X5: D-xylose; X4: xylobiose; X3: xylotriose; X2: xylotetraose; X1: xylopentose

DISCUSSION

Hemicellulose is a promising renewable raw material source with immense potential. Its annual production and wastage is humongous and if utilized sensibly it has numerous uses to offer. Xylanase, being regarded as industrially most important enzyme, if employed prudently in industry it can yield clean processes, low energy consumption and higher yields **(Kashyap *et al.*, 2014).** High concentration of salts, surfactants and solvents are required in most of the industrial processes. For instance biofuel production requires ethanol tolerant xylanase, for the bioremediation of industrial waste water contaminated with solvents requires solvent and salt resistant xylanase and deinking of recycled paper requires solvent and surfactant resistant xylanase. Moreover, solvent tolerance enables the enzyme to be recovered, recycled and reused **(Shafiei *et al.*, 2011**; **Ibara *et al.*, 2012**; **Shin *et al.*, 2004**; **Juturu and Wu, 2012**; **Kashyap *et al.*, 2014).**

In industry, the most treasured property of an enzyme is considered to be its thermostability **(Cheng *et al.*, 2014).** So, to study the strategies that an enzyme employs to become thermophilic is an intense area of research. The extraordinarily high specific activity depicted by the recombinant xylanase obtained from Geo*bacillus thermodenitrificans* C5 makes it a worthy candidate for further research.

In the presently conducted study xylanase gene was isolated from Geo*bacillus thermodenitrificans* C5 and then cloned and expressed in *E. coli* BL21 (DE3) host. The expression of this cloned gene of xylanase into the extracellular medium of *E. coli* is the most substantial part of this study which can be employed industrially for commercial xylanase production. One of the interesting facts is the greater xylanase activity of clone in *E.coli* over that of Geo*bacillus thermodinitrificans* C5 strain which may be is the result of higher protein expression. The enzyme produced by *E. coli* functionally active and proficiently degrades oat spelt xylan even in SDS-PAGE gel.

GthC5Xyl optimal reaction temperature was found to be 60ºC which is found to be even higher than that of *Paenibacillus* sp. 12-11 (55ºC) **(Zhao *et al.*, 2011).** GthC5Xyl also retained 77% of its maximum activity at 80ºC and 82 % of its maximum activity at 70ºC (Fig. 5). GthC5Xyl thermal stability was assessed by incubating the enzyme for 200 min at temperatures from 40ºC to 100ºC. The enzyme retained more than 70% of its original activity when incubated for 200 min at temperatures ranging from 40ºC to 60ºC and still retained 79% and 45% of its original activity when incubated for 200 min at 70ºC and 80ºC respectively (Fig. 7). This thermal stability is far better than most of the bacterial xylanases that are previously reported **(Shi *et al.*, 2013**; **Subramaniyan and Prema, 2000**; **Zhao *et al.*, 2011**). Maximum activity of purified GthC5Xyl was found at pH 6.0 which is lower than that of XynG1-1 (7.5) from *P. campinasensis* G1-1 **(Zheng *et al.*, 2012).** Purified enzyme retained 52 % of its activity at pH 3.0 (Fig. 6) and was found stable for 180 min at pH between 5.0 and 8.0 with retaining about 73% activity (Fig. 8). These facts revealed that GthC5Xyl has much higher stability at lower pH than most of the bacterial xylanases reported **(Beg *et al.*, 2001**; **Subramaniyan and Prema, 2000**; **Zhao *et al.*, 2011)**. The stability of this enzyme at higher temperature (60ºC-80ºC) and lower pH renders it worthy for countless industrial applications such as bioenergy conversion, food industry and animal feed.

No significant inhibition of GthC5Xyl activity occurred in the presence of different metallic ions however, it was prudently inhibited by Hg^{2+} and partially by $AlCl_3$ and $ZnSO_4$. As oxidation of indole ring occurs by Hg^{2+} it is possible that the enzyme gets inhibited by the reaction of Hg^{2+} with tryptophan residues **(Zhang *et al.*, 2007).** $MnCl_2$ and $CoCl_2$, showed no inhibitory effect on the enzyme which is similar to *Geobacillus* sp. 71 **(Canakci *et al.*, 2012).** However Mn strongly inhibited xylanase isolated from *Bacillus halodurans* **(Mamo *et al.* 2006/31)** but it differs from *Geobacillus* sp. 71 as it gets inhibited by $ZnSO_4$ whereas *Geobacillus* sp. 71 is resistant to it.

$CuCl_2$ also showed no inhibitory effect on GthC5Xyl activity. This property differed it from xylanases isolated from *Geobacillus thermodenitrificans* TSAA1 **(Verma *et al.*, 2013),** *Plectosphaerella cucumerina* **(Zhang *et al.*, 2007),** *Geobacillus thermoleovorans* **(Verma and Satyanarayana, 2012),** *Thermotoga thermarum* **(Shi *et al.*, 2013),** *Penicillium glabrum* **(Knob *et al.*, 2013),** *Penicillium sclerotiorum,* **(Knob and Carmona, 2010),** *Aspergillus ficuum* AF-98 **(Lu *et al.*, 2008)** which are inhibited by Cu^{2+}. Mn^{2+}, Co^{2+}, $AgNO_3$, and $CuCl_2$ were found to be activity stimulators of GthC5Xyl. Xylanases isolated from *Streptomyces olivaceoviridis* A1 **(Wang *et al.*, 2007)** and *Bacillus subtilis* strain R5 **(Jalal *et al.*, 2009)** had been reported to be stimulated by Fe^{2+}. Recombinant xylanase GthC5Xyl differed from them as it is inhibited by Fe^{2+}.

Sodium dodecyl sulfate (SDS) is a known protein denaturant which strongly inactivates most of the proteins. Even low concentration of SDS deactivates most of the xylanases. Only a few enzymes are reported in literature which show resistance against high concentrations of SDS. For instance xylanase BSX from alkalophilic *Bacillus* sp. NG-27, GH11 xylanase from symbiotic *Streptomyces* sp. TN119 and GH 10 xylanase XynAHJ3 from *Lechevalieria* sp. HJ3, have been reported to retain over 100% of their activities in the presence of SDS **(Bhardwaj *et al.*, 2010**; **Zhou *et al.*, 2012**; **Zhou *et al.*, 2011**; **Zheng *et al.*, 2013).** More than 85% of activity is retained by GthC5Xyl even at 1% SDS concentration and 96% and 91% at 0.1% and 0.5% of SDS concentration respectively which is far better than that of *Geobacillus thermodenitrificans* TSAA1 showing 91% at 0.1% and 72% at 0.5% of SDS concentration (Verma *et al.*, 2013). While xylanase from *Geobacillus* sp. MT-1(Wu *et al.*, 2006), *Bacillus thermantarcticus* **(Lama *et al.*, 2004)** and *Paenibacillus* sp. NF1 **(Zheng *et al.*, 2014)** is significantly inhibited even at 0.02% concentration of SDS. While xylanase from *Melanocarpus albomyces* **(Gupta *et al.*, 2014)** is partially inhibited even at low concentration 0.02% of SDS having 85% of its residual activity. Moreover, GthC5Xyl's activity was also not affected by the presence of the chelating agent EDTA which proposed that metallic cations are not required for its activity. Similar to this *Geobacillus stearothermophilus* and *Bacillus halodurans* xylanases were not inhibited by EDTA, while *Geobacillus* sp. 71 was inhibited by EDTA. CTAB and Triton X can only inhibit the activity of GthC5Xyl at high concentrations.

Substrate specificity and kinetic parameter determination of GthC5Xyl showed that it only exhibits xylanolytic activity. The thin layer chromatography analysis of oat spelt xylan hydrolysis product specified it to be endo-xylanase. Like many other xylanases, the major end products of GthC5Xyl hydrolysis of oat spelt xylan were xylobiose and xylopentose. Acellulytic xylan has added advantages in high quality pulp production. Our results showed that GthC5Xyl has cellulase-free nature.

Up to 1.2 M salt concentration is tolerated by GthC5Xyl with maximum activity of 0.8 M. Similar results were also seen with *Gordonia* sp **(Kashyap *et al.*, 2014).** *Glaecicola mesophila* KMM241 has been reported to secrete xylanase that is active at low temperatures and is salt tolerant as well **(Guo *et al.*, 2009).** *Glaecicola mesophila* retained 90% residual activity at 2.5 M NaCl where as its optimal activity was described at 0.5 M NaCl. Whereas *Bacillus pumilus* xylanase has optimum activity at 1.2 M NaCl (Menon *et al.*, 2010). *Bacillus subtilis* cho40, a marine bacterium has highest activity at at 0.5 M NaCl **(Khandeparker *et al.*, 2011).** However marine bacteria generally secrete halo tolerant enzymes. Xylanase produced by these bacteria have not been characterized for their tolerance to solvents or detergents. All these observations indicated to the fact that xylanase obtained from *Geobacillus thermodenitrificans* C5 has the utmost conditions to resist the harsh conditions of industrial processes and meet the industrial demands.

CONCLUSION

The present work reports the expression, characterization of halo thermostable xylanase from bacterium *Geobacillus thermodenitrificans* C5. It also addresses the property of xylanase such as stability in broad pH range, temperature, NaCl concentration and resistant to SDS. Thus, this strain could be good contender for

different biotechnological applications under extreme conditions. Further, improvements in enzyme production using optimization parameters by statistical approach and use in biobleaching are in progress.

Acknowledgement: Authors are thankful to TUBITAK (2216 Research Fellowship Programme) and Karadeniz Technical University, Turkey to support the project and provide necessary infrastructure to carry out the present research work.

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JMBFS

Journal of Microbiology, Biotechnology and Food Sciences

International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

RAPID AND EFFICIENT METHOD FOR ENVIRONMENTAL DNA EXTRACTION AND PURIFICATION FROM SOIL

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doi: 10.15414/jmbfs.2016.5.6.530-533

ARTICLE INFO

Received 19. 5. 2015
Revised 10. 1. 2016
Accepted 17. 1. 2016
Published 1. 6. 2016

Regular article

OPEN ACCESS

ABSTRACT

Large proportion of microbial population in the world is unculturable. Extraction of total DNA from soil is usually a crucial step considering to the difficulties of study the uncultivable microorganisms. Humic acid is considered as the main inhibitory agent in the environmental DNA studies. Here, we introduced a rapid and efficient method for DNA extraction and purification from soil. Yield of DNA extraction by the presented method was 130 ng/µl. Three conventional methods of DNA extraction including liquid nitrogen incursion, bead beating and sonication were performed as control methods. Yield of DNA extraction by these methods were 110, 90 and 50 ng/µl, respectively. A rapid and efficient one step DNA purification method was introduced instead of hazardous conventional phenol-chloroform methods. Humic acid removal percentage by the introduced method was 95.8 % that is comparable with 97 % gained by the conventional gel extraction method and yield of DNA after purification was 84 % and 73 %, respectively. This study could be useful in molecular ecology and metagenomics study as a fast and reliable method.

Keywords: DNA extraction, DNA purification, Environmental DNA, Metagenomics, Soil

INTRODUCTION

It is estimated that only less than 1% of bacteria in the world are culturable (**Bürgmann *et al.* 2001**). This limitation has let to problem that researchers lack a universal and perfect insight into microbial communities, their ecological importance and their potential role in biotechnology by standard microbial culture-based methods (**Liles *et al.* 2008**). Molecular based methods provide a useful and reliable approach to the unculturable microbial world studies (**Lakay *et al.* 2006**). One of the fundamental steps toward the study of uncultured microbial communities is the isolation of nucleic acids from the environmental samples in order to discover novel functional genes or to study the diversity and ecological aspects of selected environment (**Young *et al.* 1993**). Diverse methods have been developed and investigated to increase the yield of DNA extraction and purification (**Robe *et al.* 2003**). Type of the soil and biogeography of sampling environment greatly affect the diversity of microbial community and none of the extraction methods can be universally applied for all forms of soils (**Liles *et al.* 2008**). Various modalities including mechanical, physical and chemical enzymatic methods were used by Martin-Laurent *et al.* (**2001**). Mechanical methods such as incursion in liquid nitrogen (**Zhou *et al.* 1996**), sonication (**Yeats *et al.* 1998**), bead beating (**Miller *et al.* 1999; Bürgmann *et al.* 2001**), freeze-thawing cycles (**Degrange and Bardin 1995**) as physical methods and application of lysozyme (**Stach *et al.* 2001**) and hot-SDS approaches (**Trevors *et al.* 1992**) as chemical methods for cell lysis, are examples of DNA extraction tools. These approaches usually make the DNA sheared and fragmented so that it can make problems in proceeding (down-stream) steps such as construction of metagenomic library or performing PCR (**Frostegard *et al.* 1999**).

Humic acid compounds are difficult to remove and are usually co-extracted with nucleic acids. As a result, DNA purification and humic acid removal are the most important steps in DNA isolation from soil (**Young *et al.* 1993**).Various methods are developed for purification of DNA and humic acid removal (**Lakay *et al.* 2006**). Some of these methods are employed simultaneous with DNA extraction. Cetyltrimethylammonium Bromide (CTAB) (**Zhou *et al.* 1996**), Polyvinyl polypyrrolidone (PVPP) (**Froestegard *et al.* 1999**) and Guanidinium isothiocyanate (GITC) (**Chen *et al.* 2010**) compounds as well as high salt extraction conditions are used for DNA extraction and purification simultaneously. Agarose gel electrophoresis (**More *et al.* 1994**), Gel filtration resins including Sephadex G200 (**Kuske *et al.* 1998**) and commercial products such as DNA binding columns (**Miller *et al.* 1999**) are used for DNA purification after the extraction.

The objective of this study is to introduce the efficient high quality DNA isolation method from the soil, with less amount of DNA shearing and humic acid. Also, efficiency of extracted and purified DNA was examined by transformation efficiency of restrict digested extracted DNA as well as performance of 16S rRNA and 18S rRNA PCR.

MATERIAL AND METHODS

Soil sampling

Three soil samples 1 (clay), 2 (sandy) and 3 (loamy) were collected from Zarrinabad, Mazandaran, Iran ("36°29'64.58 N", "53°20'96.29 E"), Boroujerd, Khorramabad, Iran ("33°43'53.23 N", "48°15'96.19 E") and Geophysics institute park, University of Tehran, Tehran, Iran ("35°73'90.05 N", "51°38'71.40 E") respectively. Superior layer (3 cm) was removed and the sampling performed from 3-15 cm in depth from the surface. The samples were transferred to laboratory and stored in 4°C.

Environmental DNA extraction

For all three samples, one gram of the soil was sieved with a 1 mm mesh and roots of the plants were removed as many as possible. Then, the soil pounded with mortar and pestle. Three conventional DNA extraction methods were used as control methods, including: Method A) liquid nitrogen incursion was performed. 5 ml of DNA extraction buffer (100 mM Tris-HCl pH 8.0, 100 mM sodium EDTA pH 8.0, 100 mM sodium phosphate pH 8.0, 1.5 M NaCl, 1% CTAB) was added to the soil and lysis of microbial cells was followed by an enzymatic step in which 100 µl of lysozyme solution (25 %) was added to the mixture and incubated for 1 hour in 37 °C (**Zhou *et al.* 1996**). Method B). After the addition of 5 ml of 0.12 M sodium phosphate buffer, one gram of glass beads (0.1 mm) was added to the soil and bead beating was done for 4 minutes in 5000 rpm. Lysozyme was used as described above in method A (**Ogram *et al.* 1987**). Method C) 5 ml of extraction buffer (100 ml of 100 mM Tris-HCl pH 8.0, 100mM sodium EDTA pH 8.0, 1.5 M NaCl) added to the soil and the mixture sonicated for 20 minutes in the frequency of 37 Hz. Followed by 3 cycles of freeze-thaw in -70 and 65 °C temperature (**Yeats *et al.* 1998**). The introduced

method (D) is: first, 5 ml of Z-buffer (**Allen *et al*. 2008**) (100 mM Tris-HCl, 100 mM Na_2HPO_4, NaH_2PHO_4 1:1 solution, 100 mM EDTA, 1.5 M NaCl pH 8.0) was added to the pounded soil and the mixture carefully ground in liquid nitrogen, in the manner that layers of frozen buffer containing soil were picked up and incurred with pestle. After that, 1 gram of glass beads (0.1 mm) was added and the resulting mixture was vortexed for 10 minutes. Homogenized sample then, exposed to the lysozyme with previously described condition. Three cycles of freeze-thaw were done in temperatures mentioned before. In all of the four mentioned DNA extraction methods, the extraction proceeds with the treatment of 1.3 ml of 20 % SDS in 65 ^oC for 2hrs. But for the method D, because of the previous applied lysis steps, the time of incubation reduced to 1 hr. Then, the samples were centrifuged for 12 minutes in 13000 rpm and supernatant was transferred to 50 ml centrifuge tube. Comparison of the methods used was shown in table 1.

Table 1 Composition of various DNA extraction buffers and different lysis treatments for DNA extraction from soil, (+) addition, (-) non-addition.

Method	DNA extraction buffer			Lysis treatments						Reference
	Sodium-phosphate	Tris-HCl	NaCl	Grinding in liquid nitrogen	Glass beads	Lysozyme	Sonication	Freeze-thawing	SDS-incubation time (h)	
A	+	+	+	+	-	+	-	-	2	Zhou ***et al***. 1996
B	+	-	-	-	+	+	-	-	2	Ogram ***et al***. 1987
C	-	+	+	-	-	-	+	+	2	Yeast ***et al***. 1998
D	+	+	+	+	-	+	-	+	1	Current study

DNA Purification

The cell lysate obtained was extracted and purified using two methods:
1- Conventional gel extraction method for DNA extraction and purification
Phenol–chloroform extraction was done according to the standard protocol (**Sambrook and Russell 2001**). Precipitated DNA was then dissolved in 500 µl of TE buffer (10 mM Tris, 10 mM EDTA, pH 8.0) and stored in -20 ^oC. DNA was purified by running on agarose gel. 50 µl of DNA was loaded into agarose gel and electrophoresis was done in 60 V for 2 hr. The DNA was then recovered from the gel by DNA Gel Extraction Kit (Qiagen, USA). Purity of DNA was measured by the spectrophotometer with the ratio of A260/280. Quality of DNA was further evaluated by ligation transformation efficiency and PCR reaction.
2- One step DNA extraction and purification from soil sample
An innovative rapid method for DNA purification was tested. The resulting supernatant from lysis step in method D was mixed with the 2 volumes of NaI solution including (90.8 g NaI, 1.5 g Na_2SO_3, 6 M Guanidine thiocyanate, 140 mM MES (2-[N-Morpholino] ethanesulfonic acid) 0.006 % Phenol Red, 100 ml H_2O) and an equal volume of prepared glass bead using of silica 325 mesh powder (Sigma USA, according to the company manual). The mixture was incubated for 5 minutes at room temperature. Besides, quick spin was done to pellet the glass particles. The pellet washed with 500 µl washing buffer (10 mM Tris-HCl pH 7.5, 80% ethanol) and spined three times. After that, the pellet dried in 55 ^oC and eluted by 50 µl of elution buffer (10 mM Tris-HCl, pH 8.5).

DNA quantity and quality measurement

Total DNA extraction using control methods and introduced method in this study was examined by visualization with ethidium bromide and the purity of DNA was measured by the spectrophotometer device with A260/280 ratio (Jenway 6850, England).

Humic acid measurement

Humic acid content in the mixture was calculated by measuring the absorbance at 465 nm (**Wang and Takeshi 2011**). Humic acid (Sigma) was used as standard.
Polymerase chain reaction
Polymerase chain reaction (PCR) was performed on DNA product extracted by four methods using bacterial 16S rRNA universal primers 9F: (Forward: AAG AGT TTG ATC ATG GCT CAG) and 1541R: (Reverse: AGG AGG TGA TCC AAC CGC A) (**Zhang *et al.* 2003**), and also fungal 18S rRNA gene universal primers nu-SSU-0817: (Forward: TTA GCA TGG AAT AAT RRA ATA GGA) and nu-SSU-1536: (Reverse: ATT GCA ATG CYC TAT CCC CA) (**Prevoste-Boure *et al.* 2011**). PCR reaction was performed with PCR Master Mix (Amplicon, Korea) using Thermocycler device (SensoQuest, Germany).

Bacterial strains and vectors

Escherichia coli strain XL1 blue (Novagen, USA) was selected as a host for competent cell preparation and transformation. Plasmid pUC19 (Novagen, USA) was used as a vector for ligation and transformation.

Ligation, transformation and colony PCR

The samples were successfully digested with restriction enzyme *Bam*H1 (Fermentas, Germany) and ligated into pUC19 *Bam*H1 digested vector. Ligation reaction was performed using T_4 DNA ligase (Fermentas, Germany) with 5 µl of DNA, cloning was done using heat shock calcium chloride method. Positive white colony selection was performed on LB medium (1 % Tryptone, 0.5 % yeast extract, 0.5 % NaCl) contain X-gal, Isopropyl-β-D-thiogalactoside (IPTG) and ampicillin (**Sambrock and Russel 2001**). In order to determine the size of inserted DNA fragments in bacteria, total *E. coli* DNA extracted by boiling method (**Sambrook and Russell 2001**) and PCR was done using pUC19 specific primers B1R (Reverse: CAC ATT TCC CCG AAA AGT GC) and B1F (Forward: ACG GTT CCT GGC CTT TTG C) (**Setayesh *et al.* 2008**).

RESULTS AND DISCUSSION

Here we introduced an optimized method of DNA extraction to have sufficient amount of DNA with high quality required for molecular techniques such as cloning and PCR. Comparison between the amounts of extracted DNA from 4 different methods revealed that there are considerable differences in DNA yield between these extraction methods.

DNA extraction from the soil

For sample 3, yield of DNA extraction was the highest compare to other samples and between differenr methods by presented method was 130 ng/µl and was considerably higher than that of other methods which were 110 ng/µl for the method A, 90 ng/µl for the method B and 50 ng/µl for the method C. For other samples such pattern was also observed (Table 2). Extracted DNA is less sheared in method D comparing to the method A, but it cause more shearing in comparison with method B and C (figure 1).

Table 2 Comparison of DNA yield between different extraction methods and for three soil samples.

	Yield of DNA Extraction			
Soil Sample	**Method A**	**Method B**	**Method C**	**Method D**
1	15	20	20	70
2	80	65	40	100
3	110	80	50	130

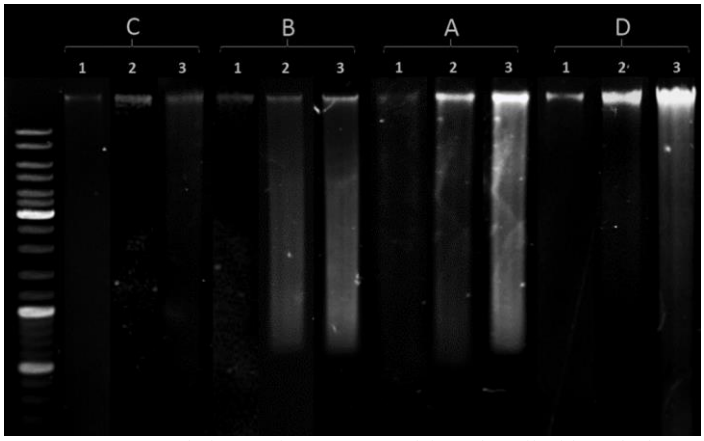

Figure 1 Agarose gel picture of DNA extracted from the soil with method C, B, A and D. C: Sonication lysis, B: Glass bead lysis, A: Liquid nitrogen based lysis as control methods and D: presented modified method.

Based on gel agarose analysis it was obvious that the highest DNA extraction yield is acquired with incursion step in liquid nitrogen. It seems that relatively harsh physical lysis steps in the extraction of DNA from the soil samples are crucial to obtain sufficient quantities of DNA. Liquid nitrogen step in method A and D was most effective lysis step through all four methods and bead beating was evidently more effective than sonication (figure 1). Bead beating was more efficient than lysis of cells with sonication and cycles of freeze-thaw. These results were relatively different from what obtained by Lakay and colleagues. They stated that bead beating method was more efficient in extracting DNA from incursion in liquid nitrogen and microwave based methods (**Lakay *et al*. 2006**). Zou ***et al***. (1996) showed 2 to 6 times higher yield of DNA extraction by grinding in liquid nitrogen comparing to the bead beating and 2 % SDS plus freeze-thaw lysis methods, respectively. Pattern of DNA shearing is different between 4 methods. Sheared DNA in method C is much less than the other methods that evaluation of its DNA fragmentation pattern could be neglected. Various studies show that harsh physical modalities like grinding in liquid nitrogen causes considerable shearing of extracted DNA (**Kabir *et al*. 2003; Zhou *et al*. 1996**). Introduced modified method minimizes the undesired damage to the DNA such as fragmentation. Comparison of method A and D, indicates that although method D has additional two mechanical steps (figure 2), glass bead vortexing and freeze thaw cycles, it cause less DNA shearing than method A and the DNA fragments have larger sizes (figure 1). It is maybe due to the modification performed by the method D of DNA extraction. Addition of buffer before incursion in liquid nitrogen makes a solid iced structure that causes delimitation of this formation during the incursion by pestle. Also, application of freeze-thaw technique after mechanical and enzymatic steps can increase the releasing of genetic content of the lysed cells. Decreasing the time of incubation with SDS in 65 °C was efficient on getting the less sheared DNA product (figure 1 and table 1).

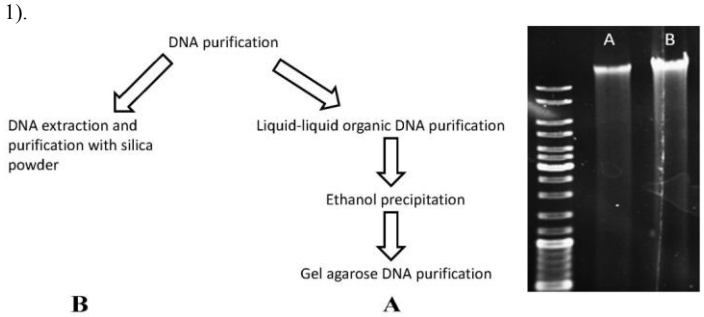

Figure 2 Comparison of two methods for purification of environmental DNA for sample 3. A: Purified DNA using conventional 3 step extraction and purification B: DNA purification by one step extraction and purification method.

DNA liquid extraction and purification

Yield and purity of extracted DNA were two important parameters to evaluate the DNA extraction and purification procedures (**Zhou *et al*. 1996**). One step extraction and purification of DNA which is introduced in this study was efficient in the term of quantity, purity and is cost effective (figure 2). The results obtained demonstrated that DNA extraction and purification with presented method was quicker and more efficient than conventional liquid-liquid organic extraction, ethanol precipitation and gel agarose purification. Purity of extracted DNA has not significant difference between new and conventional control method. For DNA extracted from soil sample 3, A280/A260 nm absorption ratio for conventional method was about 1.75 and that of our method was 1.73. Percentage of humic acid removal was 95.8 % with humic acid content of 374 ng/g for crude DNA extracts and 16 ng/g for purified DNA using introduced method. For conventional purification method, humic acid removal was 97 %. Yield of DNA recovery for introduced purification method was 110 ng/µl (84 %) and 95 ng/µl (73 %) for conventional method. DNA fragmentation shown by the gel agarose electrophoresis with one step extraction purification method was considerably lower comparing to the conventional methods and also, the fragmented DNA molecules in our improved method were larger in size (figure 2). Also, it was very rapid method comparing to the previous time consuming and hazardous methods that would reduce the quality of extracted DNA. In a similar study for DNA extraction protocol, Pushpender ***et al***. (2007) introduced a single step DNA extraction and purification procedure using Q-Sepharose which yielded 88% reduction in humic acid content comparing to the 95% reduction by our introduced method of purification. Introduced method of DNA extraction and purification removes centrifugation steps that impose excess force on DNA and make it fragmented and sheared (**Myers *et al*. 1973**). This fact is notable in Figure 2 that DNA resulted from purification step in comparison of the new and conventional control methods, showed less amount of DNA shearing with new method.

Ligation and transformation

All samples extracted were successfully digested with restriction enzyme *Bam*H1 and after ligation, transformed into the *E. coli*. Colony PCR result showed that colonies containing DNA fragments extracted by method D have larger inserts compared with those of other three methods. Average size of PCR bonds in obtained clones from method D and for sample 3 were 2.5-3 kb. In three different control methods the average sizes were 1-2 kb, almost negative and 1.5-2 kb in A, B and C, respectively (figure 3).

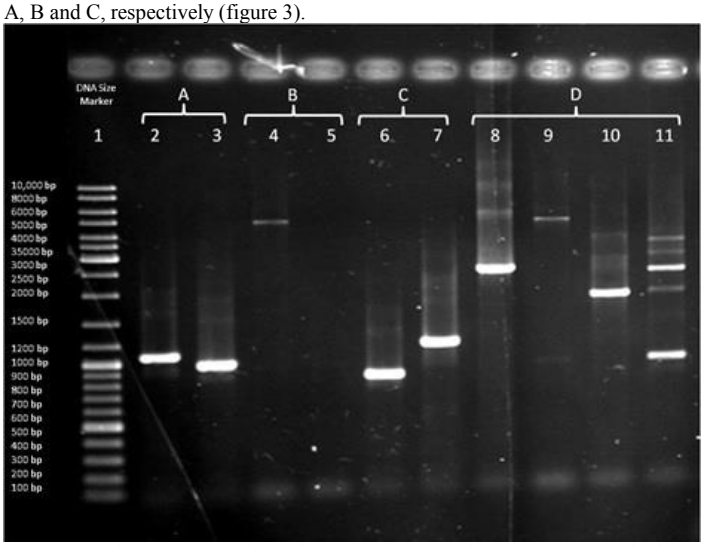

Figure 3 Colony PCR results and average PCR products size in 4 methods for sample 3. Line 1: DNA size marker, Line 2 and 3, colony PCR bands from liquid nitrogen incursion method (A). Line 4 and 5, colony PCR bands from glass bead based method (B), Line 6 and 7, colony PCR bands from sonication based method (C) Line 8-11, colony PCR bands from presented method (D).

PCR

PCR was successfully performed on DNA extracted from soil sample 3 with all four methods. Agarose gel result of PCR products showed that 16s rRNA and 18S rRNA PCR results were almost the same between four DNA extraction protocols.

CONCLUSION

Most reliable approach for study of soil microbial community is molecular based method in which the first step should be the extraction of the DNA from the soil (**Lakay *et al*, 2006**). This process often has difficulties in term of application of lysis methods on the soil and also extracted DNA has humic acid contamination which inhibits many of reactions necessary for molecular studies (**Trevors *et al*, 1992**). Here we introduced a modified liquid nitrogen based DNA extraction method and an innovative method were introduced that could make the extraction of DNA from environmental sources more easy and cheaper. This approach eliminates the need for using phenol for purification and in turn avoids the toxic and carcinogenic effects of this agent and also lessen the time needed for extraction and purification of environmental DNA.

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JMBFS
Journal of Microbiology, Biotechnology and Food Sciences
International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

COST-EFFECTIVE PRODUCTION OF THE BIO-PLASTIC POLY-β-HYDROXYBUTYRATE USING *ACINETOBACTER BAUMANNII ISOLATE* P39

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doi: 10.15414/jmbfs.2016.5.6.552-556

ARTICLE INFO

Received 15. 1. 2015
Revised 26. 11. 2015
Accepted 13. 2. 2016
Published xx.xx.201x

Regular article

OPEN ACCESS

ABSTRACT

Being biodegradable and biocompatible natural polymer, poly-β-hydroxybutyrate (PHB) drew the attention of scientists to substitute synthetic plastics in our daily lives. However, its industrial production is hampered by its high cost. In this study, an extensive screening program was done to isolate bacteria with high PHB productivity from agricultural fields and develop a cost-effective PHB production. A promising bacterial isolate *Acinetobacter baumannii* P39 was recovered and identified using 16S ribosomal gene sequencing. It produced 24% PHB per dry weight after 48 h. Several experiments were conducted to optimize the composition of the culture medium and environmental factors for the selected isolate. Results revealed that 60% aeration, 28°C incubation temperature and initial pH 7.5 showed the highest productivity. Besides, 0.7% corn oil and 0.1 g/L peptone were the best carbon and nitrogen sources, respectively. Substituting glucose with corn oil led to a 23% reduction in total input cost and an estimate price for 1kg PHB is 20.5 L.E. Strain improvement by UV mutation succeeded in improving PHB production by two fold in the selected mutant P39M2. Finally, this study valorizes usage of *Acinetobacter* isolate in PHB production in addition to solving the critical problem of high cost of production.

Keywords: Poly-β-hydroxybutyrate- bioplastic- *Acinetobacter baumannii,* biodegradable- biocompatible

INTRODUCTION

Biodegradable plastics are the ultimate solution for the everlasting problem of synthetic plastic wastes and its deleterious effect on environment **(Poirier *et al.*, 1995; Mecking, 2004)**. Biodegradable plastics, bioplastics, bacterial thermoplastic and green polymers all are names given to family of polyhydroxyalkanoates **(Holmes, 1985; Poirier *et al.*, 1995; Luengo *et al.*, 2003).** They have the same characteristics of synthetic plastics in addition to biodegradability and biocompatibility **(Luckachan and Pillai, 2011).**
Polyhydroxybutyrate (PHB) is the most abundant and well characterized member in polyhydroxyalkanoates (PHA) family. It has a widespread appearance in both Gram positive and Gram negative organisms **(Senior *et al.*, 1972).** These bacteria produce polymer when subjected to unfavorable conditions of excess carbon sources with limitation of other sources such as nitrogen or phosphorus **(Lee, 1996).** The trend nowadays is industrial production of PHB from bacteria to substitute plastics in the market. However, high cost of bacterial PHB production hampered its industrial use in comparison to its rival. Several studies assumed that the reason behind such high cost is the productivity of microorganisms, substrates used and fermentation conditions **(Nath *et al.*, 2008)**. In our previous study, 251 bacterial isolates were recovered from agricultural fields where 53 isolates were PHB producers (Elsayed et al., 2013). By comparing the PHB production among positive isolates, *Acinetobacter baumanii* isolate P39 produced the highest PHB percentage per dry weight among tested Gram negative isolates. It produced 24% PHB per dry weight after 48 hours. It was identified by microscopical examination, cultural conditions and 16S ribosomal RNA sequencing. The 16S rRNA sequences were submitted in GenBank with the following accession code KC876036. PHB production in *Acinetobacter* has not been extensively studied in literature. **Rees and his coworkers** studied PHB production in only three strains of *Acinetobacter* **(Rees *et al.*, 1992).** Also Vierkant and his coworkers (1990) reported the presence of PHB in *Acinetobacter* species **(Vierkant *et al.*, 1990).** Accordingly, the objective of our study was testing different environmental and nutritional factors affecting PHB production using *Acinetobacter baumannii* isolate P39 and strain improvement by physical and chemical mutations. The aim of this study was also to shine a new light on cost-effective PHB production using *Acinetobacter baumannii*.

MATERIAL AND METHODS

Microorganisms

Acinetobacter baumannii (accession code KC876036) is a PHB producing microorganism isolated from agricultural fields in Egypt. The organism was preserved in Luria Bertani (LB) medium supplemented with 20 % glycerol at -20°C.

Culture Media

In lab formulated basal mineral salts medium (MSM) and the newly formulated medium 39M were used in this study and their compositions are listed in Table 1. The ready-made culture media and media ingredients were obtained from Lab M (Topley house, England), Oxoid (USA) and Difco (Detroit, USA).

Production of PHB

The pre-culture was prepared by transferring a loopful from bacteria into 5 ml LB broth, incubated at 37°C and at 160 rpm for 20 hours. The flasks were inoculated with the pre-culture at 5 % v/v and incubated in a shaking incubator (200 rpm) at 37°C for 48 hours. At different time intervals (time course experiments) or at the end the incubation period (other experiments), the broth was sampled to determine biomass and PHB concentration.

Analytical Methods

Biomass determination

Cellular growth was expressed in terms of dry cell weight which was calculated from the equation of a calibration curve constructed between optical density (OD 640 nm) and dry cell weight of the tested isolate *Acinetobacter baumannii* isolate P39 **(Elsayed *et al.*, 2013).**

Table 1 Composition of MSM and newly formulated medium 83M used in PHB production

Name of ingredient	Basal medium (MSM)*	Newly formulated medium (39M)*
Carbon source (g/L)	Glucose (7.00 g)	Corn oil (7.0 ml)
Nitrogen source (g/L)	Ammonium chloride (0.10 g)	peptone (0.1 g)
Minerals (g/L)	MgSO4·7H2O (0.20 g), CaCl2 (0.01 g) Ferrous ammonium sulphate (0.06 g) Trace elements solution* (1 ml)	No minerals
Carbon source (g/L)	Glucose (7.00 g)	Corn oil (7.0 ml)
Nitrogen source (g/L)	Ammonium chloride (0.10 g)	peptone (0.1 g)
Minerals (g/L)	MgSO4·7H2O (0.20 g), CaCl2 (0.01 g) Ferrous ammonium sulphate (0.06 g) Trace elements solution* (1 ml)	No minerals

* Trace elements solution contains (amount/L) ($CoCl_2$. 6 H_2O (0.20 g), H_3B0_3 (0.30 g), $ZnSO_4$ $7H_2O$ (0.10 g), $MnCl_2$.$4H_2O$ (30.0 mg), $NiCl_2$ (10.0 mg), $CuSO_4$ $5H_2O$ (10.0 mg)
* MSM and 39M media were sterilized by autoclaving. Glucose and trace elements solution were filter sterilized and were aseptically added to the autoclaved media with the indicated concentrations.

PHB concentration determination

For PHB extraction, about 1 ml was taken from MSM growth culture in eppindorff tubes (1.5 ml) previously washed with alcohol followed by hot chloroform (to remove plasticizers) **(Law and Slepecky 1961)**.After centrifugation, the cell pellets were incubated with a sodium hypochlorite solution at 37°C for 1 hour at 160 rpm. After centrifugation, the pellets were washed with 1 ml aliquot each of water, ethanol, and acetone, respectively. About 1 ml hot chloroform was added to extract PHB. After that 10 ml concentrated sulphuric acid (98%) were added and the tubes were kept in a water bath at 100°C for 15 min. The final solution was measured spectrophotometry at 235 nm. A standard curve was constructed between crotonic acid concentrations and absorbance at 235 nm **(Elsayed *et al.*, 2013).**

Effect of different environmental and physiological conditions on PHB production

For each factor the biomass, PHB concentration and its production percent per dry weight for the tested isolate were measured as previously mentioned. The factors investigated were inoculum size, initial pH and incubation temperature.

Effect of aeration

This was done by using flasks of equal sizes (250 ml each) with varying amount of medium in each flask. MSM flasks with different medium volumes (25, 50 and 100 ml) corresponding to (90, 80 and 60 % aeration, respectively) were prepared, inoculated and incubated.

Effect of different media components

a. Effect of replacement of glucose in MSM with other carbon sources. The tested carbon sources included sugars, sugar alcohol, oils and malt extract. The carbon sources showing promising PHB percentage per dry weight were tested at different concentrations.
b. Effect of replacement of ammonium chloride in MSM with other nitrogen sources. The nitrogen sources showing promising results were tested at different concentrations.
c. Effect of different multivalent minerals: The tested minerals are ferrous ammonium sulphate, calcium chloride, magnesium sulphate and trace elements. To study the effect of these minerals both on biomass and PHB productivity, five flasks each contained 20 ml MSM were prepared as follows: One is MSM devoid of all minerals and each of the other four flasks contained one of the tested minerals. The used mineral concentration was the same as that used in MSM medium.
d. PHB production under combined selected pre-tested conditions: From the previously studied factors/ conditions that proved to be optimum for PHB production by the test isolate, a newly formulated medium was prepared and tested. In all cases, the selection criteria for the chosen factors/conditions depended on high PHB percentage per dry weight and reasonable amount of biomass formation not less than 50% of the maximum biomass attained by the respective factor within the same tested category. Time course of PHB production was done for up to 72 h incubation period

Induction of mutation using UV rays

This was done by Direct-Plate Irradiation **(Lin and Wang, 2001)**. A loopful of isolate from a nutrient agar slant was inoculated in LB broth for overnight at 37°C and 200 rpm. All UV irradiations were done in a custom-built UV chamber with a glass front (germicidal lamp 15 W UV lamp of 254 nm). All the plates were incubated at 37°C for 24 h before scoring the number of colonies. This treatment resulted in 99.9% kill as determined by viable count of the survivors. The resultant colonies were collected for PHB production assessment.

Induction of mutation using chemical mutagens

This was done according to **(Lopes *et al.*, 2001)** with minor modifications. Stock solutions of acriflavin and proflavin (each of 50 mg/ml) were prepared in phosphate buffer (50 mM, pH7.5). Aliquots of 0.5 ml from stock solutions of acriflavin and proflavin were separately added to a same volume of cell suspension of test isolate for 30 minutes. After centrifugation, about 200µl of the mutagen treated cells were surface inoculated onto nutrient agar plates then incubated at 37°C for 24 hours. The grown colonies were collected to measure PHB production.

Economic analysis

After evaluating the effect of newly formulated media on PHB production of the test isolate and its mutants, an economic analysis was done according to **Baumol and Blinder (2005).** This analysis was done to determine whether the change in culture conditions along with environmental conditions in newly formulated media affected the cost of production or not. For conducting this, the following parameters were calculated.

- Change in total cost = Total cost in newly formulated medium-total cost in basal medium where total cost included cost of both carbon and nitrogen sources in medium. The cost of included minerals was so small reaching a negligible value.

- Change in cost= $\frac{P_2-P_1}{P_1}$
  - P_1 is the basal medium cost
  - P_2 is the newly formulated medium cost.

RESULTS

As shown in figure (1), maximum PHB percentage per dry weight occurred at 60% aeration while maximum biomass in terms of dry weight occurred using 80% aeration**.** It was found also that pH of 7.5 was the best for maximum biomass and PHB percentage per dry weight. The best incubation temperature for maximum PHB percentage per dry weight without decreasing biomass was 28°C. Maximum PHB percentage per dry weight was achieved using paraffin oil. On the other side, maximum PHB accumulation was achieved using corn oil where 181 µg/ml PHB was produced . After studying different concentrations of both agents, it was found that 0.7% corn oil is the best concentration to be used in further experiments (Figures 2 and 3).
After studying effect of different nitrogen sources on the tested parameters, peptone was the best (Figure 4). The concentration of 0.1g/L was chosen for further experiments. As shown in figure (6), maximum PHB production percentage per dry weight and biomass formation occurred using MSM free from all minerals. Individual testing of respective minerals showed variable effects on all of tested parameters. After implementing all of the optimum factors in the new medium 39M, Maximum PHB percentage per dry weight occurred after 48 h (28%). Maximum biomass occurred after 24 h (Figure 7).
UV induced mutation improved the biomass to a great extent in collected two variants (39M2-M3) in comparison to wild organism. It also increased PHB productivity in one variant P39M2 by two fold (Data not shown). However it decreased PHB percentage per dry weight due to high biomass (Figure 8). On the other side chemical mutation decreased both of PHB productivity and percentage per dry weight in the collected variants. The outstanding effect of examining the PHB percentage per dry weight and biomass of selected mutant P39M2 using new medium 39M was achieving its highest values after only 24 h rather than 48 h in case of wild organism (Figure 9).

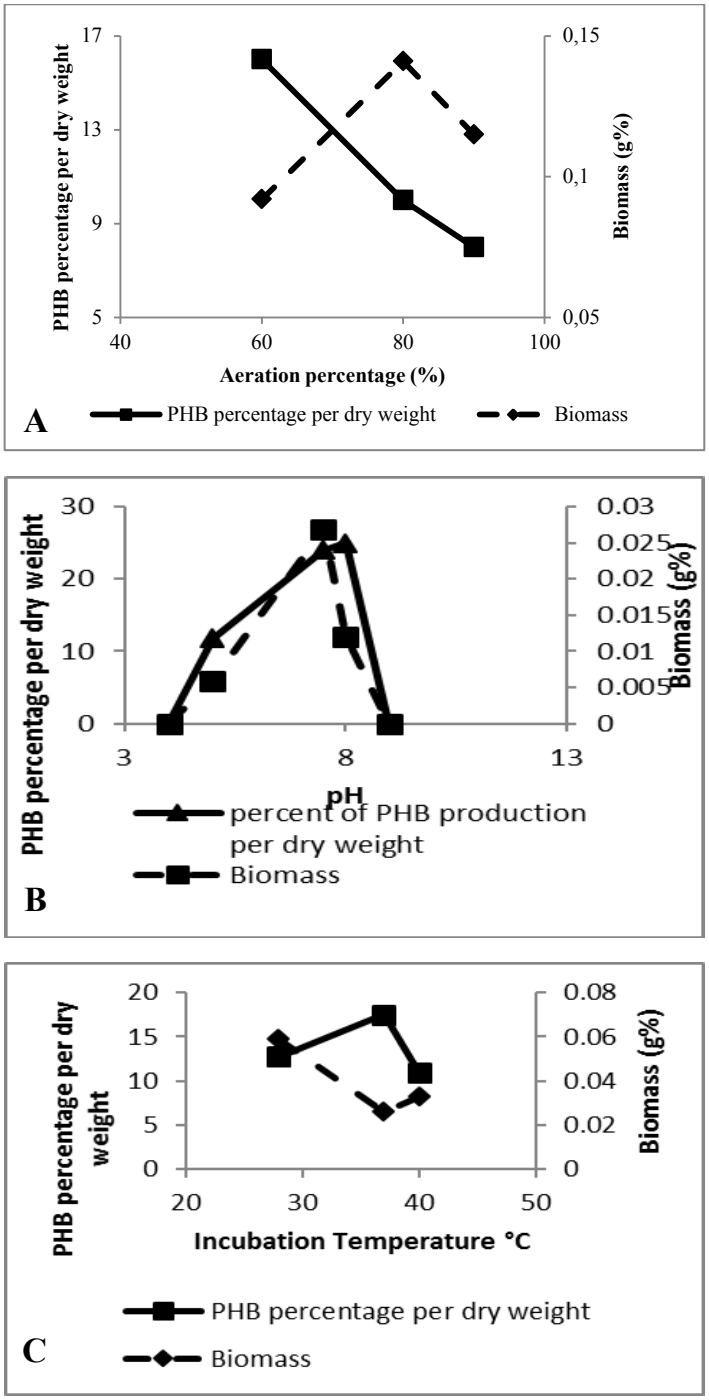


Figure 1 Effect of aeration (A), pH (B) and incubation temperature (C) on PHB percentage per dry weight and biomass of *Acinetobacter baumannii* isolate P39

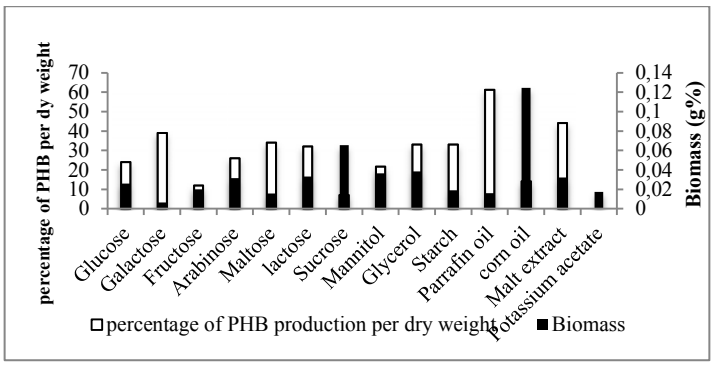


Figure 2 Effect of different carbon sources on PHB percentage per dry weight and biomass using *Acinteobacter baumannii* isolate P39

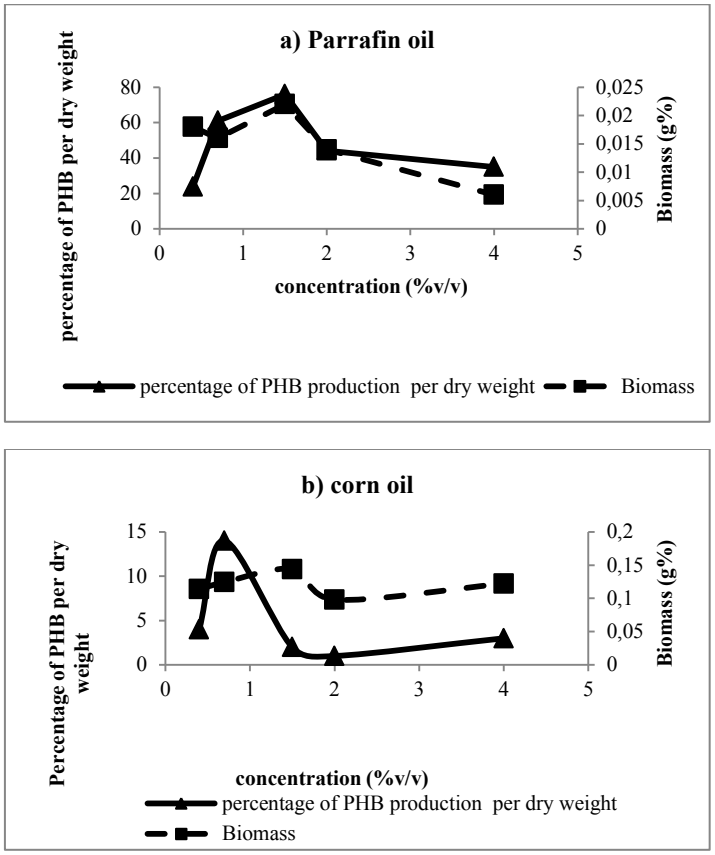


Figure 3 Effect of different concentrations of paraffin oil (a) and corn oil (b) on PHB production, biomass formation and PHB production percentage per dry weight of *Acinetobacter baumannii* isolate P39

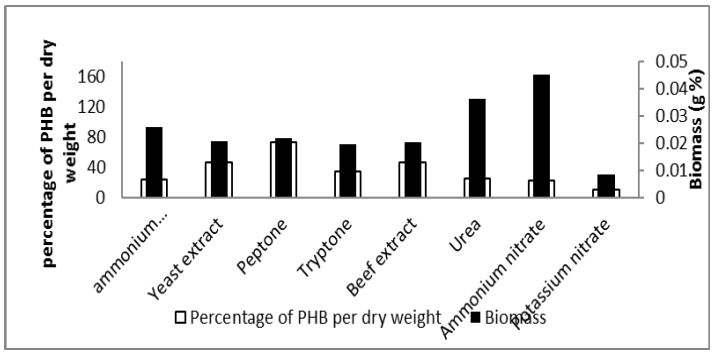


Figure 4 Effect of different nitrogen sources on PHB percentage per dry weight and biomass using *Acinteobacter baumannii* isolate P39

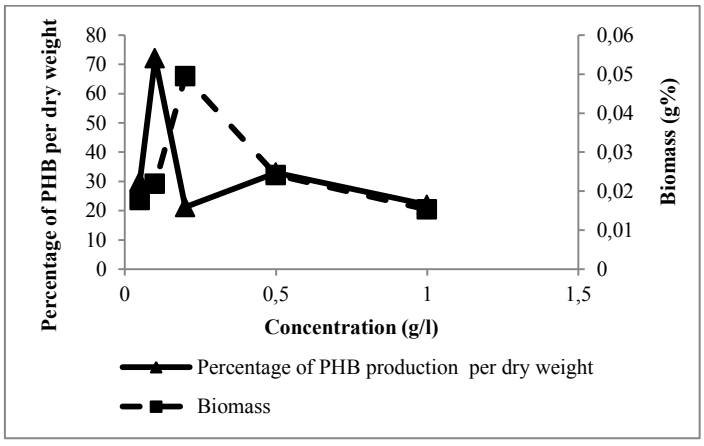


Figure 5 Effect of different concentrations of peptone on biomass formation and PHB production percentage per dry weight in *Acinetobacter baumannii* isolate P39

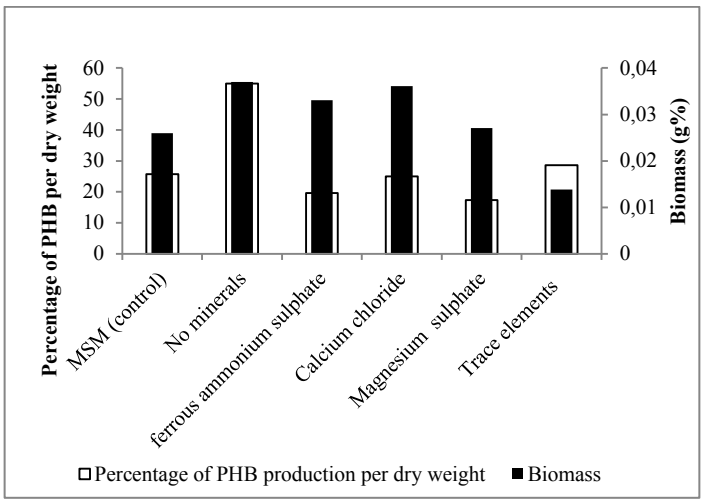


Figure 6 Effect of different minerals on PHB percentage per dry weight and biomass using *Acinteobacter baumannii* isolate P39

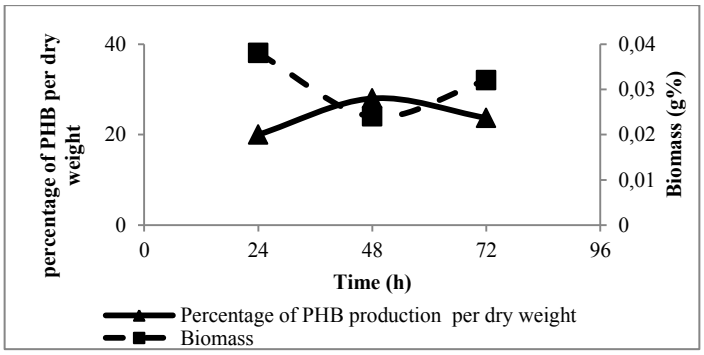
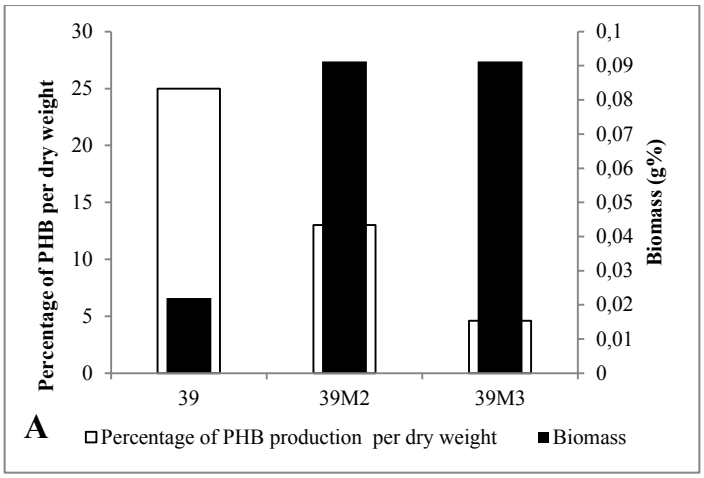


Figure 7 Time course of biomass formation and PHB production percentage per dry weight of *Acinteobacter baumannii* isolate P39 using newly formulated P39M



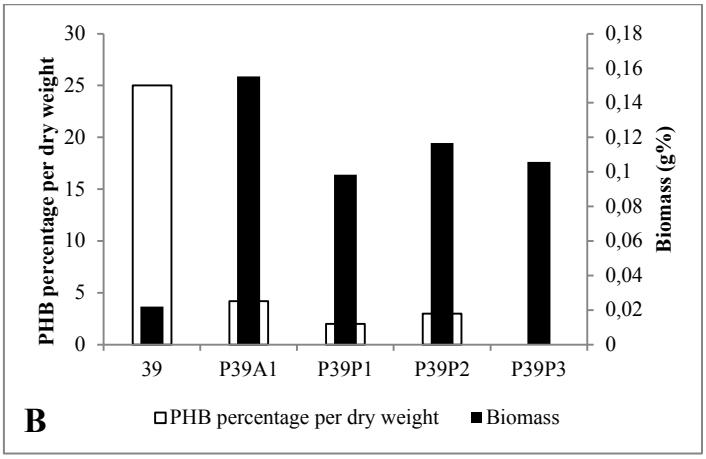


Figure 8 Effect of UV mutation (A) and chemical mutation (B) on biomass formation and PHB production percentage per dry weight of *Acinteobacter baumannii* isolate P39

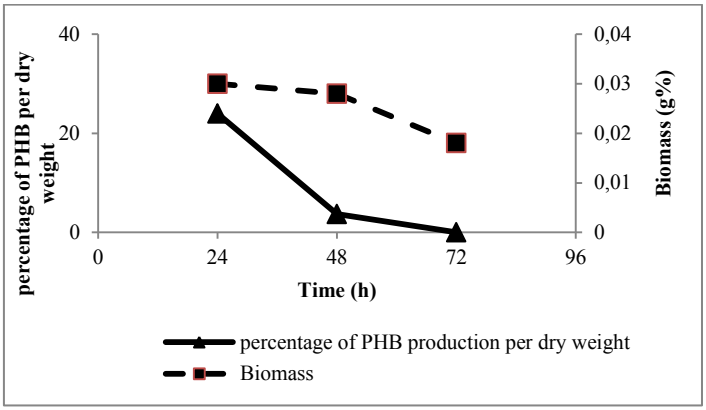


Figure 9 Time course of PHB production percentage per dry weight and biomass formation of the selected *Acinetobacter baumannii* recovered mutant P39M2 in medium 39M

Economic analysis

Table 2 The total cost of the basal medium in the tested isolate

Ingredient	Unit Price	Cost
Glucose 7.0 g/L	12 L.E per 500 g	0.1680 L.E
Ammonium chloride 0.1 g/L	16 L.E per 500 g	0.0032 L.E
	Total cost (P1)	0.1712 L.E

Table 3 The total cost of the newly formulated medium 39M

Ingredient	Unit price	Cost
Corn oil 7.0 ml/L	16 L.E per 1 L	0.1120 L.E
Peptone 0.1 g/L	99 L.E per 500 g	0.0198 L.E
	Total cost (P2)	0.1318 L.E

Change in total cost

- Change in cost= $\underline{P_2-P_1}$* 100

Price of produced PHB

Acinteobacter baumanaii isolate P39 produced 78mg/L PHB (output) using newly formulated medium (39 M).
So estimated price of 1Kg PHB produced using *Acinteobacterbaumanaii isolate P39*and corn oil as carbon source should be not less than 20.5 L.E.

DISCUSSION

After testing the effect of environmental factors, it was obvious that some factors increase biomass only and others increase PHB production in particular. As a result we tend to choose the factor that increases PHB percentage per dry weight without decreasing biomass as latter is a perquisite for PHB production. According to pH, it was found that pH 7.5 was the best for maximum PHB production. This agreed with several studies **(Sangkharak, and Prasertsan 2008; Liu *et al.*, 2011)**. Moreover some studies stated that optimum temperatures for PHB production vary from 25°C to 37°C **(Grothea *et al.*, 1999).**

This agreed with our results where incubation temperature of 28°C was chosen. Pre-culture was done in LB broth as it is rich in nitrogen so it would improve protein synthesis and accordingly increase biomass **(Ramadas *et al.*, 2009).** Best inoculum size was 5%.

Excess carbon source in the culture medium with limited nitrogen source trigger bacteria to produce PHB to be used later as carbon source during starvation. Different categories of carbon sources were tested in this study: monosaccharaides; disaccharides; polysaccharides; sugar alcohols; oils and unrefined carbon sources. In our study, maximum PHB production was achieved using corn oil while maximum PHB percentage per dry weight was attained using paraffin oil. This agreed with several studies which stated that vegetable oils can be good carbon substrate for PHB production **(Fukui and Doi, 1998; Budde *et al.*, 2010)** but, to the best of our knowledge, no other studies have mentioned the usage of paraffin oil in PHB production.This may be due to its inhibitory effect on biomass formation. After testing different concentrations of corn oil and paraffin oil, it was found that 0.7% corn oil gave maximum PHB percentage per dry weight without decreasing biomass. **Vierkant and his coworkers (1990)** studied PHB production in six strains of *Acinteobacter*. They found that glucose was optimum for three out of six strains of *Acinetobacter* and xylose for one and others did not respond to monosaccharaides **(Vierkant *et al.*, 1990).** Rees and his coworkers (1992) also studied production of PHB in two *Acinetobacter* isolates using acetate salts, only one strain produced PHB using acetate while the other not **(Rees *et al.*, 1992).**

Brandl and his coworkers (1990) stated that usually, nitrogen-limiting conditions were chosen for the experimental work, because they are easily achieved by omitting ammonia from the growth medium **(Brandl *et al.*, 1990).** **Borah and his coworkers (2002)** proposed that PHB synthesis efficiency depend on type of nitrogen source used **(Borah *et al.*, 2002)**. In our study the best nitrogen source was peptone. **Page (1992)** stated that peptone was the best nitrogen source for *Azotobacter* as it provides the cells with some amino acids so spare the need for de Novo amino acid synthesis **(Page, 1992)**.Pal and his coworkers stated that peptone is the optimum nitrogen source **(Pal *et al.*, 2009).**

By studying effect of minerals, it was found that minerals were not required for optimum PHB production. Some studies agreed with our results that absence of minerals was the optimum case **(Sangkharak and Prasertsan, 2008).**

After implementing all of optimum factors together in one newly formulated medium 39M, only a slight increase in PHB percentage per dry weight was achieved. But the fascinating result was that a cost-effective production occurred. Substituting glucose with cheap carbon source as corn oil led to a 23% reduction in total input cost and estimated price for the produced PHB will be 20.5 L.E. (tables 2 & 3) The current sale prices of PHB are between 3.1 and 4.4 USD/kg, using glucose and/or sucrose as carbon sources **(Naranjo *et al.*, 2013).** Such current sale price took in consideration all of production factors as machines and labor but our calculation was done for small scale production process only. In addition to that the selected mutant 39M2 produced 24% PHB percentage per dry weight after shorter incubation period (24 hours) in comparison to wild organism.

CONCLUSION

This study paves the way towards high PHB production using *Acinetobacter baumannii* isolate P39. Future studies will be directed towards large scale production of PHB followed by molecular characterization of genes involved in biosynthesis of PHB.

Acknowledgments: We would like to acknowledge department of Microbiology and Immunology, faculty of Pharmacy, Ain Shams University for giving us all facilities and equipment to accomplish this work. Also we thank Prof. Dr. Manal Metwally professor of economics in faculty of economics and political sciences in Cairo for her valuable assistance in economic analysis of PHB production in this research.

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JMBFS
Journal of Microbiology, Biotechnology and Food Sciences
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UTILIZATION OF AGRO-INDUSTRIAL WASTE BY HIGHER MUSHROOMS: MODERN VIEW AND TRENDS

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doi: 10.15414/jmbfs.2016.5.6.563-577

ARTICLE INFO

Received 16. 7. 2015
Revised 24. 12. 2015
Accepted 15. 2. 2016
Published 1. 6. 2016

Review

OPEN ACCESS

ABSTRACT

Waste management and providing a world population with rich in protein food are two important problems of which the utilization of agro-industrial (agriculture and food industry) waste by higher mushrooms causes the growing interest of researchers around the world. More than 150 individual types of wastes have been investigated last decade as alternative substrates alone or in various compositions (more than 450 substrates) for cultivation of 52 higher mushroom species (about 100 strains) as evidenced by the results of more than 130 considered in the review scientific publications. All waste is used as a basis for substrates and supplements thereto, are characteristic of the respective continent and region of the world. Publications containing biochemical studies of substrates and fungi confirm that fungi are grown in rich in biologically active substances unconventional substrates, provide a rich biochemical composition of fungi compared with conventional substrates (sawdust, straw, etc.). The disadvantage of many publications is the lack of mention of examined fungi strains, whereas studies of various strains of the same fungus in the same substrate show different results. The prospect of the study of agricultural residues utilization by higher mushrooms consists in the investigations of: productivity, biological efficiency of the process, morphological and biochemical indices of cultivated mushrooms, depending on the biochemical parameters of substrates and the process conditions; safety of cultivated mushrooms.

Keywords: Agro-industrial waste; higher mushrooms, utilization; substrate

INTRODUCTION

Among the world's major problems – providing a rapidly growing world population with food and waste management, including not least the agro-industrial residues (agriculture and food industry). The aim of FAO's program on the Promotion and development of non-wood forest products (NWFP) is to improve the sustainable use of NWFP in order to improve income-generation and food security, to contribute to the wise management of the world's forests and to conserve their biodiversity. One of the important group of NWFP is mushrooms. The FAO report author (**Boa, 2004**) did not took into account that world population increased on over 800 million people the last decade and reached 7.25 billion. The planet's climate is changing, and not for the better. Under these conditions, to count on mushroom wildlife resources as food, speaking at the same time about the preservation of biodiversity, is impossible. The problem of protection of the natural mushroom flora can be solved by the artificial cultivation of fungi. Wood waste (sawdust, conifer waste) is the traditional substrate for mushroom cultivation (**Stamets and Chilton, 1983; Croan, 2004; Moonmoon *et al.*, 2010; Peng, 2010; Khan *et al.*, 2012**). The using of wood waste for mushroom cultivation has two major shortcomings. A number of researchers indicate lower results of cultivation on sawdust and lower nutritional value of mushrooms in comparison with other using agro wastes (**Onuoha *et al.*, 2009; Ukoima *et al.*, 2009; Tripathy *et al.*, 2011; Govindaraju *et al*,. 2013; Dehariya and Vyas, 2013**). Furthermore, many forest trees are toxic and allergenic (**Meier, 2013**). Among substrates alternative to wood waste **Stamets and Chilton** (**1983**) mentioned for spawn making and fruit body production sugarcane bagasse, cereal straw, rye bran, rye grain, wheat bran, wheat grain, farm yard manure and some others. Meanwhile, the growth of agricultural waste in the last decades of the XX century, have attracted the researchers attention to this type of potential cheap substrates for the cultivation of mushrooms. Numerous studies have been summarized by **Poppe** (**2000**). More than 150 kinds of wastes have been proven to be useful for 45 mushroom species growing. But some listed wastes can be re-divided into at least 100 individual types of wastes. In his next worldwide survey on waste that can be used as a substrate in the Oyster mushrooms cultivation **Poppe** (**2004**) named near 90 residues (not only agro-industrial, but some forestry and industrial). In some cases, was noted the possibility of cultivation the other species of fungi in this or that waste. Last decade the situation with the aforementioned important world problems worsened. The number of undernourished and hungry people has not decreased but at the same time one third of food produced for human consumption is lost or wasted globally – a total 1.3 billion tons a year (**European Commission. Press release, 2014**).

Hundreds of studies devoted to the cultivation of mushrooms on agricultural waste have been carried out, but their ordering and review was not conducted. **Mane *et al.*** (**2007**) presented an overview of 26 substrates reported to be useful for the cultivation of various *Pleurotus* species (only 8 – in publications after 2000), 8 substrates and 44 combinations of these substrates and 3 additives for *P. sajor-caju* cultivation. The basic plant substrates that can be used for *Pleurotus* spp. cultivation are sawdust, wheat straw, rice husk, Mango, Jackfruit, Coconut, hulls, straw, stalk , paper, corn cobs, waste cotton, leaves and pseudo stem of banana, water hyacinth, duck weed, rice straw etc. (**Josephine, 2015**). Mini-review by **Kulshreshtha *et al.*** (**2014**) devoted to the achievement and current status of mycoremediation technology based on mushroom cultivation (7 species) for the remediation of waste and also focused on the safety aspects of mushroom cultivation on waste. Thus, the analysis of publications, identification of trends and prospects of utilization of agricultural waste by higher mushrooms is relevant and important. Publications (Table) should be divided into three categories. Some researchers explored primarily yield, biological efficiency (BE) and morphological parameters of fungi. Of great interest is the analysis of the biochemical composition of the substrates and mushrooms grown on them, the definition of therapeutic activity of fungi, depending on the substrate. The use of food processing waste is separate important problem.

YIELD, BIOLOGICAL EFFICIENCY AND MORPHOLOGICAL PARAMETERS OF MUSHROOMS

Agaricus bisporus is the largest commercially produced mushroom in the world. **Ram and Kumar** (**2010**) investigated morphological parameters of *A. bisporus* fruiting bodies (initiation of pin heads, harvesting of flushes, diameter and weight of fruit bodies), total yield in cultivation on different agricultural waste. Six mixture formulations were used as substrate for studies (Table 1) Maximum weight of sporophore was obtained with application of casing coconut coir pith + vermi compost + sand, where maximum length of stalk was recorded from coconut coir pith + farm yard manure + sawdust casing mixture. The total maximum yield (1199.99 g) was obtained by the application of coconut coir pith

+ vermi compost + farm yard manure + sawdust + sand, which differs significantly.

Pleurotus species (Oyster mushrooms) is the second most cultivated edible mushroom in the world. Optimal conditions for *P. ostreatus* growth, yield, biological efficiency (BE) and mushroom size have been studied by a number of authors (**Elenwo and Okere, 2007; Pathmashini *et al.*, 2008; Kumari and Achal, 2008; Nwokoye *et al.*, 2010; Samuel and Eugene, 2012; Oseni *et al.*, 2012; Govindaraju *et al.*, 2013; Yang *et al.*, 2013; Ashrafi *et al.*, 2014; Mohammed *et al.*, 2014**). Two crops of *P. ostreatus* were grown on rice straw as the basic substrate. In crop I, rice straw was mixed at spawning with 0 %, 25 %, 50 %, 75 % and 100 % of banana leaves or *Leucaena leucocephala* or maize bran or maize cobs. In crop II, rice straw was supplemented at spawning with 0 %, 1 %, 2 %, 3 %, 4 %, and 5 % of sunflower or cotton seed cake. Mushroom yield (1,040.0 g) and BE (98.5 %) were greater on a 50/50 mixture of rice straw and banana leaves. Rice straw supplemented with 2 % sunflower seed hulls (yield =1,087.5 g, BE =103.3 %) gave similar yield and BE to rice straw supplemented with 2 % cotton seed hulls (yield =1,073.8 g, BE =101.8 %), and were significantly greater than other supplement ratios. The largest mushrooms (21.0 g) were obtained from non-supplemented rice straw (**Mamiro and Mamiro, 2011**). Three strains of *P. eryngii* such as Pe-1 (native to Bangladesh), Pe-2 (germplasm collected from China) and Pe-3 (germplasm collected from Japan) were cultivated on sawdust and rice straw and their growth and yield parameters were investigated (**Moonmoon *et al.*, 2010**). Pe-1 on sawdust showed the highest biological yield and BE (73.5 %) than other strains. Also, the mycelium run rate and number of fruiting bodies were higher in Pe-1 than other two strains. The quality of mushroom strains was near about similar. On sawdust, the yield and efficiency were better than those cultivated on rice straw, however, on straw the mushroom fruiting bodies were larger in size. *P. florida* cultivation on different substrate compositions has been investigated by **Mondal *et al.*, (2010**). Highest mycelium running rate was found on banana leaves and rice straw (1:1) but the lowest in control. Completion of mycelium running time was lowest on banana leaves and rice straw (1:3 and 3:1). Number of total primordia and effective primordia, found highest in control but the maximum pileus thickness was measured from rice straw. Highest biological yield and economic yield (164.4 g and 151.1 g) was obtained from rice straw which was much higher than control. A number of papers devoted to the study of the process of *P. pulmonarius* cultivation on different mixtures of cotton waste and cassava peel (Adebayo et al. 2009), cotton waste alone and combined with rice husk (**Khan *et al.*, 2010**), coir fibre, oil palm waste, sawdust of *Gmelina arborea* and rice straw (**Jonathan *et al.*, 2013**). *P. pulmonarius* was also cultivated on agricultural wastes viz., cotton, rice straw, corn cob, corn husk and sawdust (S). Rice bran was added as a nutritional supplement to each substrate. Data collected after two weeks of incubation were diameter of the cap (pileus), length of stem (stipe) and dry matter of fruiting body. The dry matter (32.4±1.5 g) and pileus (19.2±2.4 cm) of fruiting bodies cultivated on cotton waste supplemented with rice bran (CR) was significantly higher while at the same level of significance, the stipe (18.0±1.2 cm) for corn husk supplemented with rice bran (CHR) has significance difference compared with other substrates. Supplemented substrates yielded better compared with non-supplemented substrates. CR was the best substrate followed by CHR while S was least. In addition to sawdust which is widely used by farmers, cotton waste, corn cob, husk and rice straw are possible agro-waste materials for *P. pulmonarius* production (**Adedokun, 2014**). **Mane *et al.*, (2007**) studied the yield and morphological parameters of *P. sajor-caju* fruiting bodies in cultivation on selected agro wastes viz. cotton stalks, groundnut haulms, soybean straw, pigeon pea stalks and leaves and wheat straw, alone or in combinations. Cotton stalks, pigeon pea stalks and wheat straw alone or in combination were found to be more suitable than groundnut haulms and soybean straw for the cultivation. Organic supplements such as groundnut oilseed cake, gram powder and rice bran not only affected growth parameters but also increased yield. Mycelial growth, colonization period, primordial initiation, harvesting time, yield, mushroom size and BE of *P. sajor-caju* were assessed on three different substrates namely maize stalk, pea residue (tendrils) and banana leaves with and without supplementation of rice bran and chicken manure. The faster mycelial growth and highest yield (348.13 g) with 87.03 % BE was obtained from maize stalk with rice bran and second best yield (299.53 g) with 74.88 % BE was recorded from pea residue with rice bran. Among the substrates used, maize stalk appeared best followed by pea residue and banana leaves. Rice bran showed best supplementation for mycelial growth and yield with all substrates (**Pokhrel *et al.*, 2013**). **Dehariya and Vyas (2013**) determined the effect of different agro-wastes viz. soybean straw, wheat straw, paddy straw, sugarcane bagasses, sun flower stalks, maize stalks, domestic waste, used tea leaves, fruit waste, semal flowers, news paper, bamboo leaves, sawdust and their combinations in 1:1 proportion in *P. sajor-caju* cultivation (yield and BE). Soybean straw showed significantly highest yield (with 93.3 % BE). Among all the combinations soybean straw + wheat straw showed significantly highest yield (with 87.3 % BE). The organic wastes (dry substrates which include maize cob, cassava peelings, plantain peelings and water melon pod) were used in the study of *P. tuber-regium* cultivation. Maize cob (T1) and cassava peelings (T2) supported very abundant mycelial growth and also the development of healthy fruit bodies of the fungus studied. Plantain peelings (T3) and water melon pod (T4) supported abundant and moderate mycelial growth of *P. tuber-regium* respectively but fruit bodies were not developed on them. In all parameters measured fruit bodies produced on T1 were better than those on T2 except in dry weight (DW). The DW of *P. tuber-regium* was 21.8 g on both T1 and T2. The BE of *P. tuber-regium* produced on T1 were 8.7 % and 8.5 % on T2. Corn cobs and cassava peelings which are major agro wastes abundantly found in Nigeria, have been found to excellently support the mycelial growth and fruit body formation of *P. tuber-regium* (**Stanley and Odu, 2012**). The powerful enzyme system of *Pleurotus* spp. promotes biodegradation of the wide spectrum of substrates, not only traditional sawdust and cereal straw (Table). The increasing interest in *Pleurotus* spp. mushrooms is explained also of their species diversity.

The influence of seven oak-wood sawdust substrates (OS), supplemented with wheat straw (WS) or corn-cobs (CC) on mycelium growth and sporophore production characteristics of *Lentinula edodes* (popular edible mushroom) was examined by **Philippoussis *et al.*, (2004**). Colonization rate measurements demonstrated faster colonization on OS supplemented with WS or CC in a ratio of 1:2 (OS : supplements). Similarly, higher sporophore yields were obtained on OS + CC mixtures, especially in the supplementation ratios 1:1 and 1:2. However, substrates with high OS content (2:1 ratio) appeared to promote mushroom quality and high protein content of the sporophores. *L. edodes* cultivation on hard wood sawdust, rice straw, crushed corn cobs and crushed bagasse supplemented with 20 % wheat bran, 1 % soy bean flour, 2 % gypsum has been investigated by **Hassan (2011**). Incubation period, early of harvesting, yield and BE were estimated as well as drying parameters for fruit bodies. Sawdust recorded the shortest incubation time and first harvesting day time, while bagasse showed the longest ones. Also, sawdust produced the maximum yield 297 g/ kg wet media with the highest BE, while bagasse recorded the lowest values.

Pani (2012) studied the utilization of cotton wastes and sunflower stalks either alone or in combination with paddy straw (1:3, 1:1, 3:1) for sporophore production of milky mushroom, *Calocybe indica*. The various combinations with paddy straw showed better results (BE) than single substrates (cotton wastes and sunflower stalks). There was also faster substrate colonization and primordial initiation and higher number of fruiting bodies. Cotton wastes + paddy straw (1:3) sustained the highest mushroom yield (73.2 % BE) which was statistically at par with paddy straw (71.3 % BE).

Akavia *et al.*, (2009) investigated the cultivation of five *Hypsizygus marmoreus* strains on 24 substrates (Table 1). Average number of colonized particles per day, BE, number of mushrooms and weight of mushrooms harvested during one month have been studied. The best substrate in terms of BE was corn cob with bran and olive press cake, with a BE of 85.6%. The BE of the same composition but without olive press cake was only 67.5 %.

Harvesting yield and BE of *Pholiota nameko* utilization of different substrates viz. *Eucalyptus* shaving, *Cordia* shaving, coffee husk, *Pinus* shaving, cotton seed and teff straw have been studied by **Gizaw (2010**). Wheat bran was used as an additive material 100:10 and 100:30 w:w of the main material. *Eucalyptus* shaving supplemented with 30 % wheat bran showed the best result (yield = 797.33 g, BE = 53.27 %).

The effect of pH and temperature variations on the growth of *Volvariella volvacea* cultivated on various agricultural wastes singly and in various combinations has been studied by **Akinyele and Adetuyi (2005**). A pH range of 5.5 to 8.5 recorded the maximum mycelia yield and the highest mycelia weight was recorded at pH 6.5. High mycelia growth of the mushroom was also observed between 25°C and 30°C with the highest mycelia DW of 80.0 mg obtained at 30°C. Effect of different substrates on mycelial growth and yield of *Volvariella* spp. (*V. diplasia* and *V. volvacea*) was also evaluated (**Tripathy *et al.*, 2011**). Paddy straw, oil palm fibre, sawdust, and a mixture of oil palm fibre and sawdust were screened for the cultivations of *V. volvacea*. The paddy straw served as the control as it is the traditional substrate for the growth of this mushroom. The straw naturally supported the mycelial growth and production of fruit bodies. Growth and production of fruit bodies on oil palm fibre was similar to that of paddy straw. The production of fruit bodies on the mixture of oil palm fibre and sawdust was scanty. Sawdust alone as a substrate produced few fruit bodies that were comparatively small in size (**Onuoha *et al.*, 2009**). *V. volvacea* showed that it is an active agro waste bio-destructor, not only the traditional for this fungus paddy straw.

Researchers are interested not only in *Pleurotus* spp. and *Volvariella* spp. fruit bodies (first of all) cultivation on cheap substrates, but also in best-known and very delicious edible mushrooms, such as *Agaricus* spp., *Lentinus* spp., *Hypsizygus marmoreus*.

Table 1 Utilization of agro-industrial waste by higher mushrooms

Fungal species	Wastes and some non waste components (if they are basic for substrates or control in studies)	Best results	References
Agaricus bisporus (J.E. Lange) Imbach	mung bean straw; beet pulp	mung bean straw (yield =2.56 kg/10kg)	**(Al Abttan *et al.*, 2005)**
	coconut coir pith; farm yard manure; sawdust; vermi compost (wheat straw, wheat bran, urea, potassium, phosphorus, gypsum molasses and lindane); sand	coconut coir pith +vermi compost + farm yard manure + sawdust + sand (1:1:1:1:1)	**(Ram & Kumar, 2010)**
	rice straw; rice bran; chicken manure	***	**(Peng, 2010)**
Agaricus bitorquis (Quel.) Sacc.	rice straw; rice bran; chicken manure	***	**(Peng, 2010)**
Agaricus flocculosipes R.L. Zhao, Desjardin, J. Guinberteau & K.D. Hyde	wheat straw +horse manure	yield = 1.04 g /kg	**(Thongklang *et al.*, 2014)**
Agaricus subrufescens Peck	wheat straw +horse manure	yield = 85.90 g /kg	**(Thongklang *et al.*, 2014)**
Agrocybe aegerita (V.Brig.) Singer	wheat straw; cocoa shells; wheat straw supplemented with either cocoa shells (17 %), citrus pellets (17 %), carrot mesh (17 %) or black tea pomace (17 % and 45 %)	wheat straw supplemented with black tea pomace (17 % and 45 %). BE =36 %	**(Kleofas *et al.*, 2014)**
Agrocybe cylindracea (DC.) Maire	rice bran; wheat bran	***	**(Peng, 2010)**
	wheat straw; two-phase olive mill waste; composted two-phase olive mill waste	wheat straw + 20% composted two-phase olive mill waste (yield =377.91 g)	**(Zervakis *et al.*, 2013)**
Antrodia cinnamomea T.T. Chang & W.N. Chou	citrus peel (pomelo, lemon, orange and grapefruit) extracts	lemon peel extract	**(Yang *et al.*, 2012)**
Auricularia auricula-judae (Bull.) Quél.	dry olive mill residue	increases peroxidase secretion and produced a sharp decrease in total phenolic content	**(Reina *et al.*, 2013)**
Auricularia fuscosuccinea (Mont.) Henn.	rice bran; wheat bran	***	**(Peng, 2010)**
	substrates alone and in combination (1: 1): coir; shells of cacao; banana leaves	banana leaves (mycelial growth rate = 6 mm / day)	**(Carreno-Ruiz *et al.*, 2014)**
Auricularia polytricha (Mont.) Sacc.	sawdust mixed with empty fruit bunches (50:50) + 10 % spent grain; sawdust mixed with oil palm fronds (90:10) + 15 % spent grain and compared to 100 % sawdust	sawdust mixed with oil palm fronds (90:10) + 15 % spent grain – BE = 288.9 %	**(Abd Razak *et al.*, 2013)**
Auriporia aurea (Peck) Ryvarden	amaranth flour after CO_2 extraction	high antiviral activity	**(Krupodorova *et al.*, 2014b)**
Bjerkandera adusta (Willd.) P. Karst.	dry olive mill residue	increases peroxidase secretion and produced a sharp decrease in total phenolic content	**(Reina *et al.*, 2013)**
Calocybe indica Purkay. & A. Chandra	rice bran; maize powder; wheat bran supplement to rice straw	30% maize powder supplement to rice straw (biological yield = 459.3 g/packet)	**(Alam *et al.*, 2010)**
	ten popular paddy straw varieties of Orissa	variety: CR-1014 (70.5 % bioefficiency (BE), Kanchan (69.9 % BE), Jagabandhu (69.6 % BE)	**(Pani, 2011)**
	cotton wastes and sunflower stalks alone or mixed with paddy straw (1:3, 1:1, 3:1)	cotton wastes +paddy straw (1:3)	**(Pani, 2012)**
	coir pith; maize straw; paddy straw; sugarcane bagasse; sugarcane leaves and vettiver leaves	paddy straw (protein=31.2 g/100g , carbohydrate = 58.4 g/100g), coir pith (fat = 0.85 g/100g),	**(Lakshmipathy *et al.*, 2012)**
	sorghum straw; paddy straw; sugarcane bagasse; banana leaves	sugarcane bagasse (yield)	**(Ramanathan *et al.*, 2013)**
	paddy straw; wheat straw; soybean straw; coconut coir pith; cotton waste; sugarcane bagasse	wheat straw; paddy straw	**(Vijaykumar *et al.*, 2014)**
	paddy straw; reeds; banana stem; sugarcane bagasse milled and crushed; sugarcane leaves; coir pith; sorghum husk; sunflower stem	paddy straw (BE = 91.81 %)	**(Karuppuraj *et al.*, 2014)**
Coprinellus radians (Desm.) Vilgalys, Hopple & Jacq. Johnson	dry olive mill residue	increases peroxidase secretion and produced a sharp decrease in total phenolic content	**(Reina *et al.*, 2013)**
Coprinus comatus (O.F. Müll.) Pers.	rice bran; wheat bran	***	**(Peng, 2010)**
	spent of *P. sajor-caju*, *P.ostreatus* and *P.florida* mixed with 100 g of the different enrichment types (corn grit, rice grit and rice bran)	spent of *P. sajor-caju* mixed with 100 g of corn grit (yield =11.06 g)	**(Dulay *et al.*, 2014)**
Flammulina velutipes (Curtis) Singer	rice bran; wheat bran	***	**(Peng, 2010)**
	paddy straw; palm empty fruit bunches; palm-pressed fiber	paddy straw + palm empty fruit bunches (25:75). BE = 185.09 %	**(Harith *et al.*, 2014)**
Fomes badius Cooke	coffee pulp	cellulose degradation (61.3 %); hemicellulose degradation (51.2 %)	**(Parani & Eyini, 2010)**
Fomes fomentarius (L.) Fr.	amaranth flour after CO_2 extraction	high antiviral activity	**(Krupodorova *et al.*, 2014b)**

G*anoderma applanatum* (Pers.) Pat.	milk whey; starch grits	milk whey	**(Krupodorova, 2011)**
G*anoderma lucidum* (Curtis) P. Karst	sawdust (S) + tea waste (TW) at the various levels (75S:25TW, 80S:20TW, 85S:15TW, 90S:10TW) coffee pulp milk whey; starch grits hydrolysed rye straw – 55 %; oak sawdust – 25 %; rye bran – 10 %; corn flour - 8 %; chemically precipitated chalk – 1 %; rock gypsum – 1 %. coir pith + fishery waste (1:1); woodchips + fishery waste (1:1); sugarcane bagasse + fishery waste (1:1) sawdust and rice bran + food waste compost at the various levels	80S:20TW; 75S:25TW cellulose degradation (64.3 %); hemicellulose degradation (51.2 %) milk whey *** coir pith+ fishery waste (1:1) sawdust and rice bran + 10 % of food waste compost	**(Peksen & Yakupoglu, 2009)** **(Parani & Eyini, 2010)** **(Krupodorova, 2011)** **(Patent RU No. 2,453,105, 2012)** **(Lakshmi, 2013)** **(Jo *et al.*, 2013)**
G*anoderma tsugae* Murrill	rice bran; wheat bran	***	**(Peng, 2010)**
Hericium erinaceus (Bull.) Pers.	sawdust + 20 % wheat bran + 1 % $CaCO_3$ + 1 % sugar; rice straw + 20 % wheat bran + 1 % $CaCO_3$ + 1 % sugar; wheat straw + 20 % wheat bran + 1 % $CaCO_3$ + 1 % sugar; sawdust + rice straw + 20 % wheat bran + 1 % $CaCO_3$ + 1% sugar; sawdust + wheat straw + 20 % wheat bran + 1 % $CaCO_3$ + 1 % sugar; rice straw + wheat straw + 20 % wheat bran + 1 % $CaCO_3$ + 1 % sugar rice bran; wheat bran	sawdust (yield = 184 g/kg); wheat straw (protein); sawdust + wheat straw + 20 % wheat bran + 1 % $CaCO_3$ + 1 % sugar (fat) ***	**(Hassan, 2007)** **(Peng, 2010)**
Hypsizygus marmoreus (Peck) H.E. Bigelow	corn cob; bran; olive press cake; cotton straw; crashed cotton seed; cotton waste; wheat straw; banana leaves; pea straw; mandarin peel; red wine waste; soy seed skin; hard almond shells; coffee ground; sunflower seed shells; soft almond shells; sunflower straw; coco fiber in different ratio sawdust; rice bran; wheat bran; corn flour	corn cob 60 %, bran 30 %, olive press cake 10 % ***	**(Akavia *et al.*, 2009)** **(Peng , 2010)**
Lentinus connatus Berk.	paddy straw; sorghum stalk; banana pseudostem	paddy straw	**(Rani *et al.*, 2008)**
Lentinus edodes (Berk.) Singer	Sikhae factory waste with addition of: D-fructose; D-galactose; D-glucose; sucrose; maltose; starch; D-mannose and cellulose; $MgSO_4 \cdot 7H_2O$; $ZnSO_4 \cdot 7H_2O$; KCl, NaCl; $CaCl_2$; $CuSO_4$; $FeSO_4$ oak-wood sawdust; supplemented with wheat straw or corn-cobs 50–80 % wheat straw + 20–50 % olive oil waste + 2–10 % gypsum; 50–70 % wheat straw + 30–50 % olive oil waste + 5–10 % gypsum; 50–60 % wheat straw + 30–40 % olive oil waste + 5–10% gypsum; 57% wheat straw+ 37% olive oil waste+ 6% gypsum 71-78 % hardwood sawdust + 0.1–3 % chalk+0.1-3 % gypsum + rape grain waste (the rest) rice bran; wheat bran barley straw; wheat straw; vineyard-pruning sawdust; rice straw; crushed corn cobs and crushed bagasse supplemented with 20 % wheat bran, 1 % soy bean flour, 2 % gypsum winery and apple wastes (1:1); winery wastes + wheat bran (9:1); winery wastes + rye bran (9:1); apple wastes + wheat bran (9:1); apple wastes + rye bran (9:1) apple wastes + 1.5 % barley; apple wastes + 2 % wheat bran; pear wastes + 1.5 % barley bran; plum wastes + 2.5 % barley bran; apple wastes + 3 % wheat bran oak sawdust; rice bran; beet pulp; and cottonseed hull supplemented with food waste compost at the various levels	Sikhae factory waste + 1 % sucrose + 0.05 % $MgSO_4 \cdot 7H_2O$ oak-wood sawdust + corn-cobs *** *** *** *** sawdust supplemented with 20 % wheat bran, 1 % soy bean flour, 2 % gypsum apple wastes + wheat bran (9:1) (cellulose degradation 0.7 g% d.w.) plum wastes + 2.5 % barley bran (cellulose degradation 0.7 g%) oak sawdust, rice bran, beet pulp, and cottonseed hull supplemented with 13 % of food waste compost	**(Jung *et al.*, 2001)** **(Philippoussis *et al.*, 2004)** **(U.S. Patent No.7,043,874, 2006)** **(Patent BY No.8910, 2007)** **(Peng, 2010)** **(Gaitán-Hernández *et al.*, 2011)** **(Hassan, 2011)** **(Petre & Petre, 2012)** **(Petre & Petre, 2013b)** **(Jo *et al.*, 2013)**
Lentinus giganteus Berk.	rice bran; wheat bran	***	**(Peng, 2010)**

Lentinus tigrinus (Bull.) Fr.	wheat straw (77 %), supplemented with wheat meal (20 %) and $CaCO_3$ (3 %)	BE =62 %	**(Lechner & Papinutti, 2006)**
Lyophyllum decastes (Fr.) Singer	livestock composts - one year (C-I) and five weeks (C-II), fermented with five supplements (barley bran; corn husk; a mixture of wheat, rice, barley (WRB) brans; cotton waste, and nucleic acid)	C-I with WRB bran and barley bran supplements, with BE of 59.34 % and 56.21 %, respectively	**(Pokhrel *et al.*, 2006)**
Morchella esculenta (L.) Pers.	detoxified loquat kernel extract (DLKE); neutralized loquat kernel extract (LKE); malt extract (ME) glucose (40 g/l) + yeast extract (3 g/l) + chicken feather peptone (10 g/l); glucose (40 g/l) + yeast extract (3 g/l) + tryptone peptone (10 g/l); glucose (40 g/l) + yeast extract (3 g/l) + fish peptone (10 g/l)	ME (biomass = 18.9 g/L, exopolysaccharide = 5.3 g/L), DLKE (biomass = 16.7 g/L, exopolysaccharide = 5.2 g/L) glucose + yeast extract + tryptone peptone (biomass = 16.3 g/l, extracellular polysaccharides = 4.8 g/l)	**(Taskin *et al.*, 2011)** **(Taskin *et al.*, 2012)**
Oudemansiella canarii (Jungh.) Höhn.	substrates alone and in combination (1:1): shells of cacao, banana leaves	shells of cacao (mycelial growth = 5.98 mm /day); shells of cacao + banana leaves (mycelial growth = 5.98 mm /day)	**(Carreno-Ruiz *et al.*, 2014)**
Oudemansiella tanzanica (nomen provisorum)	sawdust; sisal waste and paddy straw supplemented with rice bran or dried chicken manure	sawdust with chicken manure (5 %)	**(Magingo *et al.*, 2004)**
Phanerochaete chrysosporium Burds.	coffee pulp corn straw, rice husk; local grass powder; sugarcane leaves; sugarcane bagasse	cellulose degradation (62.1 %); hemicellulose degradation (48.7 %) grass powder (enzymes production)	**(Parani & Eyini, 2010)** **(Saratale *et al.*, 2014)**
Phellinus linteus (Berk. & M.A. Curtis) Teng	cheese-processing waste (whey)	mycelia growth rate 2.80 mm /day	**(Lee *et al.*, 2011)**
Pholiota adiposa (Batsch) P. Kumm.	oak sawdust; rice bran; beet pulp; and cottonseed hull supplemented with food waste compost at the various levels	oak sawdust, rice bran, beet pulp, and cottonseed hull supplemented with 13 % of food waste compost	**(Jo *et al.*, 2013)**
Pholiota nameko (T. Itô) S. Ito & S. Imai	*Eucalyptus* shaving; coffee husk; cotton seed and teff straw . Wheat bran was used as additive material 100:10 and 100:30 w:w of the main material	*Eucalyptus* shaving supplemented with 30 % wheat bran	**(Gizaw, 2010)**
Pleurotus citrinopileatus Singer	rice bran; wheat bran paddy straw; brassica straw; pea pod shell; cauliflower leaves and radish leaves separately and on various combinations of paddy straw and aforementioned wastes PDA (potato dextrose Agar) culture medium with aquatic extraction solution from waste material of *Auricularia auricula*	*** 70 % paddy straw and 30 % brassica straw (BE = 94.33 %), 70 % paddy straw and 30 % pea pod shell (BE = 94.33 %) PDA culture medium with 20 % aquatic extraction solution from waste material of *Auricularia auricula*	**(Peng 2010)** **(Singh & Singh, 2011)** **(Wang *et al.*, 2013)**
Pleurotus cystidiosus O.K. Mill.	rice bran; wheat bran sawdust – control; sawdust + cocoa pods waste (80%:20%; 75:25%; 70% :30%)	*** 75% sawdust+25% cocoa pods waste (BE = 64.49 %)	**(Peng, 2010)** **(Mudakir *et al.*, 2014)**
Pleurotus djamor (Rumph. ex Fr.) Boedijn	cotton waste; paddy straw; wheat straw	cotton waste (yield= 40 g)	**(Ashraf *et al.*, 2013)**
Pleurotus eous (Berk.) Sacc.	paddy straw; sorghum stalk; banana pseudostem coffee pulp soybean straw	paddy straw (BE = 55.49 %) cellulose degradation (61 %); hemicellulose degradation (53.7 %) yield = 383.81 g	**(Rani *et al.*, 2008)** **(Parani & Eyini, 2010)** **(Ingale & Ramteke, 2010)**
Pleurotus eryngii (DC.) Quel.	wheat straw (WS); cotton straw (CS); lentil straw (LS) and rice bran (RB) rice bran; wheat bran wheat straw; raw two-phase olive mill waste; composted two-phase olive mill waste 15 % cotton seed + 20 % sawdust + 45 % crushed corn + 17 % bran +1 % slaked lime +1 % gypsum powder + 1 % sugar	WS-CS (1:1) + 20 % RB (yield = 23.2 g/100 g) *** wheat straw + 40 % composted two-phase olive mill waste (yield = 363.69 g) ***	**(Kirbag & Akyuz, 2008)** **(Peng, 2010)** **(Zervakis *et al.*, 2013)** **(Patent KZ No.28780, 2014)**
Pleurotus flabellatus Sacc.	coffee pulp sugarcane baggase + cooked fish waste (head, tail, shells, fins, intestine, dead fishes and so on) = 1:1; coir pith + cooked fish waste; woodchips + cooked fish waste	cellulose degradation (54.4 %); hemicellulose degradation (46.8 %) sugarcane baggase + cooked fish waste	**(Parani & Eyini, 2010)** **(Lakshmi & Sornaraj, 2014)**
Pleurotus floridanus Singer	corn cob; corn husk; and poultry waste (used as an additive) sugarcane leaves; sugarcane bagasse; coir; rice straw; cotton waste; banana leaves cotton waste and paddy straw with supplementation	corn husk (BE = 0.36 %, mean mushroom weight = 45 g) banana leaves (protein and lipids), cotton waste (lipids) cotton waste with + wheat bran (yield =	**(Elenwo & Okere, 2007)** **(Khan *et al.*, 2008)** **(Narayanasamy *et al.*, 2008)**

	of wheat bran	74.35 g)	**(Murthy & Manonmani, 2008)**
	coffee cherry husk; coffee parchment husk; coffee silver skin; coffee spent wastes; coffee dried leaves with and without supplementation of agricultural wastes such as wheat bran	coffee parchment husk + coffee cherry husk + coffee silver skin + coffee spent wastes + coffee dried leaves + wheat bran (20 % : 20 % : 20 % : 20 % : 10 % : 10 %)	**(Ahmed *et al.*, 2009)**
	soybean straw; paddy straw; wheat straw and their combination in 1:1 proportion		**(Mondal *et al.*, 2010)**
	banana leaves and rice straw alone or mixed (1:3; 1:1; 3:1)	soybean straw (total yield = 875.66 g/kg, BE = 87.56 %)	**(Ingale & Ramteke, 2010)**
	soybean straw	rice straw; banana leaves : rice straw = 1:3	**(Jafarpour *et al.*, 2011)**
	wheat straw; barley straw; maize stem residue and lawn residue with supplementation of wheat bran and rice bran	yield = 283.21 g	**(Ramanathan *et al.*, 2013)**
	sorghum straw; paddy straw; sugarcane bagasse; banana leaves	wheat straw + rice bran (yield = 1039.00 g per 500g substrate dry weight; BE = 207.8 %)	**(Ng'etich *et al.*, 2013)**
	elephant grass; cotton seed husks; sugarcane bagasse; corn cobs; beans straw; mixtures (1:1): bagasse + maize cobs; bagasse + beans straw	paddy straw (yield)	**(Karuppuraj *et al.*, 2014)**
	paddy straw; reeds; banana stem; sugarcane bagasse milled and crushed; sugarcane leaves; coir pith; sorghum husk; sunflower stem	cotton seed husks (yield = 118 kg)	**(Ashrafi *et al.*, 2014)**
	SMS (spent mushroom substrate); sawdust; wheat bran in different compositions	sorghum husk (BE = 80.35 %), paddy straw (BE = 70.23 %)	
		SMS + sawdust + wheat bran (20 : 60 : 20) – BE = 92.7 %	
Pleurotus nebrodensis (Inzenga) Quél.	62 % cotton seed +10% bran + 25 % crushed corn + 1 % slaked lime + 1 % gypsum powder + 1% 3Ca $(H_2P0_4)_2$	***	**(Patent KZ No.28781, 2014)**
Pleurotus ostreatus (Jacq.) P. Kumm.	paddy and wheat straw (in the ratio of 1 : 1), bamboo leaves and lawn grasses	wheat straw; paddy and wheat straw (in the ratio of 1: 1)	**(Kumari & Achal, 2008)**
	food wastes extracts with concentrations 10, 20, 30, 40, 50 %	food wastes extract with concentrations 30 %	**(Lim *et al.*, 2009)**
	corn cob; cotton waste; banana leaves; elephant grass	corn cob (mycelial extension = 67 cm)	**(Nwokoye *et al.*, 2010)**
	100 – 20 % hemp shive + 0 – 80 % cereal straw;	***	**(Patent WO No. 2,011,145,961, 2011)**
	rice straw with banana leaves or *Leucaena leucocephala* or maize bran or maize cobs; rice straw with sunflower or cotton seed hulls	rice straw + 2 % sunflower seed hulls; rice straw + 2 % cotton seed hulls	**(Mamiro & Mamiro, 2011)**
	olive mill waste water mixed with garlic and maize wastes	olive mill waste water (15 %) + maize wastes	**(Lechner & Monaldi, 2011)**
	fermented pine sawdust substrate with different levels of wheat bran	pine sawdust + 15 % wheat bran (BE = 136.8 %)	**(Oseni *et al.*, 2012)**
	corn cobs; palm cones	corn cobs (yield = 146.1 g)	**(Samuel & Eugene, 2012)**
	different combinations of wastes (leaf, pseudo-stem and pseudo-stem + leaf) and banana cultivars - *Musa* spp.	pseudo-stem waste + banana cultivar Thap Maeo (BE = 61.5 %)	**(Carvalho *et al.*, 2012)**
	winery and apple wastes (1:1); winery wastes + wheat bran (9:1); winery wastes + rye bran (9:1); apple wastes + wheat bran (9:1); apple wastes + rye bran (9:1)	winery + apple wastes (1:1) (cellulose degradation 0.9 g%)	**(Petre & Petre, 2012)**
	cotton waste and rice straw with rice bran additive at varying percentage	cotton waste (BE = 93.6 %), cotton waste + 5 % rice bran (BE = 93.0 %)	**(Jonathan *et al.*, 2012)**
	rice straw; rice straw + wheat straw; sugarcane bagasse; sawdust supplemented with 10 % rice bran (except rice straw)	rice straw (control); rice straw + wheat straw	**(Sharma *et al.*, 2013)**
	apple wastes + 1.5 % barley; apple wastes + 2 % wheat bran; pear wastes + 1.5 % barley bran; plum wastes + 2.5 % barley bran; apple wastes + 3 % wheat bran	apple wastes + 1.5 % barley (cellulose degradation 0.9 g% d.w.)	**(Petre & Petre, 2013b)**
	cotton waste; paddy straw; wheat straw	cotton waste (yield = 49 g)	**(Ashraf *et al.*, 2013)**
		PDA culture medium with 60 % aquatic extraction solution from waste material of *Auricularia auricular*	**(Wang *et al.*, 2013)**

	PDA (potato dextrose Agar) culture medium with aquatic extraction solution from waste material of *Auricularia auricula*	paddy straw (yield = 611.00 g); sugarcane bagasse (yield = 348 g)	**(Govindaraju *et al.*, 2013)**
	paddy straw; sugarcane bagasse; coconut sawdust; banana leaves	cotton seed hull 80 % + wheat bran 20 %	**(Yang *et al.*, 2013)**
	cotton seed hull, wheat bran, rice straw, wheat straw in differrent proportions	solid olive mill wastes +10% wheat straw + 2% of $CaCO_3$	**(Mansour–Benamar *et al.*, 2013)**
	solid olive mill wastes + wheat straw + $CaCO_3$	sisal leaf waste, co-substrate *Panicum coloratum* (1:1) – yield =164.2 g/kg	**(Muthangya *et al.*, 2013)**
	sisal leaf waste: soaked in cold water, boiling in water, lime pretreatment, fermentation, co-substrate *Panicum coloratum*	wheat straw + 20 % composted two-phase olive mill waste (yield =410.09 g)	**(Zervakis *et al.*, 2013)**
	wheat straw; two-phase olive mill waste; composted two-phase olive mill waste	wheat germ oil meal (biomass = 24.1 g/l, BE= 44.6 %); amaranth flour (biomass = 22.5 g/l, BE = 42.0 %)	**(Krupodorova *et al.*, 2014)**
	wheat germ oil meal; rapeseed meal; CO_2-extraction waste – amaranth flour	SMS + sawdust + wheat bran (20 : 60 : 20); SMS + sawdust (40 : 60)	**(Ashrafi *et al.*, 2014)**
	SMS (spent mushroom substrate); sawdust; wheat bran in different compositions	*Agave salmiana* bagasse	**(Heredia-Solis *et al.*, 2014)**
	Agave salmiana bagasse; *Agave weberi* bagasse	coffee husk + cow dung (75 %: 25 %) – yield = 192.3 g , BE = 21.37 %	**(Mohammed *et al.*, 2014)**
	coffee husk; cow dung; poultry manure; bone meal in different compositions	tea leaves after hot water extraction (40 % –60 %) + cotton seed hull	**(Yang *et al.*, 2015)**
	tea leaves after hot water extraction + cotton seed hull		
Pleurotus pulmonarius (Fr.) Quél.		cotton waste (yield = 79.4 g)	**(Adebayo *et al.*, 2009)**
	different mixtures of cotton waste and cassava peel	un-supplemented corn cob (fresh weight of fruiting bodies = 53.2 g)	**(Stanley *et al.*, 2011)**
	corn cob substrate supplemented with rice bran	rice straw (yield)	**(Jonathan *et al.*, 2013)**
	coir fibre; oil palm waste; rice straw	wheat straw + 20 % composted two-phase olive mill waste (yield = 382.16 g)	**(Zervakis *et al.*, 2013)**
	wheat straw; two-phase olive mill waste; composted two-phase olive mill waste	cotton waste + rice bran (dry matter = 32.4 g; pileus = 19.2 cm); corn husk + rice bran (stipe = 18.0 cm)	**(Adedokun, 2014)**
	cotton waste; rice straw; corn cob; corn husk and sawdust with supplemented rice bran to each substrate		
Pleurotus sajor-caju (Fr.) Singer	waterhyacinth (*Eichhornia crassipes*) at ratios of 25, 50 and 75 % + paddy straw	paddy straw + 25 % waterhyacinth (BE = 85 %)	**(Nageswaran *et al.*, 2003)**
	cotton stalks; groundnut haulms; soybean straw; pigeon pea stalks and leaves and wheat straw, alone or in combinations; groundnut oil seed cake; rice bran; gram powder	cotton stalks + pigeon pea stalks and leaves + wheat straw + groundnut oil seed cake (yield = 914.03 g/kg)	**(Mane *et al.*, 2007)**
	rice straw, rice straw + oilseed rape straw (75:25, 50:50, and 25:75), and oilseed rape straw alone	rice straw + oilseed rape straw (25:75) – yield = 960.15 g/kg dry substrate)	**(Norouzi *et al.*, 2008)**
	rice bran; wheat bran	***	**(Peng, 2010)**
	soybean straw	yield = 303.63 g	**(Ingale & Ramteke, 2010)**
	soybean straw; paddy straw; wheat straw; groundnut straw; sunflower stalk; pigeon pea stalk	soybean straw	**(Patil, 2012)**
	maize stalk; pea residue (tendrils) and banana leaves with and without supplementation of rice bran and chicken manure	maize stalk with rice bran	**(Pokhrel *et al.*, 2013)**
	cotton waste; paddy straw; wheat straw	paddy straw (yield = 40 g)	**(Ashraf *et al.*, 2013)**
	soybean straw; wheat straw; paddy straw; sugarcane bagasses; sun flower stalks; maize stalks; used tea leaves; fruit waste; semal flowers; bamboo leaves (alone and in combinations in 1:1)	soybean straw (yield = 933.4 g/kg); soybean straw + wheat straw (yield = 873.4 g/kg)	**(Dehariya &Vyas, 2013)**
		P. angolensis sawdust + 15 % wheat chaff (yield of 31.22 g; BE= 37.39 %); *P. angolensis* sawdust (protein of 26.33 %)	**(Fakoya *et al.*, 2014)**

	Pycnanthus angolensis sawdust supplemented with 0, 5, 10, 15, and 20 % palmkernel cake, oil palmfibre, rice bran, wheat chaff , and corn cobs		
Pleurotus sapidus Sacc.	sisal leaf waste: soaked in cold water, boiling in water, lime pretreatment, fermentation; co-substrate *Panicum coloratum*	sisal leaf waste, co-substrate *Panicum coloratum* (1:1) – yield = 156.4 g/kg	**(Muthangya *et al.*, 2013)**
Pleurotus tuber-regium (Fr.) Singer	corn cob; corn husk; poultry waste (used as an additive) fermented sawdust; oil palm fruit fibre; mixtures (1:1): oil palm fruit fibre+fermented sawdust; corn waste+ fermented sawdust; millet waste +fermented sawdust maize cob; cassava peelings; plantain peelings; watermelon pod	corn cob (BE = 0.67 %, mean mushroom weight = 118.9 g) corn waste + fermented sawdust; millet waste + fermented sawdust maize cobs (yield = 28 g); cassava peelings (yield = 28 g)	**(Elenwo & Okere, 2007)** **(Olufokunbi & Chiejina, 2010)** **(Stanley & Odu, 2012)**
Schizophyllum commune Fr.	coconut water breadcrumb sunflower seed hull without and with supplementation with either wheat bran (3.75%, 7.5 %) or 1 % vegetal oils substrates alone and in combination (1:1): coir, shells of cacao, banana leaves	coconut water (production of schizophyllan at 7.71g/1000 ml) mycelial mass = 23.96 g/l sunflower seed hull with supplementation of 7.5 % wheat bran (BE = 48.3 %) coir+ shells of cacao (mycelial growth = 9.99 mm /day)	**(Reyes *et al.*, 2009)** **(Ivanova *et al.*, 2014)** **(Figlas *et al.*, 2014)** **(Carreno-Ruiz *et al.*, 2014)**
Trametes versicolor (L.) Lloyd	rice bran; wheat bran breadcrumb amaranth flour after CO_2-extraction	*** mycelial mass = 15.76 g/l high antiviral activity	**(Peng, 2010)** **(Ivanova *et al.*, 2014)** **(Krupodorova *et al.*, 2014b)**
Volvariella diplasia (Berk. & Broome) Singer	rice bran, wheat bran, rice straw, banana leaf, sugarcane bagasse supplemented with wheat	50 % rice bran with 50 % wheat (BE = 12.43 %)	**(Tripathy *et al.*, 2011)**
Volvariella volvacea (Bull.) Singer	rice husk; rice straw; cotton waste; groundnut shell; cassava peel; corn cob; oil palm pericarp; red sorghum shaft and their blends (ratio 1:1) corn cob; corn husk; poultry waste (used as an additive) oil palm fibre; paddy straw (control) palm fiber; rice husk cotton waste; rice straw rice bran; wheat bran; rice straw; banana leaf; sugarcane bagasse mixed with wheat paddy straw; cotton waste; banana leaves; corn stovers; sugarcane baggasse and pulses straw plantain leaves; maize husk; cotton waste	cotton waste + rice husk (mycelia extension 101.87 mm) ; oil palm pericarp + groundnut shell (mycelia extension 100.67 mm) corn husk (BE = 0.09 %, mean mushroom weight = 16 g) oil palm fibre (yield = 16.3 g) palm fiber (stipe height= 4.0 cm); rice husk (pileus diameter = 4.0 cm) *** 50 % rice bran with 50 % wheat (BE = 13.6 %) cotton waste (protein = 20.0 %) maize husk (yield = 24.67 g; carbohydrate, protein, lipids, ash and fiber)	**(Akinyele & Adetuyi, 2005)** **(Elenwo & Okere, 2007)** **(Onuoha *et al.*, 2009)** **(Ukoima *et al.*, 2009)** **(Peng, 2010)** **(Tripathy *et al.*, 2011)** **(Ul Haq *et al.*, 2011)** **(Adedokun & Akuma, 2013)**

* The substrate names are in accordance with those in the cited publications
** The quantitative measure of the yield is according to the cited publication, although its dimensions were different (g; g/kg of substrate, g/100 g of substrate, g/l; etc.)
*** The statement of the fact of cultivation, without comparing the results

THE STUDY OF CULTIVATION, PHYSICOCHEMICAL, BIOCHEMICAL PARAMETERS OF SUBSTRATES AND MUSHROOMS AND THEIR BIOLOGICAL ACTIVITY

Al Abttan *et al.*, (**2005**) studied chemical composition (moisture, carbohydrates, total nitrogen, C/N, crude fibers, ether extract, ash) of substrates for *A. bisporus* cultivation. Three wastes (peas, broad bean, beet pulp) were used in different levels with mung bean straw to prepare mushroom growth media. Results indicated that mung bean straw is the best substrate, it is possible to be mixed, but it should not be more than 50 % of the composition of the growth medium which is prepared by mixing with wheat at different levels.

Nutritional values of *P. ostreatus* cultivated on different agricultural wastes studied by a number of authors (**Jonathan *et al.*, 2012; Aguilar-Rivera *et al.*, 2012; Bermudez-Savon *et al.*, 2014; Alananbeh *et al.*, 2014**), as well as pH and chemical composition of the substrates also (**Amuneke *et al.*, 2011; Carvalho *et al.*, 2012; Aguilar-Rivera *et al.*, 2012; Bermudez-Savon *et al.*, 2014; Alananbeh *et al.*, 2014**). Total ash, fibre, protein, fat, carbohydrate and energy of *P. ostreatus* grown on different substrates as well as yield, size and BE have been investigated by **Sharma *et al.***, (**2013**). All the substrates (rice straw + wheat straw, rice straw + paper, sugarcane bagasse and sawdust of alder) except rice straw were supplemented with 10 % rice bran. The substrate without supplement was considered as control. Among all aspects, rice straw (control) was found as a best substrate with yield (381.85 g) and BE (95.46 %) followed by rice + wheat straw, rice straw + paper waste for the production of mushroom. The nutritional composition was also better from mushroom fruit grown on rice straw. **Kumari and Achal** (**2008**) studied the effect of paddy straw, wheat straw, mixture of paddy and wheat straw (in the ratio of 1:1), bamboo leaves and lawn grasses on the production of *P. ostreatus*. Wheat straw and a mixture of paddy and wheat straw gave the earliest colonization of fungus. The highest yield was recorded on wheat straw (29.27 g fresh weight/Kg substrate), followed by the combination of paddy and wheat straw (27.96 g/Kg). Non-enzymatic antioxidant activities were also obtained by estimating vitamins A, C and E. Significant amount of vitamin E was found in both fresh (7.23 mg/g) and dry fruit body (5.93

mg/g) of *P. ostreatus*. The effect of autoclaved sterilized and non-sterilized substrate on growth and yield of oyster mushroom was examined by **Yang *et al.*, (2013)**. *P. ostreatus* was cultivated on rice straw basal substrate, wheat straw basal substrate, cotton seed hull basal substrate, and wheat straw or rice straw supplemented with different proportions (15 %, 30 %, and 45 % in rice straw substrate, 20 %, 30 %, and 40 % in wheat straw substrate) of cotton seed hull. The non-sterilized substrate did not gave significantly higher mushroom yield and BE than the sterilized substrate, but some undesirable characteristics, i.e. smaller mushroom cap diameter and relatively long stipe length. Growth, yield, and proximate composition (fat, fibre, ash, protein, carbohydrate) of *P. sajor-caju* cultivated on *Pycnanthus angolensis* sawdust supplemented with 0, 5, 10, 15, and 20 % palm kernel cake, oil palm fibre, rice bran, wheat chaff, and corn cobs have been studied by **Fakoya *et al.*, (2014)**. *P. sajor-caju* produced maximum yield of 31.22 g on sawdust supplemented with 15 % wheat chaff. The BE ranged from 6.09 % to 37.39 %. Results also showed a maximum crude protein of 26.33% of *P. sajor-caju* cultivated on sawdust without any supplement and fat content ranging from 0.25 % to 2.21 %. Fibre content of harvested mushrooms ranged from 5.05 % to 9.29 %. Effect of different substrates on nutritional content of *P. sajor-caju* has been stadied by Patil (2012). *P. sajor-caju* was cultivated on soybean straw, paddy straw, wheat straw, groundnut straw, sunflower stalk and pigeon pea stalk. Soybean straw showed significantly highest yield (845.66 g/kg) and BE with maximum crude protein (25.33 %) content. Significantly maximum moisture and crude fiber content was recorded on sunflower stalk, i.e. 89.35 % and 7.82 % respectively. Maximum total carbohydrate (56.00 %) was recorded on wheat straw, while maximum fat and ash content of *P. sajor-caju* was recorded on groundnut straw, i.e. 2.85 % and 7.00 % respectively. **Ashraf *et al.*, (2013)** studied growth and yield, morphological parameters, chemical composition of three varieties of Oyster mushroom (*P. sajor-caju*, *P. ostreatus*, and *P. djamor*) grown on three different substrates cotton waste, wheat straw and paddy straw. The fastest spawn running, primordial initiation, harvesting stage, maximum number of fruiting bodies and maximum yield was observed on cotton waste. *P. djamor* showed the highest percentage of dry matter (17.23 %) and moisture content was found high in *P. sajor-caju* (87.37 %). *P. ostreatus* and *P. sajor-caju* showed the maximum protein (27.23 %) and fiber (26.28 %) contents. The ash contents were found maximum in *P. sajor-caju* (9.08 %). The highest fat and carbohydrate contents were found in *P. djamor* (3.07 % and 37.69 % respectively). *P. citrinopileatus* protein, total sugar and non reducing sugar content have been investigated in process of cultivation on paddy straw, brassica straw, pea pod shell, cauliflower leaves and radish leaves separately and on various combinations of paddy straw and aforementioned wastes (**Singh and Singh, 2011**). The mushroom failed to grow on pea pod shell, cauliflower leaves and radish leaves when it was cultivated separately on these vegetable wastes. However, it grew very well on paddy straw in combination with other substrates. 70 % paddy straw and 30 % other wastes combination supported maximum BE of mushroom followed by 80 % paddy straw and 20 % other wastes combination. The protein content, total sugar and non reducing sugar content was found to be higher in the mushrooms grown on paddy straw and other agro wastes combination than on paddy straw alone. Similarly, six essential amino acids i.e. leucine, isoleucine, valine, threonine, methionine and phenylalanine content was higher in the mushrooms cultivated on paddy straw and other agro wastes combination than on paddy straw alone. Nutritional composition of *P. florida* cultivated on sawdust, sugarcane leaves, sugarcane bagasse, coir, rice straw, cotton waste, banana leaves has been studied by **Khan *et al.*, (2008)**. The amount of protein found in mushroom cultivated in banana leaves was significantly higher than in any other substrate. *P. florida* was also cultivated on soybean straw, paddy straw, wheat straw and their combination in 1:1 proportion to determine the effect of these agro waste on yield, moisture content, crude protein, total carbohydrates, fat, crude fiber, ash and minerals like Ca, P, Fe content. Soybean straw showed significantly highest yield (875.66 g/Kg), BE (87.56 %) with maximum crude protein (23.50 %) and maximum phosphorus (920 mg/100 mg of dry mushroom) content. Maximum moisture (92.45 %) and crude fiber content (8.10 %) in the fruiting bodies was recorded on paddy straw cultivation. The combination of soybean straw + paddy straw showed significantly highest fat (2.60 %), calcium (310 mg/ 100gm) and iron (13.06 mg/100gm of dry mushroom) content (**Ahmed *et al.*, 2009**). **Jonathan *et al.*, (2013)** reported about the yield, mineral elements content and morphological parameters of *P. pulmonarius* cultivated on coir fibre, oil palm waste, sawdust of *Gmelina arborea* and rice straw at different rice bran level. The most abundant mineral element in *P. pulmonarius* was K (30.20 mg/100 g). This was obtained on rice straw at rice bran 10 % concentration; while the least mineral element was Cu (0.006 mg/100 g). The highest values of Ca and Mg obtained were 3.90 and 2.67 mg/100 g respectively on sawdust (rice bran 10 %) and palm wastes (rice bran 20 %). The values of Fe obtained, varies from 0.007 to 0.12 mg/g on rice straw (rice bran 10 % and 40 %). Manganese has values varying from 0.04 mg/g to 0.09 mg/g on coir fibre and oil palm waste with 40 % rice bran. Highest mean stipe length (6.68 cm) was found in *P. pulmonarius* produced from rice straw while the least mean stipe length (4.08 cm) was detected on oil palm waste. The highest pileus diameter (7.08 cm) was found on rice straw while the mean height obtained from the four substrates, were relatively close with values varying between 6.0 and 9.3 cm. Rice straw produced the highest yield with total mean weight of 93.33 g. Seven different substrates supplemented with fermented sawdust were used to produce mushrooms and sclerotia of *P. tuber-regium* (**Olufokunbi and Chiejina, 2010**). Protein content ranged from 20.59 % for fermented sawdust substrate to 25.19 % for river sand substrate. The rate of substrate colonization had a significant effect on sclerotium production. The mean dry weight yields varied from 46.26 g for mixture of rice bran and fermented sawdust substrate to 127.48 g for fermented sawdust substrate alone. The highest sclerotial protein content (8.40 %) was from mixture of rice bran and fermented sawdust substrate although it was not significantly different from those of other substrates. A mixture of river sand and fermented sawdust substrate is recommended as the best substrate for the production of *P. tuber-regium* mushrooms while a mixture of corn waste and fermented sawdust substrate is recommended for sclerotial production.

The degradation of lignocellulosic wastes such as paddy straw, sorghum stalk, and banana pseudostem was investigated during solid-state fermentation by edible mushrooms *L. connatus* and *P. eous* (**Rani *et al.*, 2008**). BE of 68.75 % was observed in paddy straw followed by sorghum stalk (46.67 %) for *L. connatus* and 55.49 was observed in paddy straw followed by sorghum stalk (45.10 %) for *P. eous*. The activity of extracellular enzymes, namely cellulase, polyphenol oxidase, and laccase, together with the content of cellulose, lignin, and phenols, was studied in spent substrates on seventh, 7th, 17th, and 27th days of spawning, and these values were used as indicators of the extent of lignocellulosic degradation by mushroom. Both the mushroom species proved to be efficient degraders of lignocellulosic biomass of paddy straw and sorghum stalk, and the extent of cellulose degradation was 63–72 % of dry weight (DW), and lignin degradation was 23–30 % of the DW. The chemical changes in barley-straw (BS), wheat-straw (WS) and vineyard-pruning (VP) substrates were determined during colonization of *L. edodes* mycelia in solid state fermentation (**Gaitán-Hernández *et al.*, 2011**). VP appeared to promote early sporophore initiation. The concentration of hemicellulose in BS and VP decreased gradually from 25.5 % to 15.6 % and from 15.8 % to 12.3 %, respectively. However in WS, hemicellulose decreased from 27.2% to 9.5%. Lignin broke down continuously in BS and WS, with 31.8 % and 34.4 % degradation, respectively; higher than that of cellulose. During the pinning stage, the C:N ratio decreased in VP and BS, but not in WS. On all substrates the phenols decreased notably throughout the first week of mycelial growth. The time elapsed (days) to pinning was positively correlated with cellulose content, total sugar and inversely correlated to lignin and phenol content.

Yield, biological efficiency (BE) and the chemical composition of substrates and fruiting bodies have been investigated in *Ganoderma lucidum* solid-state fermentation on substrate mixtures with tea waste (TW) supplement (**Peksen and Yakupoglu, 2009**).

The edible mushroom *Oudemansiella tanzanica*, which is new to science, has been studied as a potential crop to reduce agricultural solid wastes and increase mushroom production. The substrates sawdust (natural for this mushroom), sisal waste and paddy straw supplemented with chicken manure resulted in the highest biological efficiencies of any mushroom cultivated in Tanzania so far. Composition of the substrates and supplements in terms of acid detergent fiber, neutral detergent fiber, lignin, cellulose, hemicellulose, carbon, C:N ratio, nitrogen, crude protein, total solids, volatile solids, pH and crude fiber, reduction in the components lignin, cellulose, hemicellulose, carbon, nitrogen, crude protein, total solids and volatile solids on studied substrates supplemented with rice bran or chicken manure after growth *of Oudemansiella tanzanica* have been investigated (**Magingo *et al.*, 2004**).

Considerable interest of researchers is the study of substrates and mushrooms chemical composition in case of *V. volvacea* cultivation. **Akinyele and Adetuyi (2005)** investigated the effect of pH and temperature variations on the growth of *V. volvacea* cultivated on rice husk, rice straw, cotton waste, groundnut shell, cassava peel, corn cob, white afra dust, red afra dust, oil palm pericarp and red sorghum shaft and their blends (ratio 1:1). Nutritional properties of *V. volvacea* grown on plantain leaves, maize husk and waste cotton were studied by **Adedokun and Akuma (2013)**. Biochemical analysis of paddy straw, cotton waste, banana leaves, corn stovers, sugarcane baggasse and pulses straw used as substrates was conducted before and after inoculation with the of *V. volvacea* (strain *Vv pk*). The nitrogen %age, crude protein, crude fiber and ash contents were estimated in all substrates used for the cultivation of edible fungus, to make the comparison that how much this edible fungus plays role in the enhancement of these estimated contents in all the above mentioned substrates before and after treatment with *V. volvacea* culture. The mean values of the nitrogen percentage of the substrates showed that it was high in the cotton waste (3.80) followed by the pulses straw, paddy straw and sugarcane baggasse and minimum nitrogen was found in the banana leaves. The mean values of the protein percentage of the substrates showed that highest protein was present in the cotton waste (20.00 %) followed by the corn stover (10.28 %). The highest crude fiber was found in cotton waste (42.36 %), followed by the sugarcane baggasse (39.49 %). The ash contents were high in cotton waste (22.30 %) followed by the paddy straw (20.20 %) (**Ul Haq *et al.*, 2011**).

Only a few papers contain a study of heavy metals content in cultivated mushrooms: Pb (less than acceptable weekly intakes for adults) (**Alananbeh *et al.*, 2014**), Cd and Pb (the concentrations were under the detection limit of the

metod used) (**Akyüz and Kirbağ, 2010a; Akyüz and Kirbağ, 2010b**), Cd and Pb (the concentrations of Cd were under the detection limit of the metod used, the concentrations of Pb were less than acceptable weekly intakes for adults) (**Mallikarjuna *et al.*, 2013**). At the same time, the study of wild mushrooms testify to the exceeding of permissible concentrations of heavy metals (**Falandysz *et al.*, 2001; Isiloğlu *et al.*, 2001**).

Not sufficiently studied remains the question about the effect of substrate on biological activity of fungi. Antibacterial activity of *L. edodes* against *Bacillus subtilis* was evaluated in cell-free filtrates obtained after growth in 14 different culture media. The highest *B. subtilis* growth inhibition was promoted by filtrates of growth media supplemented with rice bran, vermiculite or molasses. Antibacterial activity, detected between 20 and 24 days of incubation of stationary cultures, was absent in filtrates of aerated cultures. Temperatures of 20–25^{o}C enhanced both growth and antibacterial activity. Optimum pH for *L. edodes* mycelial growth was 3.0–3.5, while for production of antibacterial substance – 4.5. The results indicated that incubation conditions that enhance mycelial growth are quite different from those necessary for production of antibacterial substance(s) by *L. edodes* (**Hassegawa *et al.*, 2005**). **Ramanathan *et al.*, (2013**) studied the antimicrobial activity of ethanol extracts of *P. florida* and *C. indica* cultivated on paddy straw, sugarcane bagasse, sorghum straw and banana leaf. *P. florida and C. indica* possessed antimicrobial property against antibiotic resistant human pathogens similar to that of the commercially available antibiotics. Antiviral activity against type A influenza virus of birds A/chicken/Kurgan/05/2005 (H5N1) and humans A/Aichi/2/68 (H3N2) was investigated (**Teplyakova *et al.*, 2012**) for aqueous extracts from mycelium of 11 basidial fungi species collected in the Altai Mountains (Altai Republic, Russia). A non-standard substrate (oat-corn water) was used in this study. Higher mushrooms mycelia cultivated on amaranth flour after CO_2 extraction (plant waste) have been investigated for antiviral activity *in vitro* (**Krupodorova *et al.*, 2014b**). All 10 investigated mushroom species inhibited the reproduction of influenza virus strain A/FM/1/47 (H1N1) in MDCK cells reducing the infectious titer by 2.0–6.0 lg ID50. Four species, *P. ostreatus*, *Fomes fomentarius*, *Auriporia aurea*, and *Trametes versicolor*, were also determined to be effective against HSV-2 strain BH in RK-13 cells, with similar levels of inhibition as for influenza.

The half of fungi species from 52 species represented in this review were described as bio-destructors in the last 12 years. Of them, 14 are edible and conditionally edible (*Agaricus flocculosipes*, *Agaricus subrufescens*, *Agrocybe cylindracea*, *Auricularia fuscosuccinea*, *Lentinus connatus*, *Lentinus giganteus*, *Lentinus tigrinus*, *Lyophyllum decastes, Oudemansiella canarii, Oudemansiella tanzanica*, *Pleurotus djamor*, *Pleurotus nebrodensis*, *Pleurotus tuber-regium*, *Volvariella diplasia*), and 7 – medicinal (*Antrodia cinnamomea*, *Auriporia aurea*, *Fomes fomentarius*, G*anoderma applanatum*, G*anoderma tsugae*, *Phellinus linteus*, *Trametes versicolor*).

One of the most important aspects of mushroom cultivation is the rational choice of the substrate. Various substrates and basic compositions were investigated and evaluated in many countries. It is obvious that the investigated substrates affected differently on mycelial growth and fructification, depending on the content of those or other nutrients in substrates and individual requirements of studied species or strains of fungi in nutrients. Some types of agro waste are characteristic for all continents of the Earth: cereal straw, cereal bran, corn, rape, rye, soy, sorghum and sunflower wastes, spent mushroom substrate, farm yard manure, chicken manure, livestock composts, vermi compost. Other wastes are characteristic of the respective continent and region of the world. To find new type of waste as a component of substrate is very difficult, therefore, the subject of the most studies mentioned in this review became different combinations of known components of substrates. Some new substrates will be discussed below.

The noticeable effect on the increase in mushroom production is explained by the development of the simple techniques of cultivation in the controlled conditions.

UTILIZATION OF FOOD WASTES (MAINLY, AFTER FOOD PROSESSING)

Food lost or wasted at the stage of processing is (by Region, 2009): North America and Oceania − 9 % (from total lost or wasted food 42 %), Industrialized Asia − 2 % (from total lost or wasted food 25 %), Europe − 5 % (from total lost or wasted food 22 %), North Africa, West and Central Asia − 4 % (from total lost or wasted food 19 %), Latin America − 6 % (from total lost or wasted food 15 %), South and Southeast Asia 4 % (from total lost or wasted food 17 %), Sub-Saharan Africa − 7 % (from total lost or wasted food 23 %) (**Lipinski *et al.*, 2013**). Significant interest of scientists focused on the ways of food processing waste management.

Sugarcane is the world's largest crop by production quantity. The worldwide harvest is more than 1.8 billion tons. For each 10 tons of sugarcane crushed, a sugar factory produces, after juice extraction, nearly 3 tons of wet bagasse. The high moisture content of bagasse, typically 40 to 50 %, is detrimental to its use as a fuel. Due to its typical (on a washed and dried basis) chemical composition (cellulose = 45–55 %; hemicellulose = 20–25 %; lignin = 18–24 %; ash = 1–4 %; waxes <1%) bagasse is of interest as a substrate for mushroom cultivation. Sugarcane baggase and six others agricultural wastes (saw dust, coir, sugarcane leaves, cotton waste, banana leaves and rice straw) were used as substrates or nutrient source for the production of *P. florida* to investigate the nutritional composition of mushroom. The protein content of *P. florida* cultivated on sugarcane baggase was one of the lowest. The amount of protein found in mushrooms cultivated in banana leaves was significantly higher than in any other substrate (**Khan *et al.*, 2008**). Elephant grass, cotton seed husks, sugarcane bagasse, corn cobs, beans straw, mixture of bagasse + maize cobs (1:1) and bagasse + beans straw (1:1) have been investigated for *P. florida* cultivation. The cotton seed husks had the greatest influence on both growth and total yield of 118 kg. It demonstrated excellent mycelia growth, greater height, stem circumference and cap diameter. The total yield on bagasse + beans straw (1:1) was the second and bagasse – the third (**Ng'etich *et al.*, 2013**). The BE of *P. florida* cultivation on sugarcane bagasse crushed was 66.66 % (the third result from 9 substrates) and BE of *C. indica* cultivation on sugarcane bagasse milled was 71.20 % (the second from 9 substrates) (**Karuppuraj *et al.*, 2014**). Selection of different substrates for the cultivation of *C. indica* shows that the lowest yield was recorded in the treatment of sugarcane baggase (515.7 gm/kg dry substrate) in comparison with wheat straw (1463 gm/kg dry substrate), soybean straw, coconut coir pith and cotton waste (1261 gm, 1087 gm and 920.7 gm/kg dry substrate). The BE was appropriate (**Vijaykumar *et al.*, 2014**). 50 % sugarcane bagasse supplemented with 50 % wheat showed the worst result (yield =760 g, BE =7.6 %) for *V. diplasia* and third result (from seven substrates) for *V. volvacea* (yield =960 g, BE =9.6 %). The best result showed the substrate 50 % rice bran with 50 % wheat for both mushrooms (**Tripathy *et al.*, 2011**). Sugarcane bagasse showed 7th result (yield =641.7, BE =64.1 %) from 13 substrates for *P. sajor-caju* cultivation (**Dehariya and Vyas, 2013**). Thus, despite the promising chemical composition, sugarcane bagasse showed rather mediocre results as substrate and additives thereto.

The sugarcane bagasse became the part of mixted substrate (with seafood processing wastes) in two studies. Seafood processing wastes were mixed with selected agro-industrial wastes (e.g., coir pith, woodchips, sugarcane bagasse) in specific ratio (1:1) (**Lakshmi, 2013**). Not taking into account «control» (sugarcane and coir pith) the highest biological yield (23.15 g/bed) of *G. lucidum* and BE showed mixture coir pith+ fishery waste (1:1). This study was continued by the examination of the utilization of seafood processing wastes for artificial cultivation of edible mushroom *P. flabellatus* in laboratory condition (**Lakshmi and Sornaraj, 2014**). The selected agro-industrial wastes such as coir pith, woodchips and sugarcane bagasse were mixed with cooked fish waste (CFW) in the ratio of 1:1 (500 g : 500 g). The substrates which were not mixed with CFW were treated as control. Not taking into account «control» (sugarcane) the highest biological yield (35.00 g/bed) of *P. flabellatus* and BE showed mixture sugarcane baggase + cooked fish waste (1:1).

Worldwide olive oil production is reported to be about 3,200,000 tons for the years 2013/2014. The liquid effluent of olive oil process, the olive mill wastewater (OMWW), amounts to 0.5–1.5 m^3 per 1000 Kg of olives. **Lechner and Monaldi (2011**) examined the use of OMWW at different concentrations for moistening the garlic and maize wastes to produce basidiomes of *P. ostreatus* and to compare with substrate without OMWW. *P. ostreatus* was cultivated in garlic and maize wastes mixed with 15, 30, 45 and 60 % of OMWW. Bags with 0 % OMWW (control) and 100 % OMWW were also inoculated. The BE (129.5 %) and the total yield (388.5 g) obtained permitted to conclude that the best substrate utilized for *P. ostreatus* production was a mixture composed of OMWW (15 %) and maize wastes. The effect in the growth of *P. ostreatus* on garlic wetted with 15 and 30 % of OMWW was the same as for control. OMWW is characterized by high degree of organic pollution chemical oxygen demands. **D'Annibale *et al.*, (2004**) showed that *Panus tigrinus* and *L. edodes* removed toxic phenols from OMWW. **Lakhtar *et al.*, (2010**) investigated sixteen strains of *L. edodes* for their tolerance to OMWW, apical growth rate, and biomass production on agar media. The highest biomass yields were recorded in four strains (Le118, Le119, Le121, Le122) grown in the presence of 20% OMWW. Fifteen fungal strains belonging to five species (Basidiomycota): *Agrocybe cylindracea* (strains IK10 (Greece), IK21 (Greece), and SIEF0834 (China), *P. cystidiosus* (strains LGAM P50 (Greece), LGAM P100 (Greece), and D415 (USA), *P. eryngii* (strains LGAM63 (Greece), LGAM101 (Greece), and UPA10 (Italy), *P. ostreatus* (strains LGAM60 (Greece), LGAM106 (Greece), and LGM850402 (Hungary), and *P. pulmonarius* (strains LGAM10 (Greece), LGAM26 (Greece), and LGM850403 (France), were evaluated for their efficacy to colonize media composed of two-phase olive mill waste (TPOMW), which was used either raw or composted in mixtures with wheat straw in various ratios. Qualified strains exhibited high values of BE (e.g., 120–135 % for *Pleurotus* spp. and 125 % for *A. cylindracea*) and productivity in subsequent cultivation experiments on substrates supplemented with 20–40 % composted TPOMW or 20 % raw TPOMW. The substrates hemicellulose content was negatively correlated with mycelium growth rates and yields and positively with earliness; in addition, cellulose: lignin ratio presented a positive correlation with mycelium growth and mushroom weight for *A. cylindracea* and with earliness for all species examined (**Zervakis *et al.*, 2013**).

The olive oil production is typical for Mediterranean countries, sunflower, rapeseed and other oilseeds (soybean, amaranth, wheat germ) are typical for East Europe countries. The intensity of *P. ostreatus* biomass accumulation (18–24.1

g/l) and high conversion of substrates (33.3–44.6%) have shown prospects for this mushroom cultivation on new substrates such as wheat germ oil meal, CO_2-extraction waste – amaranth flour and rapeseed meal. The optimum concentration of selected substrates were 70 g in 1 liter of distilled water for wheat germ oil meal and amaranth flour, 60 g/l – for rapeseed meal. It was found 17 amino acids, including 9 essential in fungi biomass hydrolyzate. Significant influence of cultivation substrate on quantitative composition of amino acids has been established. To all biomass samples the prevalence of glutamic and aspartic acids, arginine among the nonessential amino-acids, leucine, lysine and cystine among the essential amino-acids were common. Endopolysaccharides content in mushroom biomass and exopolysaccharides in culture liquid were slightly different depending on the selected substrates (**Krupodorova *et al.*, 2014a**). The content of proteins, lipids, amino and fatty acids was investigated in mycelium and culture broth of medicinal mushrooms *Cordyceps sinensis*, *P. ostreatus*, and *Schizophyllum commune* cultivated on amaranth flour (**Krupodorova *et al.*, 2012**). Seven essential amino acids were present in the proteins of all mushroom samples, with aspartic (6.34 %−14.29 %) and glutamic (15.12 %−17.51 %) acids predominating in culture mycelium and glutamic acid (16.3 %−19.1 %) in culture broth. Lipids in the mycelium of species *C. sinensis*, *P. ostreatus*, and *Sch. commune* consisted of 10 fatty acids and 12 fatty acids in culture broth in our experiments. Major acids in culture mycelium and culture broth of fungi were linoleic (42.43 %−67.41 %), oleic (10.47 %−32.54 %), and palmitic (16.43 %−20.33%). The proteins and lipids in culture broth of studied species contained a higher level of total non-essential amino acids and unsaturated fatty acids as compared to those in culture mycelium. **Krupodorova and Barshteyn (2012)** studied the ability of medicinal and edible mushroom species from different systematic and ecological groups for biotransformation of CO_2-extraction (*Echinacea purpurea*, *Humulus lupulus*) and food industry (broken vermicelli, flour milling production – grits, confectionery industry – cacao shell) waste. The perspective alternative substrates for 17 mushroom species cultivation have been determined according to biomass accumulation criteria. Sunflower seed hull, an abundant and cheap by-product of the edible oil industry, was used as a substrate for growing *S. commune* (**Figlas *et al.*, 2014**). Mushroom mycelial growth rate on substrates prepared with sunflower seed hull, in absence or presence of supplements (barley, wheat bran, sunflower or olive oil), was evaluated. The growth analysis on sunflower seed hull (37.5 %) substrate showed a mycelial run length of 3.8 cm in seven days. In comparison, supplementation with either wheat bran (3.75 %, 7.5 %), barley (3.75 %, 7.5 %), or 1 % vegetal oils (sunflower or olive oil) improved, but showed no significant differences on mycelial growth. BE and productivity on sunflower seed hull based substrate containing 7.5 % wheat bran (BE = 48.3 %, productivity = 1.6 %/day) were significantly greater than those obtained on sunflower seed hull substrate (BE = 40.7 %, productivity = 1.1 %/day).

Whey (milk and cheese-processing) is one of the main dairy industry waste. Milk whey and starch grits have been studied as substrate (submerged conditions) for the production of several strains of *G. applanatum* and *G. lucidum*. Micro morphological characteristics of vegetative mycelia, biomass yield and exopolysaccharides were investigated. Nutrient medium with milk whey was optimal for biomass growth and synthesis of polysaccharides for investigated cultures. Maximal content of biomass (17.2±0.1 g/l) was produced by *G. applanatum* on the 11th day of cultivation, *G. lucidum* – 29.6 g/l on then 5 day. On the 11th day of growth the highest amounts of exopolysaccharides in *G. lucidum* was 10.0 g/l and in *G. applanatum* – 9.1 g/l (**Krupodorova, 2011**). A medicinal mushroom, *Phellinus linteus*, was successfully cultivated using a cheese-processing waste, whey, and the optimal utilization conditions for the maximum mycelial growth rate was also estimated through solid-state cultivation experiments. The results proved a good potential of whey to serve as an alternative growth medium for cultivating *P. linteus* mycelia. The maximum mycelia growth rate was redacted to be 2.80 mm/day (**Lee *et al.*, 2011**).

The world coffee production increasing rapidly and, consequently, the husk amount as this production waste. Coffee industry wastes: coffee cherry husk, coffee parchment husk, coffee silver skin, coffee spent wastes, coffee dried leaves with and without supplementation of agricultural wastes such as wheat bran were used for cultivation of *P. florida*. When these substrates were used individually, the mushrooms yield was very low. Among individual substrates, highest yield was observed with coffee charry husk. The best yield (220 g) was observed in combination: coffee parchment husk (20 %) + coffee cherry husk (20 %) + coffee silver skin (20 %) + coffee spent wastes (20 %) + coffee dried leaves (10 %) + wheat bran (10 %) (**Murthy and Manonmani, 2008**). It has been studied the suitability of coffee husk for cultivation of *P. ostreatus* after compositing with different main substrate combinations. Composting of coffee waste (husk) was conducted with cow dung, poultry manure and bone meal in the ratio of 3:1. The highest yield (192.3 g) and BE (21.37 %) was obtained from combination of coffee husk (75 %) and cow dung (25 %) on 20 days composting. Therefore, better yield of Oyster mushroom was obtained after utilization of this cost-effective and cheap agro-waste of coffee husk (**Mohammed *et al.*, 2014**).

Tea leaves after hot water extraction (to obtain water-soluble components) are still contains nutrients and are interesting as alternative substrate for mushroom cultivation. Tea waste (TW) was investigated as a new supplement for substrate mixtures in *G. lucidum* cultivation in solid-state fermentation. Sawdust (S) based substrates were supplemented with TW at the various levels (75S:25TW, 80S:20TW, 85S:15TW, and 90S:10TW). The substrate formulations producing highest yield and BE were 80S:20TW (87.98 g/kg substrate and 34.90 %) and 75S:25TW (82.30 g/kg substrate and 31%). Yield and BE of substrates containing TW were generally higher than that of the control (80 sawdust : 18 wheat bran : 1 sucrose : 1 CaCO3). Nitrogen, potassium, iron, and manganese contents and C:N ratios of substrates were strongly correlated with yield. BE showed positive and significant correlations with potassium, iron and manganese. Moisture content, potassium, magnesium, calcium, iron, and zinc contents of the fruiting bodies were affected by both strain and substrate. It was concluded that TW can be used as a supplement for substrate preparation in *G. lucidum* cultivation (**Peksen and Yakupoglu, 2009**). Used tea leaves showed 5th–6th result (yield = 655.0, BE = 65.5 %) from 13 substrates for *P. sajor-caju* cultivation (**Dehariya and Vyas, 2013**). **Yang *et al.* (2015)** studied Oyster mushroom cultivation using tea waste as substrate. Substrate containing 40 % – 60 % of tea waste obtained the highest yield.

Several studies devoted to the utilization by mushrooms of waste products of traditional Korean and Mexican beverages. Mycelia of *L. edodes* ASI 3046, which is regarded as the most suitable strain for sawdust cultivation, were cultured on six kinds of previous known media and Sikhae Factory Waste (SFW). As the seven kinds of media were applied, a SFW was most excellent in growth. The dried mycelial weight in SFW was almost four times as much as that in the other media. In the flask culture, optimum culture conditions for the mycelial growth were obtained after 13 days of cultivation. SFW must be a remarkable medium for *L. edodes* because of its simple preparation and low cost (**Jung *et al.*, 2001**). The chemical composition and elemental analysis of *Agave salmiana* and *Agave weberi* bagasse (lignocellulosic residues of mexican Mezcal industry) showed a content of 3.70 % and 3.17 % for protein, 5559 mg/l and 3.23 mg/l for total reducing sugars, 0.73 % and 0.54 % total nitrogen, 3.46 % and 1.95 % calcium respectively. The BE was 70 % in *A. salmiana* bagasse and 40 % in *A. weberi* bagasse, the use of these residues for the cultivation of *P. ostreatus* is feasible (**Heredia-Solis *et al.*, 2014**).

A significant series of investigations (**Petre and Petre, 2011; Petre and Petre, 2012; Petre and Petre, 2013a; Petre and Petre, 2013b; Petre and Teodorescu, 2011; Petre and Teodorescu, 2012; Petre *et al.*, 2014**) devoted to the utilization of fruit, wine making industry and fruit trees wastes. The screening the optimal biotechnology of medicinal mushroom cultivation from the solid-state fermentation and the submerged one by using different kinds of wastes coming from cereal crop processing as well as the agro-food industry is the aim of **Petre and Teodorescu (2011)** study. The both fermentation technologies were tested through the controlled cultivation of the medicinal mushrooms *G. lucidum* and *L. edodes* on different growing substrates made of cereal, fruit and vegetable wastes. Among the five nitrogen sources examined, wheat bran was the most efficient upon the mycelia growing and fungal biomass production of *L. edodes* and *P. ostreatus*, at 35–40 g% fresh fungal biomass weight, closely followed by malt extract at 25–30 g%. The best mineral source was $CaCO_3$ that yielded the optimal mycelia growing as well as fungal biomass production at 28–32 g%. The final fruit body production by the two mushroom species was registered between 1.5–2.8 kg per 10 kg of solid composts made from winery wastes. The biotechnological controlled cultivation of edible mushrooms *L. edodes* and *P. ostreatus* was tested (**Petre and Petre, 2013b**) through the submerged fermentation of different fruit wastes from organic horticulture that provided a fast growth as well as high biomass productivity of investigated strains in comparison with the sample. All culture media used in experiments were prepared from different sorts of organic fruit wastes such as juice and pulps, resulted from the industrial processing of apples, pears and plums. The submerged fermentation was carried out inside the culture vessel of an automatic laboratory-scale bioreactor. The microbial strains of *B. subtilis* and *P. ostreatus* were used in pairs as well as separately to compare the efficiency of their biological potential in utilization of fruit wastes into protein biomass (**Petre *et al.*, 2014**). These strains were tested both in monocultures and co-cultures for growing on two variants of culture substrates made of apple and plum wastes mixed with cereal wastes. The optimal temperatures for both bacteria and mycelia cultures to produce microbial biomass through controlled submerged fermentation as mono- and co-cultures, were registered between 23–25°C, corresponding to initial pH levels of 4.5–6.0 and the agitation speed was tested in the range of 30–90 rpm. The registered results revealed an increasing of reducing sugars correlated with the significant level of protein content analysed as total nitrogen for the microbial biomass of co-cultures, in comparison with the control samples represented by the monocultures of the same bacterial and fungal species used in experiments.

A new substrate, breadcrumbs, was investigated for biomass accumulation, the pH of the cultural broth, the formation of primary metabolites such as the proteins and endopolysaccharides of *S. commune* and *T. versicolor*, as well as its utilization efficiency. The results showed that *S. commune* gives more mycelial mass (23.96 g/l) and in a shorter period (4 days) than *T. versicolor* (15.76 g/l) (in 5 days). The pH values changed from the initial 6.1 to 3.6 in *S. commune* cultural broth and to 4.4 in *T. versicolor* cultural broth. Maximal endopolysaccharide content in the mycelia of *S. commune* and *T. versicolor* were 7.13 % and 6.42 %, correspondingly. Crude protein content in *S. commune*

mycelium was 18.83 % on the 4th day of cultivation, and 20.03 %, in the mycelium of *T. versicolor*, on the 6th day of cultivation (**Ivanova *et al.*, 2014**).
Three food wastes residues (peas, broad bean, beet pulp) were used in different levels with mung bean straw to prepare mushroom growth media for *A. bisporus*. The total yield was: mung bean straw (2.56 kg/10 kg), mung bean straw + broad bean = 3:1 (2.51 kg/10 kg) (**Al Abttan *et al.*, 2005**).
The aim of the next study (**Yang *et al.*, 2012**) was to evaluate the feasibility of adding citrus peel (pomelo, lemon, orange and grapefruit) extracts to enhance the formation of bioactive metabolites in the submerged culture of *Antrodia cinnamomea*. With the exception of grapefruit, citrus peel extracts tested were proved to be beneficial to mycelial growth and to the production of intracellular polysaccharide. Lemon was the most effective for enhancing bioactive metabolite production. With an addition of 2 % of lemon peel extract, the mycelium biomass concentration and intracellular polysaccharide content rose from 11.96 g/L of the control and 123.6 mg/g to 21.96 g/l and 230.8 mg/g, respectively, on day 8. The production of triterpenoids also increased from 86.7 to 282.9 mg/l.
P. ostreatus hypha has been cultivated on the food wastes (rice, cabbage pickles, egg soup, pumpkin, lettuce, seasoned vegetables, instant noodle, bean sprout, egg) extracts with concentrations 10, 20, 30, 40, and 50 %. The initial pH were set variously with 4, 5, 6, and 7. These were cultured for 9 days at the temperature of 25^{o}C and the rotation rate of 120 rpm. The result is that the mushroom hypha has been grown best at the concentration of fluid – 30 % and the optimal pH was 5 and 6 (**Lim *et al.*, 2009**).
Water from matured coconut was evaluated for schizophyllan production (**Reyes *et al.*, 2009**). *Sch. commune* ATCC 38548 was used as the test strain. Results of the investigation showed that coconut water could stimulate the growth of *Sch. commune* with subsequent production of schizophyllan at 7.71g/1000 ml 4 days after incubation which is a day earlier than in the two semi-synthetic media. The basal semi-synthetic and the triple sugar- enriched media yielded 6.69g and 3.99g of schizophyllan per 1000 ml of the medium, 5 days after incubation, respectively.
The contents of Ca, Mg, Na, and K in fruiting bodies (FB) of *G. lucidum*, *L. edodes*, and *Pholiota adipose* have been determine in **Jo *et al.***, (**2013**) study. The objectives of this study were to evaluate applicability of food waste compost (FWC) as a substrate for cultivation of *G. lucidum*, *L. edodes*, and *P. adipose*. FB yield per substrate in FWC-free controls was 53 g/Kg for *G. lucidum*, 270 g/kg for *L. edodes*, and 1,430 g/Kg for *P. adipose*. Substrates supplemented with FWC showed the highest FB production at FWC content of 10 % for *G. lucidum* (64 g/Kg), 13 % for *L. edodes* (665 g/Kg) and *P. adipose* (2,345 g/Kg), which were 1.2~2.5 times higher than the values for the controls. *P. adipose* contained higher amounts of mineral elements than the other species. Ca, Mg, Na, and K content in FB did not show a significant relation to FWC content.
Large amounts of food waste attached to actuality of the search for possible utilization, in this case – by higher fungi. New components of substrates: milk whey, fishery waste, fruit waste, winery wastes, oil industry wastes, and food waste compost are characteristic for the majority of the countries of the word and therefore are promising for their utilization by higher fungi. Utilisation of food wastes using Macromycetes is a perspective direction, oriented to satisfaction of the global demand for food protein, nutritional supplements and natural drugs.

CONCLUSIONS

The dynamic increase of agricultural production can not keep up even more rapid growth of the world population, but leads to the accumulation of large amounts of waste. Waste management and providing a world population with rich in protein food is two important problems of which the utilization of agro-industrial (agriculture and food industry) waste by higher mushrooms causes the growing interest of researchers around the world. Not all mushrooms are edible, but many of not edible mushrooms exhibit various types of therapeutic activity, and at the same time are capable for wastes biodegradation. More than 150 individual types of wastes have been investigated as alternative substrates alone or in various compositions (more than 450 substrates) for cultivation of 52 higher mushroom species (about 100 strains) as evidenced by the results of more than 130 considered in the review scientific publications. All waste is used as a basis for substrates and supplements thereto, are characteristic of the respective continent and region of the world. Alternative substrates will be an integral part for the waste management technology.
Majority of mushrooms listed in the review are wood-decay one's, so the basis of most substrates is lignocellulosic feed stocks (different kinds of sawdust, woodchips, straw, grasses, hulls, etc.). Extremely difficult to determine the regularities that affect the morphological parameters, yield and biochemical composition of different mushrooms, depending on the qualitative and chemical composition of the substrate. Good results are obtained with the combined substrates. Publications containing biochemical studies of substrates and fungi confirm that fungi are grown on unconventional substrates rich in biologically active substances, provide a rich biochemical composition of fungi compared with conventional substrates (sawdust, straw, etc.).
The disadvantage of many publications is the lack of mention of examined fungi strains, whereas studies of various strains of the same fungus on the same substrate show different results.
A very important problem escapes the attention of researchers. Most agricultural wastes (various kinds of sawdust, straw, leaves, grasses, etc.) contain toxic substances which can be accumulated by mushrooms, primarily – heavy metals, herbicides, pesticides, the amount of which in grown mushrooms is not determines. Food waste is most often free from this drawback, as the food raw material supplied according to regulatory documents, including the safety performance.
The prospect of the study of agricultural residues utilization by higher mushrooms consists in the investigations of: productivity, biological efficiency of the process, morphological and biochemical indices of cultivated mushrooms, depending on the biochemical parameters of substrates and the process conditions; safety of cultivated mushrooms.

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JMBFS
Journal of Microbiology, Biotechnology and Food Sciences
International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

AGRO-INDUSTRIAL WASTE BASED GROWTH MEDIA OPTIMIZATION FOR BIOSURFACTANT PRODUCTION BY *ANEURINIBACILLUS MIGULANUS*

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doi: 10.15414/jmbfs.2016.5.6.578-583

ARTICLE INFO

Received 10. 12. 2015
Revised 24. 1. 2016
Accepted 16. 2. 2016
Published 1. 6. 2016

Regular article

OPEN ACCESS

ABSTRACT

The present work aimed to optimize a molasses and tuna-processing by-products based new economic medium for biosurfactant (BS) production by a promising strain of *Aneurinibacillus migulanus*. A culture medium based on a mixture of molasses and supernatants generated from tuna by-products supplemented with oligoelements solution was optimized using the mixture design methodology. Biosurfactant (BS) production and emulsification index (E24) were evaluated. Maximal BS of 2.95 g/l was obtained with a 95:5 (v:v) mixture of molasses and tuna by-product supernatant. However, higher level of E24 (62%) was recorded with medium containing the proportion 5:95 (v:v) of molasses and tuna by-product supernatant. The predicted responses from these mixture proportions were also validated experimentally. Interestingly, oligoelements supplements were not needed to prepare the culture medium. Molasses and tuna-by-product, non-conventional substrates, can be used efficiently for BS production by *A. migulanus*.

Keywords: Biosurfactant, *Aneurinibacillus migulanus*, Emulsification index, Molasses, Tuna-by-product

INTRODUCTION

Surfactants are amphipathic molecules with hydrophilic and hydrophobic regions. These molecules can reduce surface tension at the air-water interface between two immiscible liquids or between the solid-water interfaces. They can adsorb at interface of the system and decrease interfacial free energy (**Yu and Huang, 2011**). This characteristic confers excellent detergency, emulsifying, foaming and dispersing traits, making surfactant an interesting chemical for versatile process (**Reis, *et al.*, 2013**). These components have applications in various industries such as petrochemical, oil, pharmacy, medical, cosmetics, food and pharmaceutics (**Babu *et al.*, 1996**; **Banat *et al.*, 2010; Makkar and Cameotra, 2002; Muthusamy *et al.*, 2008; Soberón-Chávez *et al.*, 2011**). In 2008, the annual global production of surfactants was 13 million metric tons and it is expected that the average annual growth of the global surfactant market will be 4.5 % by 2018, resulting in revenues of more than US$ 41 billion (**Ashby *et al.*, 2013**). However, the currently used surfactants are generally chemically synthetic or derived from petroleum like alkylbenzene sulfonate, quaternary ammonium chloride, salt of long chain amine, sulfobetaine and polyoxyethylenated alkylphenol (**Rosen and Kunjappu, 2012**). These chemicals are often toxic and non-biodegradable, representing an additional source of contamination (**Reis *et al.*, 2013**). For example, the introduction of surfactants into the soil environment, for the purposes of soil remediation, can lead to contamination concerns. Consequently, the toxicity of the surfactant and its potential degradation products needs to be carefully considered prior to its use (**Van Hamme *et al.*, 2006**). In recent years, researchers are interested in microbial BS due to their diversity and their proprieties (lower toxicity, higher biodegradability, the ability to act in high temperatures, low pH and different salinity levels, higher foaming, etc.) (**Reis, *et al.*, 2013**). BS have the ability to be synthesized by microorganisms with numerous potential applications in the environmental processing (crude oil recovery, heavy metal removal, etc.), in health care and in food-processing industries (**Cameotra and Makkar, 2010**). Consequently, BS are preferred to synthetic and chemical surfactants (**Dehghan-Noudeh *et al.*, 2009**; **Deleu and Paquot, 2004**). Microbial BS are a structurally diverse group of surface active molecules including glycollipids, lipopeptides, phospholipids, fatty acids, neutral lipids, polymeric compounds, etc. (**Reis *et al.*, 2013**). These molecules with hydrophobic and hydrophilic parts are either anionic, cationic or neutral. The hydrophobic part, which is less soluble in water, is based on long-chain fatty acids, hydroxy fatty acids or α-alcyl -β- hydroxy-fatty acids. The hydrophilic portion, which is more soluble in water, can be a carbohydrate, amino acid, cyclic peptide, phosphate, carboxylic acid or alcohol (**Chayabutra *et al.*, 2001; Chen *et al.*, 2007; Volchenko *et al.*, 2007**). These molecules reduce surface and interfacial tensions in both aqueous solutions and hydrocarbon mixtures, which makes them potential candidates in various sectors as cited above such as the bioremediation processes (enhance oil recovery from wells, reduce the heavy oil viscosity, clean oil storage tanks, increase flow though pipelines, stabilize fuel water-oil emulsions, etc.) (**Mulligan, 2005**). A wide variety of microorganisms (*Pseudomonas aeruginosa, Bacillus sphaericus, Staphylococcus sp, Arthrobacter*, etc.) can produce BS using various substrates including sugars, alkanes and wastes such as frying oils, distillery, curd whey by-products (**Elazzazy *et al.*, 2014; Geys *et al.*, 2014; Dubey and Juwarkar, 2001**). Nevertheless BS have shown their potential applications, their use is limited because of the lack of cost effective production processes **(Reis, *et al.*, 2013)**. Generally, the BS production costs can be reduced through process optimization of various control factors such as the culture medium composition or/and the growth conditions including limiting nutrients, the trace elements, the addition of inducer, pH, temperature, etc. (**Elazzazy *et al.*, 2014**). In the context, many studies have been conducted in order to improve the microbial genetics, the production process and the commercial applications of BS (**Kuyukina *et al.*, 2001**). The nature and the productivity of BS by microorganisms are controlled mainly by the carbon source used during culture. In order to reduce the production cost, the use of cheaper carbon source is needed. In this perspective, many waste materials such as corn oil, molasses, whey and lipids have been used as substrates for BS production (**Joshi *et al.*, 2008; Makkar and Cameotra, 1997; Mukherjee *et al.*, 2006; Ramani *et al.*, 2012; Rocha-e-Silva *et al.*, 2014; Santos *et al.*, 2013**). However, no studies have examined the feasibility of using tuna processing waste in the formulation of microbial growth media for BS production. In this context, molasses and tuna-by-product based-growth media supplemented with oligoelements were optimized for BS production by *A. migulanus* using mixture design methodology.

MATERIAL AND METHODS

Tuna by-products sampling, characterisation and treatment

Tuna (*Thunnus thynnus*) by-product (heads, viscera, skin, some muscle tissue and bones) were collected from fish processing industry located in Sfax region

(Tunisia). Samples were grinded with a grinder, mixed with water (500 g.L^{-1}) and heated at 100°C for 20 min. After heat pre-treatment, insoluble material was removed by centrifugation (10000 rpm for 30 min). The obtained supernatant was stored at -20°C until use. Supernatant was subject to characterisation according to the AOAC methods (**AOAC, 1990**), water content was quantified by drying samples at 100°C, lipid by Soxhlet extraction, nitrogen by Kjeldahl procedure, and ash by incineration in a muffle furnace at 550°C. Protein content was calculated using a rate of 6.25% nitrogen to protein (**AOAC, 1990**).

Molasses sampling and characterisation

Molasses were sampled from the sugar refining industry (Tunisian Society of Sugar Beja, Tunisia) and stored at 4°C until use. Sample was subject to chemical characterisation as described in the AOAC methods (**AOAC, 1990**).

Microbial strain and culture conditions

A. migulanus NCTC TSA 7092 was used throughout this study. *A. migulanus* was maintained at 4°C on Luria Broth (LB) solid medium (10 g.L^{-1} tryptone, 5 g.L^{-1} yeast extract, 10 g L^{-1} NaCl and 15 g.L^{-1} agar, pH 7.0) and inoculum preparation was conducted in Erlenmeyer flask containing 50 mL of liquid LB medium (the flask was sterilized at 121ºC for 20 min and incubated at 30°C overnight on a rotary shaker at 200 rpm).

Microbial growth was studied in media based on molasses solution (34.5 g.L^{-1}), supernatant generated by boiling tuna by-product and oligoelements solution (composed of in g.L^{-1}: KH_2PO_4, 1; K_2HPO_4, 1; $MgSO_4$, $7H_2O$, 0.2; $CaCl_2$, $2H_2O$, 0.02 and $FeCl_3$, $6H_2O$, 0.05). Experiments were conducted in 500 mL Erlenmeyer flasks each containing 100 mL of medium. The initial pH of the medium was adjusted to 7.0. Then, culture media were sterilised at 121°C for 20 min. Flasks were inoculated with 4% (v/v) of the inoculum and growth was performed for 72 hours under the same conditions used to prepare the inoculum.

Emulsification index (E24)

Emulsification assays of the BS were performed using the method described by **Cooper and Goldenberg (1987)**. The emulsification activity of the supernatant was measured by adding 3 mL petroleum ether to 3 mL of the culture supernatant in a test tube, vortexing for 2 min, and then leaving it to settle for 24 h. E24 was estimated as the height of the emulsion layer, divided by the total height, multiplied by 100.

Biosurfactant determination

BS was extracted from the culture medium after cell removal by centrifugation at 8500 rpm for 10 min at 4°C.The supernatant pH was adjusted to 2.0 with 1.0 N HCl solution. Pellet thus precipitated was collected by centrifugation (8500 rpm for 20 min at 4°C). The precipitate was then re-dissolved in distilled water and collected by centrifugation (8000 rpm for 45 min at 4 °C). The operation was repeated twice. The yield of isolated BS was expressed in g.L^{-1} (**Chander *et al.*, 2012**).

Experimental design and statistical analysis

The Design-Expert (7.0) Software (Stat-Ease Inc., USA) was used to determine the optimum proportions of culture medium formulation. The mixture components consisted of volume of molasses (X_1), volume of tuna by-product supernatant (X_2) and volume of oligoelements solution (X_3). All components had the same range, between 0 and 1. Components proportions were expressed as ratios of each compound volume to the mixture (sum $X_1+X_2+X_3 = 1$). These three components levels were used to investigate their effect on BS production and E24. The design-expert software generated 15 runs for each culture medium and responses (BS production and E24) were determined experimentally for each one.

The regression models of responses (BS production and E24) were established through second order polynomial equation and were presented as follows (Eq. 1):

$$Y = b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{123}X_1X_2X_3 \quad (1)$$

Where Y is the response (BS production and E24), X_1, X_2 and X_3 were the levels of variables (molasses, tuna by-product supernatant and oligoelements, respectively), b_1,b_2, $b_3$were coefficients of linear term and b_{12},b_{13},b_{23}, b_{123}were the interaction coefficients.

The statistical and mathematical analyses were evaluated using Design Expert 7. The effects of the three variables were calculated, as well as their possible interactions on the BS and E24. The significance of each variable was evaluated using analysis of variance (ANOVA).

RESULTS AND DISCUSSION

BS are of considerable commercial interest in various commercial applications in the petroleum, pharmaceuticals, biomedical and food processing industries (**Reis *et al.*, 2013**). Due to their beneficial properties such as biodegradability, BS were proposed to replace chemical surfactants. However, BS production depends especially on the raw material cost, which represent about 10–30% of the global production cost (**Cameotra and Makkar, 1998**). Consequently, the choice of low-cost raw materials for the preparation of the microbial growth media is an important way to ensure the economy of the BS process. Interestingly, many strategies were used for economical BS production using selected bacteria and economic growth media (**Fox and Bala, 2000; Makkar and Cameotra, 1999**). In this context, a mixture design was applied to determine the optimum conditions for BS productions and to maximize the E24 by *A. migulanus* growing on media based on molasses supplemented with supernatant generated from boiled tuna by-product and with oligoelements. Molasses is a co-product of refining of sugar beets into sugar. The extensive use of molasses as carbon source is related to its low price compared to other sources, and the presence of several other compounds and vitamins which are valuable for microbial growth (**Gaurav *et al.*, 2014**). Interestingly, fish by-product, which are an easily available substrates generated in large amount by the Tunisian industries, provide an excellent source for microbial growth media (especially nitrogen and minerals), which can be exploited in producing various high added value metabolites (**Ben Rebah and Miled, 2013**). The integration of both molasses and tuna-by-product in microbial growth media for BS production, can lower the bioprocess cost and reduce environmental problems associated with agro-waste materials and propose another environmental friendly disposal way.

Raw material composition

The composition of raw materials (molasses and supernatant generated by boiled tuna processing by-product) was determined (Table 1). There were significant differences ($P < 0.05$) in proteins, lipids, ash and carbohydrates contents between supernatant generated by boiled tuna processing by-product and molasses. Tuna by-product contained the highest protein, lipid and ash contents (46.41±2.42; 5.33±1.10% and 41.30±2.54 of total dry weight (w/dw), respectively). However, molasses showed the highest levels in carbohydrates (86.84±2.51%). Generally, fish processing by-product contain growth factors offering good potential as culture media for microbial growth (**Ben Rebah and Miled, 2013; Dehghan-Noudeh *et al*., 2009**). In order to enhance the soluble protein fraction and ash contents, various pre-treatment processes (heat treatment, chemical and enzymatic treatment, etc.) have been applied to fish wastes before being used as growth media (**Ben Rebah *et al.*, 2008; Huang *et al.*, 2011; Poernomo and Buckle, 2002**) As reported by **Ben Rebah *et al.* (2013)** applying heat treatment (100°C, 20 min) on fish by-product for microbial growth use, may has numerous advantages (simpler process, reduction of energy requirement, and consequently the cost production). Hence, the heat treatment allows the solubilisation of minerals contained in bones for tuna by-product (**Ben Rebah and Miled, 2013; Ben Rebah *et al.*, 2008**). Furthermore, high temperature treatment may affect the quality of the boiled product, such as the structure and the solubility of proteins (**Ben Rebah and Miled, 2013; Niamnuy, 2002; Poernomo and Buckle, 2002**) allowing an enhancement of the alkali-soluble protein fraction as reported while treating shrimp by-product (**Niamnuy *et al.*, 2008**). Although, fish processing waste may be considered as potential nitrogen source and salts, in some cases the presence of lipids in this waste may inhibit the microbial growth as reported by **Ben Rebah *et al.* (2013)**.

Table 1 Chemical composition of molasses and tuna by-product; means of three replicates (% dry weight).

	Proteins	Lipids	Ash	Carbohydrates**
Molasses	2.27 ± 0.52	1.26 ± 0.36	9.63 ± 1.62	86.84 ± 2.51
Tuna by-product supernatant*	46.41 ± 2.42	5.33 ± 1.10	41.30 ± 2.54	6.96 ± 2.02

*Supernatants obtained after heat treatment (100 ºC; 20 min) of raw materials.
**Carbohydrates were calculated by the difference [100% − (proteins + lipids + ash)].

Experimental design data and analysis of the models

The optimal conditions for BS production were predicted using the optimization function of the Design Expert software. To improve the economic competitiveness of microbial BS production, tuna-by-product and molasses based-growth media were optimized. Oligoelements solution was also added to the growth media. In this study, BS production and E24 were maximized by mixture proportions given in Table 2.

The response data (E24 and BS production) in Table 2 were converted into two polynomial equations with three independent variables. Consequently, the polynomial models describing the correlation between responses and variables were (Eq. 2-3):

$$Y_{E24(in\ \%)} = 49.12\ X_1 + 62.55\ X_2 + 67.10\ X_3;\text{with adjusted } R^2 = 0.545 \quad (Eq.\ 2)$$

$$Y_{BS\ (in\ g/l)} = 3.22\ X_1 + 0.91\ X_2 + 1.93\ X_3 - 5.40\ X_1X_2 - 9.93\ X_1X_3 - 3.91\ X_2X_3 + 36.92\ X_1X_2X_3;\text{ with adjusted } R^2 = 0.816 \quad (Eq.\ 3)$$

Where Y_{E24} and Y_{BS} are the predicted responses of E24 and biosurfectant production, respectively. X_1, X_2 and X_3 are the proportions of molasses, tuna by-product supernant and oligoelements solution, respectively.

Table 2 Mixture design matrix with the observed and predicted values.

	Experimental Condition			E24 (%)		Biosurfactant (g/l)	
Run	Molasses (X_1)	Tuna by-product supernatant (X_2)	Oligoelement solution (X_3)	Observed	Predicted	Observed	Predicted
1	0.95	0.05	0	50	49.8	3.2	2.8
2	0.05	0.95	0	60	61.9	0.6	0.8
3	0.05	0.05	0.9	70	66.0	1.7	1.3
4	0.5	0.5	0	51	55.8	0.6	0.7
5	0.5	0.05	0.45	64.6	57.9	0.7	0.1
6	0.05	0.5	0.45	64	63.9	0.7	0.2
7	0.35	0.35	0.3	60	59.2	1.5	1.1
8	0.65	0.2	0.15	54	54.5	1.1	0.8
9	0.2	0.65	0.15	65	60.6	1.2	0.1
10	0.2	0.2	0.6	53	62.6	0.6	0.1
11	0.05	0.05	0.9	62	66.0	1.2	1.3
12	0.05	0.5	0.45	68	63.9	0.6	0.2
13	0.95	0.05	0	48	49.8	2.6	2.8
14	0.5	0.5	0	59	55.8	0.8	0.7
15	0.05	0.95	0	61	61.9	0.8	0.8

ANOVA was also performed (Table 3). The associated *p*-value was used to estimate whether *F*-value was large enough to indicate statistical significance. A *p*-value below 0.05 indicates that the model was statistically significant. As indicated in table 3 for both E24 and BS production, linear mixture components were significant model terms. The values of R^2, a measurement for fitness of the regressed Eq. 2 and Eq. 3 were 0.61 and 0.89, respectively. These results indicated that the experimental data were in a good agreement with predicted values.

Table 3 ANOVA and regression analysis of the model for E24 and biosurfactant production.

Source	Sum of Squares	Degrees of freedom	Mean Square	F-value	p-value	
E24						
Model	387.524	2	193.762	9.397	0.0035	significant*
Linear Mixture	387.524	2	193.762	9.397	0.0035	
Residual	247.425	12	20.619			
Lack of Fit	172.925	7	24.704	1.658	0.2988	not significant
Pure Error	74.500	5	14.900			
Cor Total	634.949	14				
R-Squared = 0.610						
Biosurfactant production						
Model	7.669	6	1.278	11.364	0.0015	significant*
Linear Mixture	4.268	2	2.134	18.970	0.0009	
X_1X_2	1.603	1	1.603	14.250	0.0054	
X_1X_3	2.224	1	2.224	19.768	0.0022	
X_2X_3	0.234	1	0.234	2.081	0.1871	
Residual	0.876	1	0.876	7.789	0.0235	
Lack of Fit	0.900	8	0.112			
Pure Error	0.550	3	0.183	2.618	0.1631	not significant
Cor Total	0.350	5	0.070			
R-Squared = 0.895						

*Statistically significant at 95% of confidence level

The regression coefficients for all terms in optimized models were analyzed. In the case of E24 (Eq.2), the effect of X_3 (67.10) was more important than that of X_2 (62.55) and X_1 (49.12). However, for BS production (Eq.3), the influence of X_1 (3.22) was more important than that of X_2 (0.91) and X_3 (1.93), indicating that the molasses proportion (X_1) was the main factor controlling the higher BS production.

Positive coefficients for a three-component blend mean that the three components were complementary. This is the case of BS production when combing molasses with tuna byproduct supernatant and oligoelements (Eq.3). Also, the fact that two-component blends have negative coefficients, means that the two components were non-complementary (Eq.3). This was the case of the interactions of X_1X_2 , X_1X_3 and X_2X_3.

The best way to predict the relationships between responses and the growth medium compositions is to analyze the contours diagrams or the three dimensional surface plot generated from the estimated models. The contours

diagrams (A) and response surface (B) of E24 and BS production were depicted in figure. 1 and 2, respectively.

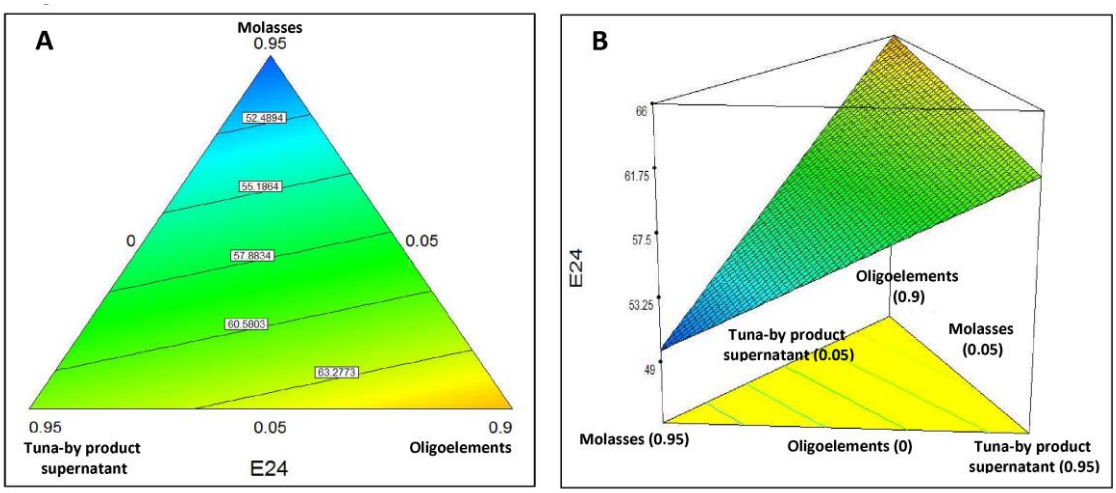

Figure 1 Contour diagrams (A) and response surface (B) for E24 resulted of growing *A. migulanus* as a function of the added molasses, tuna-by product supernatant and oligoelements.

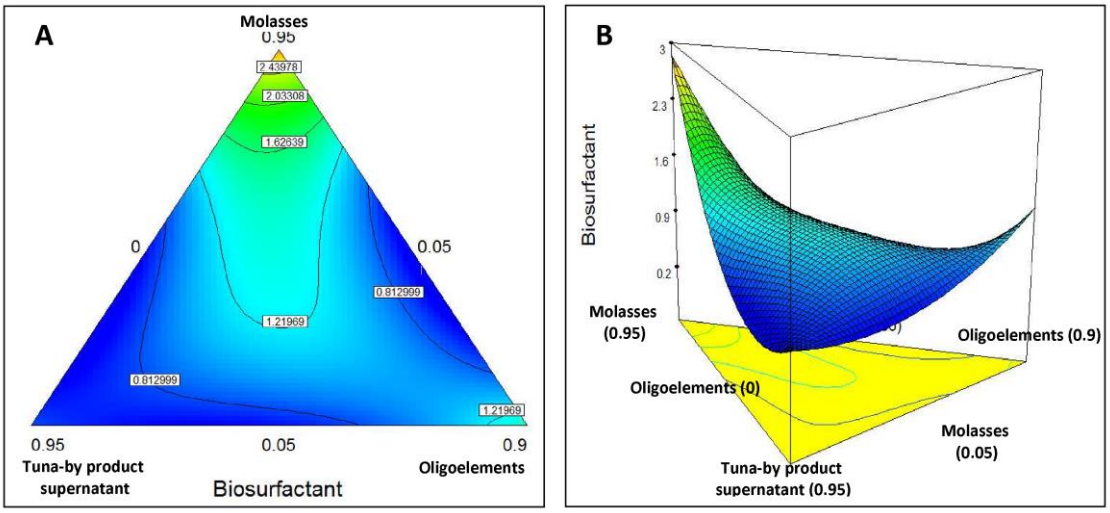

Figure 2 Contour diagrams (A) and response surface (B) for biosurfactant produced by *A. migulanus* as a function of the added molasses, tuna-by-product supernatant and oligoelements.

E24 decreased gradually with the added amount of molasses. Similarly, adding tuna-by-product supernatant reduced E24, but with lesser extent while compared to molasses effect. Interestingly, the addition of oligoelements (up to 90%) to the medium increased E24 and values remained between 50 and 70%. The variation of the E24 may be explained by the variation of the BS concentration in the culture medium or/and by the nature of BS morphology. According to **Ron *et al.* (2002)** low molecular weight BS effectively reduce the interfacial tensions, while the high molar mass polymers such as lipopeptides are less effective in reducing the interfacial tensions. Moreover, the E24 was evaluated using the culture supernatant and its composition may have an effect on the E24 value. Hence, the production of secondary metabolite and the remained nutrient form the growth medium could interfere with emulsion formation (**Bonilla et *al.*, 2005**). Moreover, the morphology of BS can be significantly affected by pH variations, which may control the surface tension and dispersion rate (**Shin *et al.*, 2004**). The ionic strength or salinity of the medium could also affect the process (**Abouseoud *et al.*, 2010**).

BS production decreased progressively with the enhancement of tuna-by-product supernatant rate in the culture medium. This may be explained by the inhibition of the BS production which is related to the microbial growth. Lipids content in tuna-by-product may affect the bacterial growth and consequently the BS production as reported for other microorganisms cultivated in fish waste-based media (**Vazquez, 2004**). In contrast, it was reported that agroindustrial waste with high content of carbohydrates, or lipids meet the requirement for use as substrate for BS production (**Makkar and Cameotra, 1999**).

Generally, results indicated that molasses and tuna-by-product contain nutrients necessary to sustain the growth of *A. migulanus* and consequently the BS production. The positive effect of molasses may be explained by its higher carbohydrates and amino-acids contents. Slight addition of tuna-by-product supernatant may increase the BS production. However, an over addition of tuna supernatant decreases both BS production and E24 this may be related to the unbalanced nutrients concentrations. The beneficial use of molasses as a carbon source supplemented with yeast extract, or other nitrogen source and some metal ions for BS production has been reported by many studies (**Dubey and Juwarkar, 2001; Patel and Desai, 1997**). However, according to **Joshi *et al.* (2008)**, the only use of molasses without addition of nitrogen source, or metal supplements allow acceptable yield of BS production by Bacillus strains. It seems that BS production depends on the used species (having different nutrient requirements) and on molasses characteristics which vary depending on its origin and this may affect the microbial growth and the BS production. In this perspective, for example in the study of *P. Aeruginosa* strain (**Dubey and Juwarkar, 2001**), it was reported that an industrial waste based media should have optimum carbon, nitrogen, phosphorus and iron concentrations with C/N, C/P and C/Fe ratios suitable for maximum production of BS. Therefore, it is very important to determine in molasses and in fish by-product the specific factors, the nature of nitrogen-containing compounds such as the amino acid composition and the small-size peptides that might be vital factors for *A. migulanus* growth and BS production.

Optimization of mixing proportion for responses and validation of the model

The optimal conditions for E24 and BS production were predicted using the optimization function of the Design Expert software. The formulation of an economic and competitive medium and maximization of both emulsification index and biosurfactnat production were satisfied by mixture proportions given in Table 4. These solutions provide E24 of 61.88 ± 4.541% and 2.4 ±0.335 g/l of BS. Experiments were conducted under optimal conditions in order to assess the validity of regression models (Table 4). The result demonstrated that the experimental data were in good agreement with the predicted values, confirming the validity and the adequacy of the predicted models. Interestingly, in optimized media, no additional oligoelements were required. However, the addition of oligoelements considerably stimulated cell growth and BS production (**Reis *et***

al., 2004). Indeed, in the present study, tuna-by-product and molasses were used as based media to the growth of *A. migulanus*. These two media contained an appreciable level of ash (41.30 ± 2.54% and 9.63 ± 1.62%, respectively) which was generally correlated to the salt content. Therefore, oligoelements is provided by molasses or/and tuna-by-product media.

Table 4 Solutions for optimal conditions as generated by the Design Expert Software

Experimental Condition			Response			
Nutrient source (mL)			Biosurfactant (g.L^{-1})		E24	
Molasses (X_1)	Tuna by-product supernatant (X_2)	Oligoelement solution (X_3)	Observed value	Predicted Value	Observed value	Predicted value
95	5	0	2.95 ± 0.353	2.4 ±0.335	-	-
5	95	0	-	-	62 ± 1.553	61.88 ± 4.541

CONCLUSION

In this study we show that molasses and tuna-by-product, non conventional substrates (agro-industrial by-product), can be used efficiently for BS production by *A. migulanus*. The BS production process using these materials is a relatively inexpensive and economic process, which can be easily adapted for various environmental applications. Moreover, we demonstrated that the mixture design methodology can be used to determine the optimum medium mixtures based on molasses and supernatant generated by boiling tuna-by-product, allowing to maximize BS production and E24. These studies will give insights into the potential of using industrial wastes. However, more investigations are needed to determine effects of others factors (temperature, pH, oxygenation, etc.) related to the bioprocess. Additionally the recent availability of the genome sequences of the strain used in this study and another strain of *A. migulanus* (**Alenezi *et al.*, 2015a, b**) will help identify genes that control biosynthesis of BS and the regulatory mechanisms underlying their biosynthesis.

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Journal of Microbiology, Biotechnology and Food Sciences

International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

IDIOSYNCRASY OF LOCAL FUNGAL ISOLATE *HYPOCREA RUFA* STRAIN P2: PLANT GROWTH PROMOTION AND MYCOPARASITISM

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doi: 10.15414/jmbfs.2016.5.6.593-598

ARTICLE INFO

Received 16. 12. 2014
Revised 11. 2. 2016
Accepted 23. 2. 2016
Published 1. 6. 2016

Regular article

OPEN ACCESS

ABSTRACT

Trichoderma viride an anamorph of *Hypocrea rufa,* is a known bio-control agent against various fungal phytopathogens. In the present study, *H. rufa* strain P2 was tested for plant growth promoting (PGP) traits and antifungal activity against *Fusarium oxysporum, Alternaria alternata, Aspergillus niger, Sclerotium rolfsii*. *In-vitro* assessment of *H. rufa* strain P2 showed maximum IAA production of 68 µg ml^{-1}, solubilised tri-calcium phosphate up to 72 µg ml^{-1} and showed production of chitinase enzyme 120 U ml^{-1}. In order to determine *in-vivo* plant growth promotion, talc based formulation of *H. rufa* strain P2 was prepared and tested on *Arachis hypogaea* L. using seed and soil application. After 15 days, treated plants showed six-fold increases in the fresh and dry root mass whereas, fresh and dry shoot mass was increased up to two folds. The result indicates the local isolate *H. rufa* strain P2 can be categorized as phyto-friendly fungi which can be used as both, bio-control agent as well as phyto-augmenting bio-fertilizer.

Keywords: *Hypocrea rufa* strain P2; Plant Growth Promoting Fungi (PGPF); Chitinase; talc based bio-formulation; Peanut (*Arachis hypogaea* L.)

INTRODUCTION

Rhizospheric fungi have the ability to stimulate plant growth are designated as 'Plant Growth Promoting Fungi' (PGPF) (**Hyakumachi, 1994**). PGPF is non-pathogenic soil inhabiting saprophytes, which have been reported for growth promotion in several crop plants and providing protection against diseases (**Shivanna *et al.*, 1996**). Such PGPF belongs to various genera, including *Penicillium*, *Trichoderma*, *Fusarium,* and *Phoma*. Few species of PGPF have been reported to trigger systemic resistance against numerous phytopathogens (**Shoresh *et al.*, 2005**). *Hypocrea rufa* is a common inhabitant of the rhizosphere and decisively recognized as a bio-control agent of soil-borne plant pathogens (**Harman *et al.*, 2004**). A praiseworthy amount of research has been focused on the mycoparasitic nature of *H. rufa* and its contribution in plant growth promotion. Antibiosis, competition, and mycoparasitism are the different mechanisms by which *H. rufa* controls plant-pathogenic fungi (**John *et al.*, 2010**). The complex process of mycoparasitism requires the production of plenty of cell-wall-degrading enzymes, such as chitinases, cellulases, polysaccharide lyases, proteases, and lipases, which digest the fungal cell wall (**Witkowska and Maj, 2002; Gruber and Seidl-Seiboth, 2012; Phitsuwan *et al.*, 2013**).
The mechanism of PGPF mediated plant growth promotion involves the production of various phytohormones like indole acetic acid (IAA) (**Contreras-Cornejo *et al.*, 2009**), gibberellic acid and cytokinins (**Salas-Marina et al., 2011**). Moreover, plant growth promotion is also supported by the production of siderophore, mobilization of insoluble phosphate, induction of different plant pathogen defense-related enzymes (i.e. β-1, 3 glucanase, chitinases). Production of such enzymes contributed to their biocontrol characteristics (**Dey *et al.*, 2004; Chandler *et al.*, 2008**). Reduction in severity of plant diseases by application of *H. rufa* under field conditions was also reported by **Hermosa *et al.* (2012)**. Field trials using talc-based bio-formulation of *H. rufa* was reported in India for the management of several soil-borne diseases applied through seed treatment and soil application (**Jeyarajan, 2006; Mukherjee *et al.*, 2012**).

Groundnut (*Arachis hypogaea* L.) is an important oilseed crop in India. Gujarat is the principal producer state of groundnut in the country. The area and production of groundnut in the state found about 30.9 per cent and 37.1 per cent respectively in India (**Swain 2013**). The yield of groundnut is dropping by 25 per cent from 1,170 kg per hectare to 1,560 kg per hectare in Gujarat (**SEA crop survey 2014**). Various biotic and abiotic factors are responsible for this loss. There is 28 to 50 % of mortality observed due to plant fungal pathogen (**Ghewande et al., 2002**). Various remedies like crop rotations, use of recommended chemical fungicide etc. are available to effectively control fungal disease. However, these types of strategies affect human health, environmental pollution, development of pathogen resistance to fungicide and the production cost (**Patel *et al.*, 2015b**). Apart from all these strategies, an unconventional approach is by inoculating crop seeds and seedlings with plant growth promoting organisms (**Patel *et al.*, 2015a**).
In spite of the well-documented history of *H. rufa* as a biofertilizer and biocontrol agent, very few researchers have aimed to test multiple traits from a single isolate simultaneously. Therefore, in the present study, multiple traits of PGPF were accessed in a local isolate *H. rufa* strain P2 along with its antagonistic activity against several fungal pathogens. *In-vivo* plant growth promoting the activity of *H. rufa* strain P2 was determined using *Arachis hypogaea* L. as a test plant.

MATERIALS AND METHODS

Sample collection and Isolation

Farm soil sample was collected randomly at 15 cm soil depth using a cylindrical tube, from Anand, Gujarat (22˚53'N, 72˚96'E). One gram of soil was suspended in 9 ml of sterile distilled water. The serial aliquot of 0.1 ml was plated on selective medium, Rose Bengal agar with pentachloronitrobenzene (PCNB), and incubated at 28±2°C for 5-7 days. Isolate has shown green conidia was used further for experiments and identified using 18S rRNA gene sequencing. Pure culture was maintained and stored at 4°C on PDA.

Identification of isolate and morphological characterization

Isolate was grown for 5 days at 28±2°C in 100 ml of potato dextrose broth. Mycelia were collected by centrifugation. Fungal DNA extraction was done using GeNei™ Fungal Genomic DNA Extraction kit (Bangalore Genei, India). PCR amplification of 18S rRNA gene from the purified genomic DNA was carried out using the following primers (forward primer 5'-GGAAGTAAAAGTCGTAACAAGG-3' and reverse primer 5'-TCCTCCGCTTATTGATATGC-3'). Thermal cycler conditions involved an initial denaturation step at 95°C for 5 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min, and a final extension at 72°C for 1 min, followed by holding at 4°C. Amplified gene product was sequenced at 1st BASE (Agile Life Science Technologies India Pvt. Ltd.). The BLASTn search program (http://www.ncbi.nlm.nih.gov) was used to look for nucleotide sequence homology. The gene sequence was submitted to GenBank under accession number KC351188. The similar sequences from the database obtained were then aligned by ClustalW using MEGA 4.0 software (**Tamura *et al.*, 2007**) and a neighbor-joining (NJ) tree with bootstrap value 500 was generated. Morphological characteristics of the isolate were studied. Arrangements of conidia were visualized under a light compound microscope.

Selection of medium showing optimum growth of the fungal isolate

Sterile 100 ml Potato Dextrose Broth (PDB), Czapek Dox Broth (CDB) and Malt Extract Broth (MEB) were prepared in 250 ml flasks. All flasks were inoculated with 10 mm plug of *H. rufa* strain P2. All flasks were incubated at 28±2°C at 130 rpm. After completion of 3 and 6 days, the content of the flasks was filtered using pre-weighed Whatman filter paper No.1 and dried in hot air oven at 95˚C until three constant weights of mycelia were obtained.

Assessment of plant growth promoting (PGP) traits

IAA production by isolate was determined using a method of **Bano and Musarrat (2003)**. The quantitative estimation of a tri-calcium phosphate solubilisation by the isolate in the liquid Pikovskaya's medium was estimated using a method described by **Pikovskayas (1948)**. The pattern of change in the pH of the medium was recorded along with the tri-calcium phosphate solubilization using a pH meter. The concentration of the soluble phosphate was estimated from the supernatant using stannous chloride method after 13 days of incubation given by **Usharani and Lakshmanaperumalsamy (2010)**. For ammonia production, the isolate was inoculated in peptone water and incubated for five days at 28±2°C. Biomass was separated through filtration and supernatant was used for estimation of ammonia production by the method given by **Demutskaya and Kalinichenko (2010)**. The concentration of ammonia was estimated against a standard curve of ammonium sulfate in the range of 0.1-1 µmol ml^{-1}. For the qualitative estimation of HCN production, Picrate assay was performed (**Kang *et al.*, 2010**).

Biocontrol activity

The antagonistic capability of isolate P2 was assessed against prominent phytopathogens including *Fusarium oxysporum* (MTCC 3930), *Alternaria alternata* (MTCC 6572), *Aspergillus niger* (MTCC 2196), *Sclerotium rolfsii* (MTCC 6052) using dual culture technique (**Patel *et al.*, 2015b**) with slight modification. Briefly, 10 mm diameter plug of the mycelial disc of a pathogenic fungus was taken from 6th-day old culture plates and put it on PDA plates at one end in an inverted position and simultaneously, the other end was inoculated with the isolate. Plates were incubated at 28±2°C for five days. Inhibition percentage of a plant pathogen was calculated using formula % inhibition = [(I1-I2)/I1] x 100. Where I1 = radial growth of isolate, I2 = radial growth of a pathogen in dual culture experiments (Radial growth of the fungal strains were measured in mm).

Establishment of association between biomass and Chitinase enzyme

One agar plug of 10 mm size obtained from the four-day-old growth of *H. rufa* strain P2 was inoculated in 100 ml of sterile malt extract broth in 250 ml Erlenmeyer flasks and incubated at 28±2°C at 130 rpm. On each successive day, dry mycelial weight was determined as described earlier. The filtrate was stored at 4˚C till assay was performed. Chitinase assay was performed with the slight modification of the method given **Lingappa and Lockwood (1962)**. Briefly, the assay mixture consists of 200 µl broth, 300 µl of 0.1 M sodium acetate buffer (pH 5.0) and 500 µl of 1.0% colloidal chitin and incubated at 37˚C for 1 hour. Then after 200 µl of 1 N NaOH were added to the each tube followed by centrifugation at 11000 rpm for 10 minutes at 4˚C. 1 ml of Schales' reagent (0.5 M Sodium Carbonate + 2.0 mM Potassium Ferricyanide) was added to the 500 µl supernatant was taken from each of tube followed by incubation in boiling water bath for 10-15 minutes. The absorbance was immediately measured at 420 nm using a spectrophotometer. The enzyme activity was calculated from a standard curve based on known concentrations of N-acetyl-β-D-glucosamine. One unit of chitinase activity was defined as the amount of enzyme that liberated 1µmol of N-acetyl glucose amine per hour.

***In vivo* pot study**

The talc-based formulation of the isolated fungal strain was prepared (**Soe and De Costa, 2012**). Isolated strain was evaluated for its effects on the growth, yield and vegetative parameters of peanut (*Arachis hypogaea* L.). For ensuring complete sterilization, soil (medium black, pH 7.4) was sterilized at 121°C and 15 lbs for 1 h, thrice in autoclavable bags and 1 kg of soil was filled in a plastic pot of 16 cm diameter. Pots were watered at regular intervals. For bio-formulation application, the pot study was divided into two groups; (A) seed coating and (B) soil application. Different vegetative parameters like shoot length, numbers of leaves, numbers of branches, tap root length, lateral root length, fresh and dry root mass, fresh and dry shoot mass were measured after 15 days after sowing (DAS). Pot without application of talc-based bio-formulation of the isolate was used as a control.

Statistical analysis

Analysis of Variance (ANOVA) was carried out using triplicate value to identify a significant difference in each vegetative parameter between treated (seed coating, soil application) and non-treated seeds (control). Mean values of triplicates were compared at significance levels of 5%, 1%, and 0.1% LSD.

RESULTS

Morphological characteristics of the isolate

The isolate was grown on Rose Bengal agar amended with pentachloronitrobenzene (PCNB) as a green colony. One colony from the plate was transferred to PDA which was matured within 5 days at 28±2°C. Initially, isolate grew as a white color colony which turned into scattered blue-green or yellow-green patches after conidia formation. The colony was wooly and became compact; the patches were sometimes observed as concentric rings. The isolate was more readily visible on PDA and characterized rapidly by postulated branched conidiophores with lageniform phialides and green conidia born in slimy heads (Fig. 1) which were arranged repeatedly.

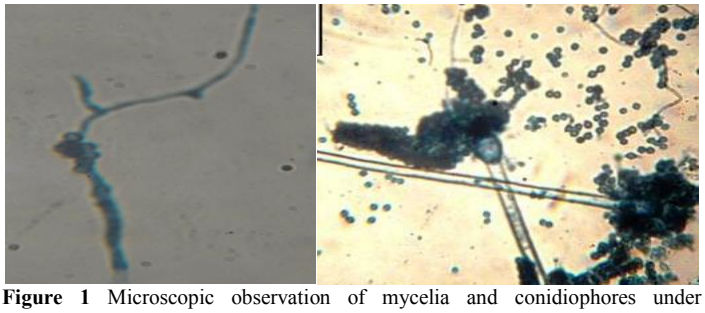

Figure 1 Microscopic observation of mycelia and conidiophores under compound microscope

Identification of isolate

Amplified 18S rRNA gene product was sequenced at 1st BASE (Agile Life Science Technologies India Pvt. Ltd.). After performing BLASTn, 18S rRNA sequences of organisms showing maximum similarity were aligned by using ClustalW and an NJ tree was developed using software MEGA 4.0 with Bootstrap values based on 500 replications, which are listed as percentages at the branching points (Fig. 2). Gene sequence has been deposited in the GenBank nucleotide sequence database under the accession number KC351188. 18S rRNA sequence of *H. rufa* strain P2 showed maximum similarity with *H. rufa* strain W63 (JN935058).

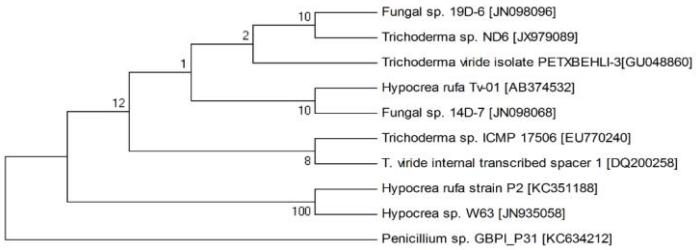

Figure 2 Phylogenetic analysis based on 18S rRNA gene sequences available from European Molecular Biology Laboratory (EMBL) library constructed after multiple alignments of data by ClustalW. Distances and clustering with the neighbor-joining method were performed using MEGA 4.0 software package.

Bootstrap values based on 500 replications listed as per percentages at the branching points

Selection of the medium for the growth of organism

Liquid mediums viz., MEB, PDB, and CDB, were used to compare the growth of isolate which was measured as dry mycelia weight (DMW). Out of these three media, MEB best supported the growth of isolate. More than four-fold increase in DMW was obtained in MEB when compared to CDB and PDB on the third day. Even on the sixth day, DMW was more than two-fold higher in MEB (Fig. 3).

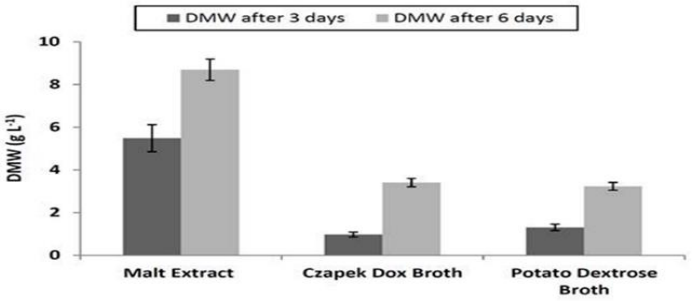

Figure 3 Comparative analysis of Dry Mycelial Weight (DMW) using fluid mediums for the growth of *H. rufa* strain P2 at 3rd and 6th day

Plant growth promoting traits

The isolate was assessed for IAA production. *H. rufa* strain P2 in PDB medium showed the maximum of 72 µg ml^{-1} production of IAA after 120 h (Fig. 4). To verify the relation between IAA production by the strain and the concentration of its precursor L-tryptophan, increasing the amount of this amino acid was added to the culture medium and production of IAA was estimated. The concurrent increase in IAA production was observed with increasing amount of L-Tryptophan supplementation (Fig. 4).

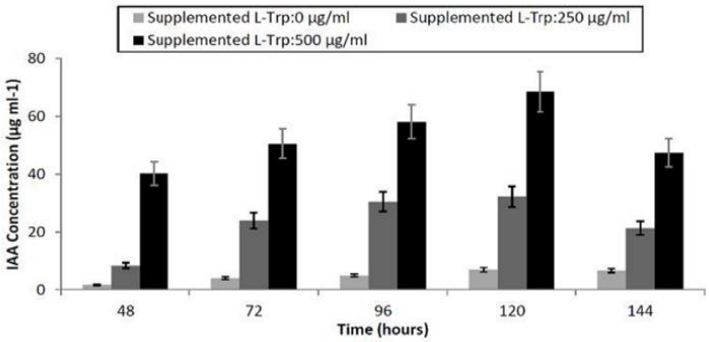

Figure 4 Correlation between IAA production and supplemented L-Tryptophan in the PDB medium at different time intervals by *H. rufa* strain P2

Isolate has shown the promising result for solubilisation of tri-calcium phosphate (Fig. 5). Isolate is capable of solubilizing inorganic phosphate by the production of organic acid. Isolate was capable of solubilizing maximum of 72 µg ml^{-1} of tri-calcium phosphate after 11 days of incubation in the liquid Pikovskaya's broth. Initial pH of the Pikovskaya's medium prior to inoculation of the isolate was adjusted to 7.0 but the pH was decreased to 4.21 after 11 days of incubation indicating the production of organic acids. Isolate produced 2.35 µmol ml^{-1} of ammonia in peptone water. However, HCN production was not detected.

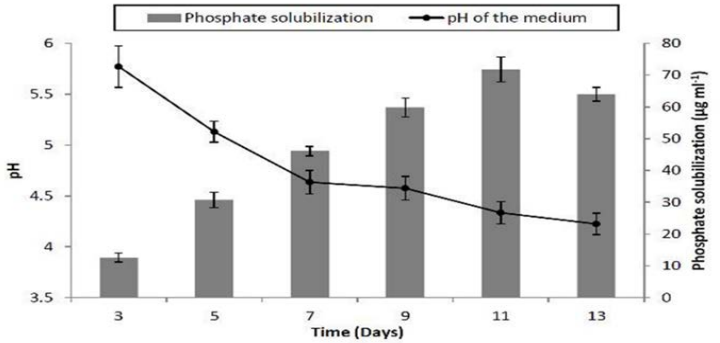

Figure 5 Correlation between tri-Calcium Phosphate solubilization and change in pH at different time intervals from *H. rufa* strain P2 in Pikovskaya's broth

Biocontrol activity

Isolate successfully inhibit potent plant pathogenic fungi viz., *F. oxysporum* (MTCC 3930), *A. alternata* (MTCC 6572), *A. niger* (MTCC 2196), *S. rolfsii* (MTCC 6052) under *in-vitro* conditions. The percentage inhibition varied with the pathogen. The percentage of inhibition was 57.6%, 48%, 34%, and 30% respectively for *F. oxysporum* (MTCC 3930), *A. alternata* (MTCC 6572), *A. niger* (MTCC 2196), *S. rolfsii* (MTCC 6052) (Fig. 6e).

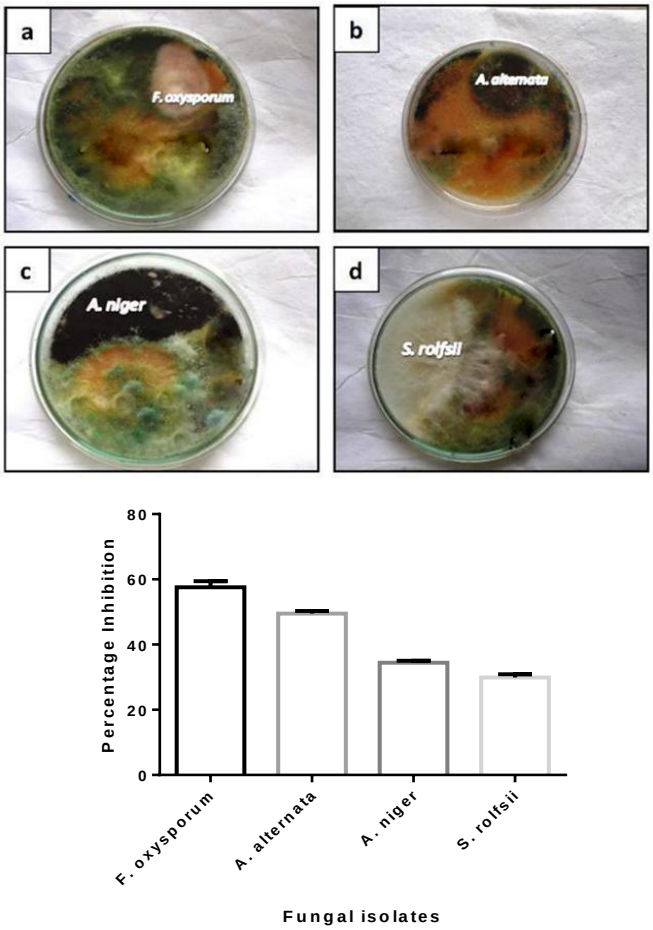

Figure 6 *In-vitro* Antagonistic activity of *H. rufa* strain P2 against (a) *Fusarium oxysporum* (b) *Alternaria alternata* (c) *Aspergillus niger* (d) *Sclerotium rolfsii* (e) antagonistic activity of the isolate as bar diagram

Establishment of association between biomass and Chitinase enzyme activity

In this study, biomass increased up to the 6th day and later gradually depleted till the 13th day. Moreover, isolate showed characteristic antagonistic activity. Antagonistic activity is a key attribute for the bio-control agent. Chitinase is one of the important enzyme responsible for the inhibition of fungal pathogens. The cell wall of fungi made up of chitin. Therefore, corresponding changes in chitinase activity were estimated and related to the growth of the fungi to determine the association between a decrease in biomass and chitinase activity. In the present study, results showed a clear association between biomass and chitinase activity. There was an increase in biomass up to the 6th day when the chitinase activity was at the basal level, later on, chitinase activity rapidly increased with the consequent depletion of the biomass due to its own degradation by the enzyme. A similar trend was observed on day 11th (Fig. 7).

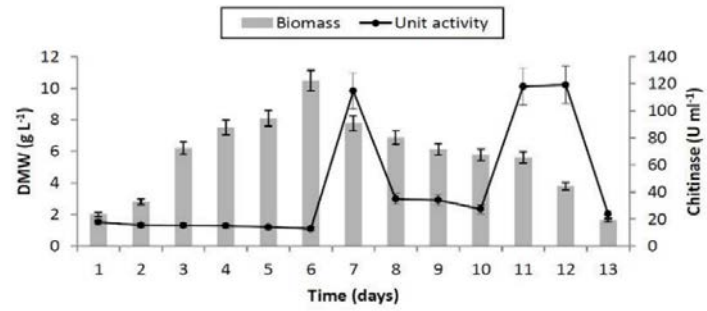

Figure 7 Association between biomass production and activity of chitinase by *H. rufa* strain P2

***In vivo* pot Study**

Pot trials were carried out using *Arachis hypogaea* L. as a test plant. A significant difference in vegetative parameters of the control plant and treated plants (i.e. Seed coating application and soil application of talc based formulation of isolate) was observed. Seed coat and soil application of talc based formulation of isolate resulted in up to 2 fold increase in the numbers of leaves (Table 1). Similar growth promotion was also observed in the numbers of branches (2 folds) for seed application and (1.8 fold) for soil application treatment where the P value was found to be less than 0.01. Furthermore, significant growth promotion in shoot length (117% for seed application and 135% for soil application) was observed for test plantlets as compared to control. Moreover, 1.25 fold and 3 fold increment in lateral roots of test plant were observed for seed and soil application treatment respectively (Fig. 8).

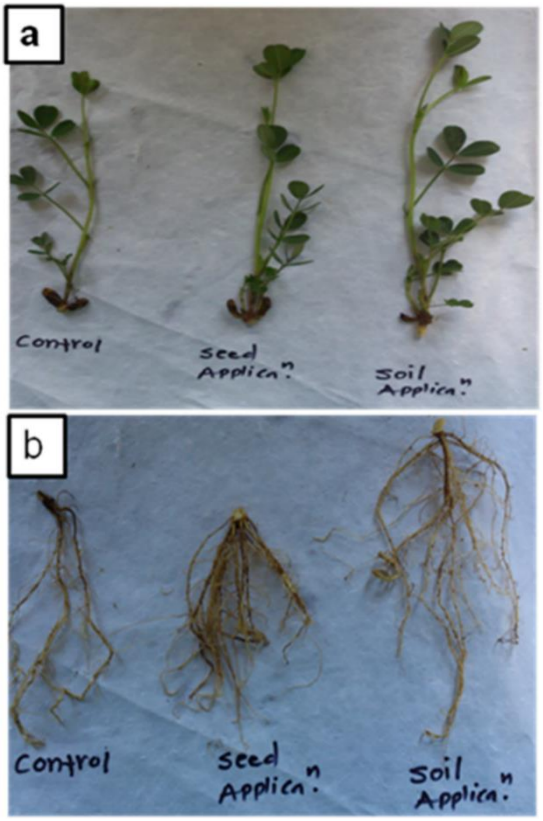

Figure 8 Difference in (a) the size of seedling and (b) in root growth between non-treated controls with *H. rufa* strain P2 treated soil and seeds.

Increased fresh root mass (1.68 fold in seed application and 2 fold in soil application) along with significant increment in dry root mass (3.78 and 6 fold in seed and soil application respectively) were achieved for treated plantlets. Increase in shoot fresh and dry mass was observed for both the treatments in test plants (Table 1).

Table 1 Effect of talc-based bio-formulation of *H. rufa* strain P2 on the germination of Peanut after 15 DAS (Days after sowing) in pots with two ways of application. Values are the mean of triplicates with standard deviation.

Vegetative parameters	Control	Seed Application	P-value	Soil Application	P-value
Numbers of leaves	20.00±8	41.3333± 6.1101*	0.02137	36.00±4*	0.036278
Numbers of branches	5.00±2	10.3333±1.5275*	0.02138	9.00±1*	0.3628
Shoot length (cm)	17.00±2.6457	20.100±2.0074	0.18125	22.6666±2.5166	0.05478
Numbers of tap root	2.33±0.5773	9.333±1.5275**	0.00176	1.00±0.000*	0.01613
Tap root length (cm)	14.83±2.2546	10.933±1.1015	0.05455	14.7667±2.1126	0.97198
Lateral root length (cm)	3.53±0.5033	4.600±1.0148	0.17825	7.0666±2.3860	0.06608
Fresh root mass (mg)	650.00±150	1092.000±228.0066*	0.04856	1337.6667±209.7721**	0.00989
Dry root Mass (mg)	50.00±4	189.000±28.5831**	0.00113	303.33±47.2581***	0.00076
Fresh shoot mass (mg)	1238.00±88.42	1946.667±261.0715*	0.01122	2441.00±206.64***	0.00075
Dry shoot mass (mg)	240.00±15	330.000±26.4575**	0.00686	453.33±15.2752***	0.00007

* (P value between 0.05 to 0.01) Significant at 5% compared to control (ANOVA)
** (P value between 0.01 to 0.001) Significant at 1% compared to control (ANOVA)
*** (P value less than 0.0001) Significant at 0.1% compared to control (ANOVA)

DISCUSSION

Several species of *Trichoderma* are widely known for their ability to be used as a bio-fertilizer and as a bio-control agent. Efforts are being made to commercialize isolates belonging to this genus. Therefore in the present study, the role of *H. rufa* strain P2 as biofertilizer and as an antagonist to several fungal phytopathogens was determined. Also, for commercialization, it is a very essential to know the optimum growth medium which can support maximum biomass production. Therefore, we also studied the biomass of the fungus produced in several commercially available growth media. MEB supported maximum growth of *H. rufa* strain P2. From the data obtained it is essential to understand the growth medium and the growth requirements of *H. rufa* strain P2. Malt extract contains components like glucose, oligomers, inorganic salts, protein. Malt extract contains carbohydrates 91 g carbohydrates per 100 g (monosaccharides 10%, disaccharides 42-43%, oligosaccharides 38-39%), inorganic salts 1.8%, proteins 7.0%, and vitamins 0.2% of the total composition. This indicates that malt extract is not just providing carbon but also providing essential macro and micro nutrients. *H. rufa* strain P2 is saprophytic fungi that use a wide range of compounds as carbon and nitrogen sources. The carbon and energy requirements of *H. rufa* were similar to the *Trichoderma* sp. can be satisfied by monosaccharide and disaccharides (**Papavizas, 1985**). Amino acids, urea, nitrate, ammonium are most readily utilized sources of nitrogen in buffered media which support the vegetative growth of *Trichoderma* sp. (**Danielson and Davey, 1973**). Therefore, malt extract supported the higher growth of *H. rufa* strain P2 by producing up to 10 g $L^{-1}$of biomass.

H. rufa is categorized as L-Tryptophan dependent IAA producer dependent IAA producer (**Gupta *et al.*, 1999**). So, here we proved this claim as we showed that *H. rufa* produces a higher concentration of IAA if the supplemented concentrations of L-tryptophan is also high. The isolate in this study showed several desirable features for PGPF and multiple action mechanisms, which

suggest its potential for growth promotion in *Arachis hypogaea* L. One of these beneficial features can be observed as high levels of IAA produced after 120 h by fungus *H. rufa* strain P2, which are 12 fold high when compared to *T. atroviride* (**Gravel *et al.*, 2007; Mukherjee *et al.*, 2012**) and about two-fold higher than *T. harzianum* which produce IAA in range of 26-50 µg ml^{-1} (**Akladious and Abbas, 2012**). In comparison with other fungi viz., *Aspergillus* CMU-LPS019, *Fusarium* CMU-BK001, CMU-JT007 and *Paecilomyces* CMU-CM009 which produced IAA in the range from 1.6-26.1µg ml^{-1}, *H. rufa* strain P2 showed at least 2.5 fold higher production of IAA than these widely studied PGPF (**Ruanpanun *et al.*, 2010**). Tryptophan is the most important precursor for IAA synthesis, although several tryptophan-independent pathways have been described. The synthesized IAA by PGPF mostly affects the root system, which depends upon the increasing the size and number of adventitious roots and root ramifications, as a result root exudates are able to cover larger soil volume, which in the end providing a large amount of nutrients to the plant. However, the effects of IAA may vary according to the concentrations that are released into the root system and, depending on the plant variety.

The amount of P in the soil is 0.5% although only 0.1% is available to the plant. The solubilisation of Tri-calcium-phosphate observed in the present study is related to decrease in pH of the culture medium through the production of organic acids by *H. rufa* strain P2 by the utilization of glucose as a carbon source. Phosphate solubilizing fungi like *Trichoderma* sp. and *Aspergillus* sp. produces different kinds of organic acids viz., lactic, maleic, malic, acetic, tartaric, citric, fumaric and gluconic acid (**Akintokun *et al.*, 2007**). Deficiency of P in turn severely restricts plant growth and yield. *Trichoderma* isolates solubilizing insoluble tricalcium phosphate (TCP) to various extents, *T. viride* TV 97 (9.03 µg ml^{-1}), *T. virens* PDBCTVs 12 (9 µg ml^{-1}), and *T. virens* PDBCTVs 13 (8.83 µg ml^{-1}) was able to solubilize only 70% of the amount solubilized by *Bacillus megaterium* (12.43 µg ml^{-1}) (**Rudresh *et al.*, 2005**). Superior ability to improve phosphate availability by *H. rufa* strain P2 used in the present study was evident through 1 to 6 fold more TCP solubilizing activity compared to previous reports.

One of the important cause for the *H. rufa* strain P2 to be characterized as an antagonist to fungal phytopathogens is chitinase production which can easily degrade fungal cell wall. *Trichoderma* sp. is an antagonistic fungus, which prevents the crops from diseases viz. root rots, wilts, brown rot, damping off, charcoal rot and other soil borne diseases in crops. *Trichoderma* sp. suppresses more than 60 species of pathogens including *Pythium*, *Botrytis*, *Sclerotium*, *Fusarium*, *Ascochyta*, *and Alternaria* on different plants like cucumbers, tomatoes, cabbages, peppers, peanut, coffee, sugarcane, apples, cauliflower, citrus, Chinese cabbage, sweet potatoes etc. Some of the fungal plant pathogens were inhibited by *H. rufa* strain P2 under present study is demonstrated (Fig. 6). The mycoparasitic activity of *Trichoderma* sp. against various phytopathogens and oomycetes are due to the lytic activity of cell wall-degrading enzymes (**Howell, 2003**). Enzymes such as chitinase, glucanase produced by the biocontrol agents are responsible for suppression of the plant pathogens. These enzymes functions by breaking down the polysaccharides, chitin, and β-glucans that impart rigidity of fungal cell walls resulted in the destroying cell wall integrity (**Mukherjee *et al.*, 2012**).

One of the important cause of the *H. rufa* strain P2 to be characterized as an antagonistic to fungal phytopathogens is chitinase production which can easily degrade fungal cell wall. One of the interesting results suggesting a significant decrease in biomass on the 7^{th} day and then after an 11^{th} day in the growth medium could be due to autolysis of its own cell wall by own chitinase production (Fig. 7). *H. rufa* strain P2 is an anamorph of *Trichoderma viride*, so it behaves similarly to *Trichoderma* sp. Mechanism of parasitism by *Trichoderma* sp. is destructive, causing death of the host fungus attributed by the action of chitinase (**Mukherjee *et al.*, 2012**). The process of autolysis allows the remodeling of its growth by utilizing its own nutrients released into the medium by the action of chitinase on its own cell wall. During this process, cell wall polysaccharides are apparently exposed and consequently degraded. It is also possible that during other processes in fungal colony development, e.g. hyphal branching and fusion, the localized accessibility and de-protection of chitin and another cell wall polysaccharides is a determining factor which plays important role in establishment of an association between chitinase, biomass production and shifting of biomass towards the production of chitinase (**Gruber and Seidl-Seiboth 2012**). In the present study, a similar trend was observed. Exo-chitinase can control the growth of soil-borne pathogens through the degradation of cell wall made up of chitin (**Solanki *et al.*, 2011**). *Trichoderma* sp. produces secondary metabolites including gliotoxin, gliovirin and paptaibols with known antimicrobial activities that have been shown to act synergistically with lytic enzymes to enhance the destruction of host cell walls (**Djonovic *et al.*, 2006; Mukherjee *et al.*, 2012**).

Biofertilizers are live formulations of agriculturally beneficial microorganisms. There are various ways of application i.e. seed, root or soil. Biofertilization improve nutrient status of the plant by various means including associative nitrogen fixation, phosphorus solubilization, siderophores production, altering the permeability and transforming nutrients in the rhizosphere resulting in the increasing their bio-availability. Biofertilizers can mobilize the nutrients availability to improve the soil health by their biological activity. Biofertilizer colonizes the rhizosphere and promotes plant growth through increased supply of primary nutrients for the host plant. (**Goswami *et al.*, 2014**). Some of the biofertilizers act as a phytostimulator which has capacity of the production of various phytohormones like IAA, GA, Cytokines, and ethylene (**Lugtenberg *et al.*, 2002; Somers *et al.*, 2004; Pindi *et al.*, 2014**). Biofertilizer plays a role like bio-pesticide by the way of production of antibiotics, siderophores, HCN (**Vessey, 2003**), while production of hydrolytic enzymes is also correlated with the mechanism of biofertilizer (**Somers *et al.*, 2004**). Furthermore, the plant growth promotion by controlling phytopathogenic agents by means of acquired and induced systemic resistance (**Chandler *et al.*, 2008**). *In-vitro* tests showed the presence of all these important traits which makes the local isolate P2 an efficient biocontrol as well as biofertilizer. Further, pot experiments confirmed the proposed scheme. *H. rufa* strain P2 used in the study showed 1.21-1.37 fold higher plant height after 14 DAS as compared to isolate studied by **Nawangsih et al. (2012)** for growth promotion and control the bacterial wilt disease in Peanut with PGPB biofertilizer (Fig. 8). Thus, from the entire study, it can be confirmed that *H. rufa* strain P2 had several potentials which promote its use as a bio-fertilizer with the trait of mycoparasitism.

CONCLUSION

Isolate *H. rufa* strain P2 exhibited potential plant growth-promoting traits *in-vitro* and could be a potential candidate for enhancing the growth of the plant and protect the plant from infection by pathogenic fungi. Seed application significantly improves vegetative parameters of *Arachis hypogaea* L. However, soil application showed clear edge with the more significant difference compared to control. Further evaluation in field condition is required concurrently with plant defense induction potential to evaluate candidature of isolate *H. rufa* strain P2 in field conditions.

Acknowledgments: Authors are thankful to Ashok and Rita Patel Institute of Integrated Study and Research in Biotechnology and Allied Sciences (ARIBAS) and Charotar University of Science and Technology (CHARUSAT) management for providing necessary facilities.

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JMBFS
Journal of Microbiology, Biotechnology and Food Sciences
International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

PURIFICATION AND CHARACTERIZATION OF SOLVENT STABLE LIPASE FROM A SOLVENT TOLERANT STRAIN OF *GEOBACILLUS STEAROTHERMOPHILUS* PS 11

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doi: 10.15414/jmbfs.2016.5.6.602-605

ARTICLE INFO

Received 4. 3. 2014
Revised 26. 2. 2016
Accepted 1. 3. 2016
Published 1. 6. 2016

Regular article

OPEN ACCESS

ABSTRACT

An extracellular organic solvent stable lipase produced by solvent tolerant strain of *Geobacillus stearothermophilus* PS11 was purified and characterised. The overall purification was 8.04 fold with a yield of 22.6%. The molecular weight of purified lipase was approximately 27.5 kDa. The purified lipase activity was stable (745 EU/ml at 72h incubation) in presence of toluene, benzene, propanol, methanol etc. The enzyme activity was maximum (764 EU/ml) when assayed under optimum temperature and pH of 50^{0}C and 10.0, respectively. The enzyme showed stability at a wide range of temperature from 10^{0}C to 60^{0}C. This solvent stable lipase can be a novel tool for biodiesel industry.

Keywords: Solvent stable, lipases, *Geobacillus stearothermophilus*

INTRODUCTION

Diesel fuels are the key to the industrial economy of a developing country. With the industrialization bloom in the whole globe, the world is confronted with the twin crises of fossil fuel depletion and environmental degradation **Van. (2005)**. In order to resolve these problems, researchers are interested to find an alternative environmental friendly reproducible diesel fuel. Biodiesel is an alternative diesel fuel that is produced from vegetable oils or animal fats. It have the following advantages over diesel fuel: produce less smoke and particulates, have higher cetane numbers, produce lower carbon monoxide and hydrocarbon emissions, are biodegradable, and nontoxic engine lubricity to low sulfur diesel fuels. With this huge range of advantages, it is gaining more and more importance as an alternative fuel. Lipase is the key enzyme used to produce biodiesel **(Marchetti *et al.* 2005, Barnwal and Sharma 2005)**. Only organic solvent tolerant lipase, especially tolerating methanol and ethanol, can distinctly improve the production of biodiesel **(Kaieda *et al.* 2001)**. Till date, the number of bacteria producing organic solvent tolerant lipase is limited, and most of the isolated organic solvent-tolerant strains belong to the genera are *Pseudomonas* isolated from soil or marine samples [5]. We have previously isolated a solvent tolerant strain of *Bacillus thermophilus* PS11 (now known as *Geobacillus stearthermophilus*) that could grow in presence of wide range of solvents. In this work, we report that the bacterial strain is capable of producing lipase that is stable in presence of various solvents such as toluene, n-octanol, propanol, methanol, benzene etc. This will open novel and simpler routes for the synthetic processes and in turn pave a route to alternative biodiesel production.

MATERIAL AND METHODS

Isolation of lipase producing solvent tolerant bacterial strains

Solvent tolerant strain of *Geobacillus stearothermophilus* PS11 (accession no KC311354), previously isolated from soil is used in this study **Sarkar and Ghosh (2012)**. It was plated on nutrient agar supplemented with 1% tributyrin. After 48 h of incubation at 37°C, the lipolytic activity was confirmed by the formation of a clear zone around the colonies.

Measurement of bacterial growth

Overnight grown culture of PS11 strain was inoculated in nutrient broth overlaid with 5% tributyrin and incubated at 37 °C under shaking condition at 140 rpm. Growth and dry cell mass of the isolate was determined according to the process described by **Sarkar and Ghosh (2012)**.

Production media and enzyme preparation

A 1% (v/v) bacterial suspension was transferred from an overnight nutrient broth seed culture to the basal production medium (LPM-1) composed of (g l^{-1}): peptone 5, beef extract 3, sodium chloride 2, tween 80 5 and olive oil 10, pH 8. Bacterial cells were grown at 37 °C under shaking condition at 140 rpm for 96 h. After every 24 h cells were removed by centrifugation and the supernatant was desalted and used for measurement of lipase activity.

Lipase assay and protein content determination

Lipase activity was measured on the basis of hydrolysis of p-nitro phenyl palmitate (pNPP). A 2.5 ml of the assay mixture consisted of, 2.4 ml of 50 mM Tris HCl (pH 9) with pNPP and 0.1 ml concentrated enzyme preparation. Incubation of the reaction mixture at 60^{o}C for 10 minutes was followed by addition of 200 µl of 1 M calcium chloride. The precipitation of free fatty acids with calcium chloride was used to detect lipase activity. Release of p-nitrophenol (pNP) from pNPP was measured as the increase in absorption at 410 nm. One lipase unit is defined as the amount of enzyme that liberated 1 µM p-nitro phenol per minute under the assay conditions.

Optimization of production media

Optimization of process parameters and manipulation of media composition are the most important techniques used for the overproduction of lipase to meet industrial demands. Optimization was carried out through modification of several growth parameters. For these, the bacterial cells were grown for 96 h at 37 °C under shaking condition. The cells were removed by centrifugation and the supernatants were used for measurement of lipase activity. The various parameters optimized for obtaining maximal lipase yield were, incubation time (24, 48, 72 and 96 h), agitation speed (100 – 200 rpm). Substrate specificity was determined using 1% (v/v) rice bran oil, sunflower oil, soybean oil, olive oil, and mustard oil. Further the effect of various carbon sources (1% w/v) such as glucose, sucrose, lactose, maltose, and nitrogen sources (1% w/v), that is, soybean meal, sodium nitrate, peptone, beef extract, yeast extract were also examined for production of lipase. For each step lipase activity was assayed under standard condition to know the optimal yield.

Purification and Molecular weight of crude enzyme

The bacterial isolate was cultured in LPM-1 for 72 h at 37°C. Bacterial cells were removed by using cooling centrifuge (4°C) at 5000 rpm for 10 min. The enzyme was precipitated by adding solid ammonium sulphate (0-40%) at 4°C for 24 h. The precipitate was collected by centrifugation at 10,000 rpm for 40 min and dissolved in 50 mM Tris HCl buffer (pH 9.0). It was then desalted by dialysis with a cellulose dialysis bag (3500 Da pore-size) in same buffer. The desalted enzyme preparation was loaded on to DEAE-sephacel column. The active fractions were collected, lyophilized and applied on sephadex G75 column. The active fractions were used to determine the molecular weight. Protein content (mg ml -1) was determined by Lowry method **Lowry *et al.* (1951).**
Molecular weight of lipase was detected on 15% polyacrylamide slab gel using mini gel system Bio-Rad. Low molecular weight protein standard ranged from 20-97 KD was used to determine the molecular weight of the purified lipase.

Effect of organic solvent on lipase stability

In order to study the effect of organic solvents on enzyme stability, suitably diluted purified lipase in 50 mM Tris–HCl (pH 9.0) was mixed with different organic solvents to yield a final concentration of (25%, v/v) and then the mixture was incubated on a shaking incubator (180 rpm) at 4 ◦C for 48 h. The residual lipase activity was measured using p-NPP method. The used organic solvents were hexane, xylene, toluene, benzene, dichloromethane, diethylether, ethylacetate, isopraponal, acetone, acetonitrile, methanol and dimethyl sulfoxide (DMSO). The initial lipase activity (without containing organic solvents) was considered to be 100%.

Effect of pH on activity and pH stability of PS11 lipase

The optimum pH of purified lipase was determined by incubating it with various buffers at a pH range of (4.0–12.0) and lipase activity was determined under standard assay conditions. The pH stability of the enzyme was characterized by preincubating the purified enzyme with different buffers at pH range from 4.0 to 12.0 at 4 ◦C for 2 h and assayed for lipase activity. Buffer systems were used at a concentration of 50 mM: acetate–HCl buffer (pH 4.0–5.0), sodium hydrogen phosphate–NaOH buffer (pH 6.0–7.0), Tris–HCl buffer (pH 7.0–9.0), disodium hydrogen orthophosphate–NaOH buffer (pH 9.0–11.0), and glycine–NaOH buffer (pH 10.0–12.0).

Effect of temperature on activity and temperature stability of PS11 lipase

The effect of temperature on purified lipase was determined by incubating the reaction mixture at a temperature range of 10º-80º C under standard assay conditions. The thermostability of the enzyme was determined by incubating the enzyme at different temperatures from 10º to 80º C for 2 h and lipase activity was determined.

RESULTS AND DISCUSSION

Measurement of bacterial growth and lipase production

Time course of cell growth and lipase production by PS11 in production medium are shown in figure 1. The bacteria exhibited a minimal lag phrase. The exponential phase lasted up to 18 h and the stationary phase continued till 90 h. A slight decline in biomass was noted after 96 h probably due to nutrient depletion. The findings are similar with results reported by **Abada (2008)**.
Lipase production was noted from the early stationary phase (24h) but the production attained maximum level (760 EU/ml) during the late stationary phase (72h) of the microorganism suggesting that the extracellular lipase is a secondary metabolite. Furthermore, the enzyme activity gradually decreased after 72h. Therefore, the optimum incubation periods was maintained throughout the studies.

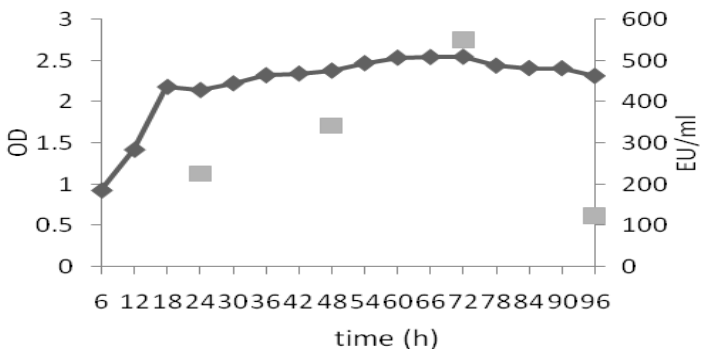


Figure 1 Determination of bacterial growth ( ) and enzyme ( ) production

Optimization of lipase production media

Different sources of nitrogen were tested in order to determine their influence on the synthesis of lipase. Peptone showed maximum enzyme activity (310 EU/ml) as shown in figure 2a. **Gunasekaran and Das (2004)** has also reported about peptone to augment lipase production
Since each microorganism requires a different carbon source to produce lipase at its maximum level, the influence of different carbon sources was tested. Glucose (367.5 EU/ml) was the best carbon source (figure 2b). In some cases glucose has been found to cause repression of lipase production but the case was opposite for PS11 where glucose acted as an inducer. The present study is in agreement with the findings of (**Lakshmi *et al.* 1999** and **Banerjee *et al.* 1985)** who reported maximum production of lipase in medium containing glucose.
Lipidic sources seem to be essential for obtaining a high lipase yield. Among the various lipidic carbon source tested rice bran oil (370 EU/ml) best supported lipase production by PS11 (figure 2c.). The high content of fatty acids specifically PUFA in rice bran oil (38% monounsaturated, 37% polyunsaturated, and 25% saturated) might support high level of lipase synthesis.
The production of lipase was observed till 96h of incubation and it was highest at 72h (765 EU/ml) during stationary phrase. The results of several other studies have shown the optimum lipase production at varying time period between 12 to 24 h **Dharmsthiti *et al.* (1998)** which was in contrast with our results. However it was noted by **Kumar *et al.* (2005**) that a high biomass was obtained at 48 h of incubation and high lipase activity was found in 72 h of incubation time in strains of *Bacillus cereus.* Most *Bacillus* species required more than 2 days for the maximum lipase activity to occur **(Joseph *et al.* 2006, Wang *et al.* 2009)**.

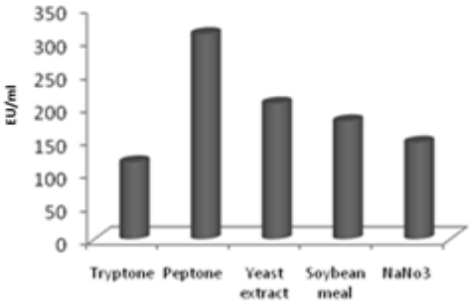
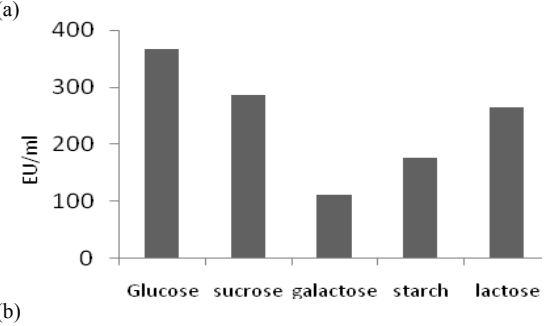


Figure 2(a)



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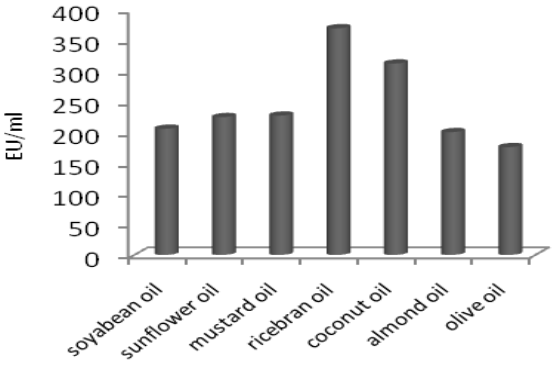

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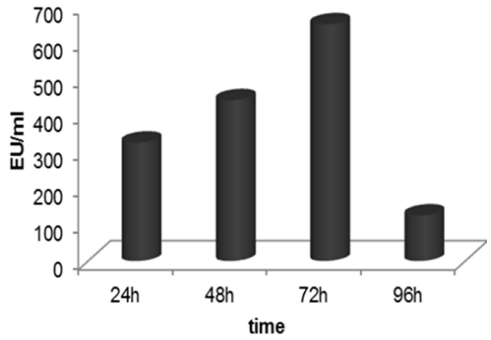

Figure 2(d)

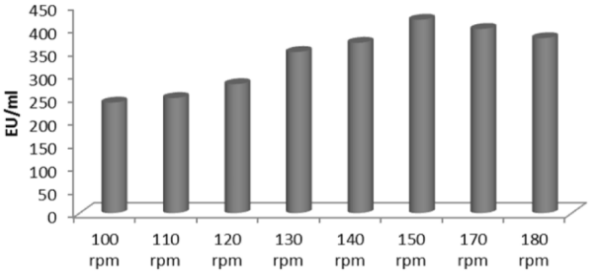

Figure 2(e)

Figure 2 Optimization of lipase production. a. nitrogen source, b. carbon source, c. substrate, d. incubation time, e. agitation rate

Agitation rates influenced the lipase yield and cell growth. Lipase production increased with increase in agitation speed and reached a maximum at 150 rpm (420 EU/ml) due to sufficient supply of dissolved oxygen in the media. Further increase in agitation lowered the production of lipase (figure 2e) probably because at a high agitation rate, the structure of enzyme would be altered Micro-organisms vary in their oxygen requirements as oxygen acts as a terminal electron acceptor for oxidative reactions to provide energy for cellular activities. The variation in the agitation speed influence the extent of mixing in the shake flasks or the bioreactor, oxygen transfer rate, surface area of contact with the media components, dispersability of the carbon source and the nutrient availability.

Purification and molecular weight determination

Lipase was purified from the extracellular medium by ammonium sulfate precipitation followed by different column chromatography. A large amount of lipase was lost after ammonium sulfate precipitation; less than 5O% of the precipitated lipase was resolubilized. Our three step purification protocol to purify the lipase enzyme from PS11 involving ammonium sulfate precipitation, ion exchange chromatography, and gel filtration chromatography resulted in 8.04 fold purified lipase with 22.6% recovery rate (Tab 1) and the characterizations of the purified enzyme revealed a molecular mass of 27.5 kDa in SDS-PAGE (Figure 3).

Table 1 Purification of lipase from PS11 strain

Step	Total EU	Total Protein(mg)	Specific activity (EU/mg)	Purification fold	Yield (%)
Crude	323.60	28.72	11.26	1	100
Ammonium sulphate	152.60	2.90	52.62	4.67	47.00
DEAE-sephacel	83.60	1.46	56.96	5.05	25.83
Sephadex G-75	73.37	0.81	90.58	8.04	22.67

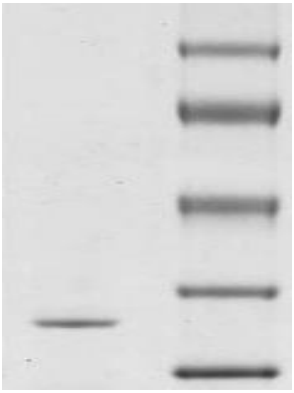

Figure 3 SDS-PAGE of purified lipase from *Geobacillus stearothermophilus* PS 11.
Lane a: purified lipase from *Geobacillus stearothermophilus* PS 11. Lane b: protein standard : phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa) and soyabean trypsin inhibitor (20.1 kDa).

Organic solvent stability of lipase

To date, some organic solvent stable lipases have been reported. The toxicity of the organic solvents is usually measured by log P value (the partition coefficient of the given solvent in an equimolar mixture of octanol and water). The effect of different organic solvents on purified lipase is shown in Table 2. In this study, organic solvents with various log P value ranging from low to high were selected. The lipase was stable and restored almost 90% of its activity when p-xylene, benzene, toluene, and hexane (25% v/v each) were added to the purified enzyme for 2h at C and 1 0 rpm. In presence of methanol and ethanol, the residual activity was 42% and 46%, respectively.

Table 2 Effect of different organic solvent on lipase stability

Organic solvents	Log P	Relative activity (%) at concentration of 25% (v/v)
Control	—	100
Methanol	-0.76	90
Acetonitrile	-0.33	30
Ethanol	-0.24	76
Acetic acid	-0.23	18
Benzene	2.0	81
Toluene	2.5	92
P-xylene	3.1	91
n-Hexane	3.1	80
Hexane	3.6	86

25% (v/v) of organic solvents were added to the enzyme solution and incubated for 48 h in a rotary shaker (180 rpm) at 4 ◦C.

A similar result was shown by the lipase from *Staphylococcus saprophyticus* M36; the residual activity was 32% and 36%, respectively, when methanol and ethanol (25% v/v) were added to the enzyme up to for 15 days at 30 C and 160 rpm **Fang *et al.* (2006)**.
The stability of the PS11 lipase in organic solvents did not follow the log *P* trends. The greater is the polarity, the lower the log P value and the greater the toxicity of solvent. It is well known that water acts as a lubricant that affords a high conformational fexibility to enzyme molecules. If one follows the trends of log*P*, the lower the log *P* values, the less hydrophobic the solvent, so the enzyme is less stable and there may be change in the conformation of the enzyme molecules. However, different organic solvents showed different tolerance profiles to the PS11 lipase. It is well-known that the effect of organic solvents on enzyme activity differs from lipase to lipase **Sugihara *et al.* (1993)**. There was no clear correlation between the solubility of an organic solvent in water and stability of lipase in its presence **Ogino *et al.* (2000).**

Effect of pH on activity and pH stability of PS11 lipase

PS11 was inoculated in the lipase production medium and incubated at a wide range of pH (4- 12). At pH 10, maximum lipase activity of 417.5 EU/ml was observed (Figure 4.).The enzyme activity gradually increased from pH 5 to its maximum at pH 10.

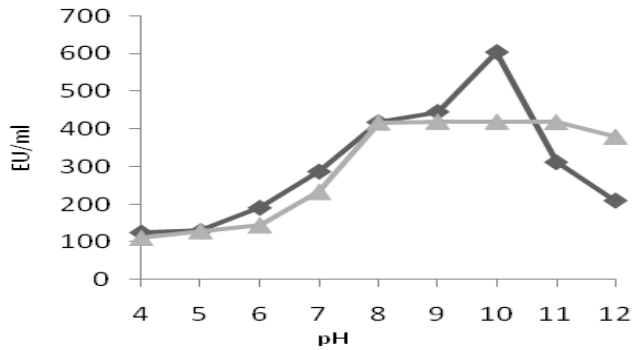

Figure 4 Determination of optimum pH (◆) and pH stability (△) of PS11 lipase

The pH of the production medium plays a critical role for the optimal physiological performances of the bacterial cell and the transport of various nutrient components across the cell membrane aiming at maximizing the enzyme production. **Moon and Parulekar (1991)** reported that the pH of culture has been shown to strongly affect many enzymatic processes and transportation of various components across the cell membrane. The effect of pH on the stability of lipase was tested by incubating the enzymes over a range of pH values. The enzyme also exhibited stability in high alkaline pH which is an important industrial attribute.

Effect of temperature on activity and temperature stability of PS11 lipase

Temperature is a critical parameter which needs to be controlled and this usually varies from organism to another. The optimum temperature for the lipase production was 50°C, although at 40°C the enzyme activity was good. The lipase activity gradually decreased and reached a minimum at 70^0 C (Figure 5).

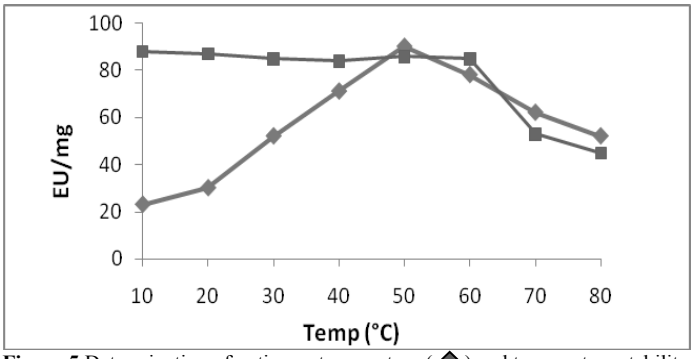

Figure 5 Determination of optimum temperature (◆) and temperature stability (■) of PS11 lipase

The enzyme stability was tested by exposing the enzyme to different temperatures for 2 h. PS 11 showed highest activity at 50^{o}C and its activity remained stable from 10^{o}C to 60^{o}C. The activity further declined as the temperature increased to 70^{0}C showing that the enzyme was not stable at high temperatures. The studies by **Frankena *et al.* (1986)** showed that there was a link between enzyme synthesis and energy metabolism in bacteria, and this was controlled by the temperature and oxygen uptake. As for the extra-cellular enzymes, temperature was found to influence their secretion, possibly by changing the physical properties of the cell membrane. It is a well-known fact that the protein conformation is changed or degraded at higher temperatures, and hence a decrease in the lipase activity was noted at higher temperatures.

CONCLUSION

The major challenge of biodiesel catalyzed by lipase is that its activity significantly reduces in presence of organic solvents, especially methanol and ethanol. Therefore, in this study the solvent tolerant lipase of 27.5kDa was purified from *Geobacillus stearothermophilus* PS11 that could be very useful for biodiesel production as it is stable in presence of methanol and ethanol.

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JMBFS

Journal of Microbiology, Biotechnology and Food Sciences

International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

ENHANCED PRODUCTION OF POLYHYDROXYBUTYRATE (PHB) FROM AGRO-INDUSTRIAL WASTES; FED-BATCH CULTIVATION AND STATISTICAL MEDIA OPTIMIZATION

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doi: 10.15414/jmbfs.2016.5.6.606-611

ARTICLE INFO

Received 20. 9. 2014
Revised 7. 2. 2016
Accepted 1. 3. 2016
Published 1. 6. 2016

Regular article

OPEN ACCESS

ABSTRACT

Bacillus megaterium SW1-2 showed enhanced growth and polyhydroxybutyrate (PHB) production during cultivation on date palm syrup (DEPS) or sugar cane molasses. FT-IR and NMR spectroscopic analyses of the polymer accumulated during growth on DEPS revealed specific absorption peaks characteristic for PHB. 1.65 g/L of PHB (56.9% CDW) was produced during growth on medium supplemented with 2 g/L of DEPS. Approximately, 36.1% CDW of PHB were recorded during growth on sugar cane molasses. Six runs of different fed-batch cultivation strategies were tested, the optimal run showed approximately 6.87-fold increase. Modified E2 medium was prefered recording 10.11 and 11.34 g/L of total PHB produced for runs 1 and 2, at the end of 96 h incubation period, respectively. Decrease in PHB was recorded during growth on complex medium (run 3 and run 4). In another independent optimization strategy, ten variables were concurrently examined for their significance on PHB production by Plackett-Burman statistical design for the first time. Among variables, DEPS-II and inoculum concentration followed by KH_2PO_4 and $(NH_4)_2SO_4$ were found to be the most significant variables encourage PHB production. Indeed, DEPS-II or Fresh syrup is more significant than commercial syrup DEPS-I (*p*-value= 0.05). RPM, incubation period have highly negative effect on PHB production. Role of ago-industrial wastes, especially DEPS, in enhancement of PHB production was closely discussed.

Keywords: Date syrup or DEPS, *Polyhydroxybutyrate* (PHB), *Bacillus megaterium*, Plackett-Burman design, fed-batch cultivation, optimization

INTRODUCTION

Among the most commonly known biopolymers, bacterial polyhydroxyalkanoates (PHAs) polyesters gain more interest. It is synthesized and intracellular accumulated as storage materials to be used as energy source during unbalanced growth **(Anderson and Dawes, 1990; Steinbuchel 2001; Du and Yu, 2002; Ojumu *et al.*, 2004)**. PHB production by microorganisms attracted attention of many scientists due to close resemblance to synthetic petroleum–based plastics such as polypropylene **(Mokhtari-Hosseini *et al.*, 2009)**, biodegradability and biocompatiblility **(Madison and Huisman, 1999; Ojumu *et al.*, 2004; Khanna and Srivastava, 2005)**. Several applications in medicine, veterinary practice, tissue engineering materials, food packaging and agriculture have been reported **(van der Walle *et al.*, 2001; Zinn *et al.*, 2001; Luengo *et al.*, 2003; Chen and Wu, 2005; Bucci *et al.*, 2005; Urtuvia *et al.*, 2014)**. Many bacilli have been reported to accumulate PHA and co-polymers of 3HB during growth on different substrates **(Belma et al., 2002; Tajima et al. 2003; Valappil *et al.*, 2007; Adwitiya *et al.*, 2009; Reddy *et al.*, 2009)**. In spite the privilege of microbial PHAs production compared with petroleum-derived plastics, costs of the feedstock are the main limiting factor for their mass production **(Ojumu *et al.*, 2004; Nikel *et al.*, 2005)**. Economically, the use of agro-industrial wastes, improved fermentation strategies and downstream processes have positive contribution on the overall biopolymer production costs **(Liu *et al.* 1998; Tamer *et al.* 1998; Kim, 2000; Halami, 2008)**.

Recently, scientists have been exploring different cultivation strategies involving inexpensive, renewable carbon substrates in order to reduce production cost and obtain high productivity **(Ojumu, 2004)**. Many carbon sources derived from wastes like whey, cane molasses, sugar beet molasses and date syrup can be used in production **(Beaulieu *et al.*, 1995; Lee *et al.*, 1997; Liu *et al.*, 1998; Omar *et al.*, 2001; Khanafari *et al.*, 2006; Albuquerque *et al.*, 2007; Hamieh *et al.*, 2013)**. Nowadays, PHB proved to be produced from relatively cheaper substrates such as methanol **(Kim et al., 2003; Mokhtari-Hosseini *et al.*, 2009)**, carbon dioxide **(Ishizaki *et al.*, 2001)**, and several agro-industrial by-product such as rice bran, pulp, paper and cardboard industry, whey, dairy wastes, sea water and municipal wastes **(Law *et al.*, 2001; Nikel *et al.*, 2005; Nath *et al.*, 2008; Santimano *et al.*, 2009; Pandian *et al.*, 2010; Bhuwal *et al.*, 2013; Hamieh *et al.*, 2013; Singh *et al.*, 2013; Watanabe *et al.*, 2014)**. Furthermore, fed-batch cultivation in chemically defined medium for optimization of PHB production has been applied **(Wang and Lee., 1997; Liu *et al.*, 1998)**. Investigations have also focused on reducing the total cost of PHB through optimizing fermentation processes. Recently, application of statistical methods has gained a lot of impetus for medium optimization and understanding the interactions among various physiochemical parameters involved in biopolymer production **(Khanna and Srivastava, 2005; Nikel *et al.*, 2005; Sharma *et al.*, 2007; Mokhtari-Hosseini *et al.*, 2009; Pandian *et al.*, 2010; Berekaa and Al Thawadi., 2012; Hamieh *et al.*, 2013)**.

The main objective of this study was to investigate the possible production of PHB from several agro-industrial wastes mainly; date palm syrup (DEPS) and sugar cane molasses. Produced polymer was identified by chemical characterization using FT-IR, C^{13}NMR and H^1NMR spectroscopy. Furthermore, optimization of PHB production by application of six different fed-batch cultivation strategies was closely investigated. Special emphasis was given to the application of statistical experimental design (Plackett-Burman) for optimization of PHB production from DEPS for the first time.

MATERIAL AND METHODS

Microorganism

Group of bacilli previously isolated, maintained and screened for PHB production **(Berekaa and Al Thawadi, 2012; Berekaa, 2012)**, were tested for PHB production from agro-industrial wastes namely; date syrup (DEPS) and sugar cane molasses. The potent PHB producing bacterial strain *Bacillus megaterium* SW1-2 used in this study was identified by 16S DNA gene analysis as previously reported **(Berekaa and Al Thawadi, 2012).**

Growth and production conditions

The bacterium was grown in 50mL aliquot of nutrient broth dispensed in 250 mL Erlenmeyer flask and incubated at 37°C for 24h or 48h at (150 rpm). 1.5% inoculums of the overnight culture was used to inoculate modified E2 medium of

the following composition (g/L): ammonium sulfate; 2.5, KH_2PO_4; 1.5, Na_2HPO_4; 3.5, $MgSO_4.7H_2O$; 0.2, traces of yeast extract and 1 mL of trace element solution ($FeSO_4.4H_2O$, $CaCl_2.2H_2O$, $MnSO_4.4H_2O$, $ZnCl_2$ 1 mM each) at 37°C. To test the possible production of PHB, date palm syrup (DEPS) or sugar cane molasses was used as a sole carbon source at different concentrations. Two types of date syrup (DEPS) were used in this study, the commercial (DEPS-I) composed mainly of (per 100 g); carbohydrates 55 g (mainly sucrose), vitamin B complex 0.57 mg, calcium 685 mg, phosphate 75 mg, iron 16 mg and magnesium 258 mg) and Freshly prepared date palm syrup (DEPS-II) as following; 500 g of date palm (Khalas from Kaseem province) were placed in 1.5 L dist. water and boiled for 90 min with mixing. At the end of incubation period the extract was filtered in cloth for 3-4 times. Subsequently, the extract kept in oven at 60°C till net volume of 200 mL. Finally, the fresh date syrup was kept in refrigerator till use. Sugar cane molasses used in this study were obtained from an industrial sugar manufacturing plant, Egypt. Sugar content of molasses was 76% (w/v), and composed mainly of sucrose (62%), and fructose (38%). At the end of incubation period, PHB was determined and the cell dry weight was estimated.

Fed-batch cultivation and PHB production strategies

For optimization of PHB production from date syrup (DEPS), fed-batch cultivation was applied. During fed-batch cultivation, two different feeding strategies were applied. The first pulsed feeding of DEPS-I was carried out after 24 and 60 h time intervals, while the second was performed after 48 and 84 h, respectively, with final concentration of 15 g/L. Furthermore, three basal media were tested in this study namely; modified E2 medium, nutrient broth medium and a mixture of nutrient broth and modified E2 medium (50% w/w each) with the sum of six different fed-batch cultivation strategies (run 1 to run 6). In each experiment, sample was taken during different time intervals, PHB was determined and the cell dry weight was estimated.

Extraction of PHB from the isolate

PHB was extracted from the cell masses by using modified hypochlorite method as previously described **(Rawte and Mavinkurve, 2002; Berekaa and Al Thawadi, 2012)**.

Analytical procedures

Cell dry weight

After centrifugation of the culture medium, supernatant was discarded and cell pellet was washed with distilled water. The washed pellet was resuspended in 1 mL distilled water, transferred to pre weighed boats and dried to constant weight at 60°C.

Chemical analysis of polymer

Characterization of PHB by FT-IR

The presence and characterization of PHB in dry cell matter was confirmed by Fourier Transform Infrared Spectroscopy (FT-IR) **(Hong *et al.*, 1999)**. Dry PHB polymer from *B. megaterium* SW1-2 was used to prepare KBr discs. Spectra between 400 and 4000 cm^{-1} were recorded using Nicolet 6700 FTIR spectrometer from the Nicolet Instrument Corporation, USA.

Characterization of PHB by C^{13} NMR and H^1 NMR analysis

Extracted PHB biopolymer from *B. megaterium* SW1-2 was characterized by spectroscopic analysis. H^1NMR spectrum was recorded on a JEOL JNM-LA 500 MHz spectrometer at 30°C in $CDCl_3$ as solvent. While, C^{13} NMR spectral experiments were performed at 125.65 MHz with the following acquisition parameters: 32 k data point, 0.967 s acquisition time, recycle delay 1 s and contact time 4.50 ms.

Fractional factorial design

Plackett-Burman experimental design

Screening design namely; Plackett-Burman experimental design was applied to investigate the significance of various medium factors on PHB production **(Plackett and Burman, 1946)**. 10 chemical and environmental independent variables were examined in two levels: –1 for low and +1 for high level based on Plackett-Burman design (Table 1). According to the matrix shown in Table 2, the independent variables were screened in 14 combinations. The main effect of each variable is the difference between average of measurements at high setting (+1) and average of measurements observed at low setting (-1) of that factor. Plackett-Burman experimental design was based on the first order model (Equation 1):

$$Y=\beta 0+\Sigma\ \beta iXi \quad (1)$$

Where, Y is the predicted response, β0 and βi are constant coefficients, and Xi is the coded independent variables estimates or factors. The quality of fit of the polynomial model equation was expressed by the coefficient of determination R^2.

Table 1 Variables and their settings employed in Plackett-Burman design for optimization of PHB production by *Bacillus megaterium* SW1-2 during growth on date syrup or DEPS.

Medium Composition	Levels	
	-1	1
DEPS-I (%)	2	4
DEPS-II (%)	2	4
$(NH_4)_2SO_4$ (g/L)	1.5	3
Yeast Extract (g/L)	0.5	1
Na_2HPO_4 (g/L)	2	4
KH_2PO_4 (g/L)	1	2
Inoculum conc. (%)	1	2
Temperature (°C)	28	37
RPM	60	120
Incubation Period	48	72

Table 2 Effect of different environmental conditions on PHB production from *Bacillus megaterium* SW1-2 during growth on date palm syrup or DEPS

Exp. No.	DEPS-I (%)	DEPS-II (%)	$(NH_4)_2SO_4$ (g/L)	Yeast Extract (g/L)	Na_2HPO_4 (g/L)	KH_2PO_4 (g/L)	Inoculum (%)	Temperature (°C)	RPM	Incubation Period (h)	PHB (% CDW)
1	4	4	3	0.5	4	2	1	37	60	48	**46**
2	4	2	3	1	2	2	1	28	60	72	**9.8**
3	1.5	1.5	2.5	0.5	3.5	1.5	1.5	37	120	48	**31**
4	4	2	3	0.5	2	1	2	37	120	48	**27.4**
5	4	4	1.5	1	2	1	1	37	120	72	**11.5**
6	2	2	1.5	1	4	2	1	37	120	48	**7.1**
7	2	2	3	1	4	1	2	37	60	72	**32.7**
8	2	4	3	1	2	2	2	28	120	48	**53.6**
9	2	4	3	0.5	4	1	1	28	120	72	**10.6**
10	4	2	1.5	0.5	4	2	2	28	120	72	**21.3**
11	4	4	1.5	1	4	1	2	28	60	48	**42.4**
12	2	2	1.5	0.5	2	1	1	28	60	48	**23.8**
13	1.5	1.5	2.5	0.5	3.5	1.5	1.5	37	120	48	**28.6**
14	2	4	1.5	0.5	2	2	2	28	60	72	**46.1**

Statistical analysis of the data

Data of the PHB production were subjected to multiple linear regressions using MICROSOFT EXCEL 97 to estimate t-value, P-value and confidence level. The significance level (*P*-value) was determined using the Student's t-test. Factors having highest t-value and confidence level over 95% were considered to be highly significant on PHB production. Data presented in this study measured in duplicate.

RESULTS AND DISCUSSION

Growth of *Bacillus megaterium* SW1-2 on date syrup (DEPS)

Among a group of polyhydroxybutyrate-producing bacilli explored for potential production of PHB biopolymer from an agro-industrial waste namely; date palm syrup (DEPS), *Bacillus megaterium* SW1-2 showed potent growth on DEPS or date syrup as a sole source of carbon. Elemental analysis revealed that 70% of the polymer accumulated during growth on date syrup or DEPS was carbon (data not shown).

PHB accumulation by *Bacillus megaterium* SW1-2 cultivated on DEPS-I

For economic visibility of PHB biopolymer production by *B. megaterium* SW1-2 date syrup or DEPS was used as a natural carbon source. Monitoring of PHB accumulation in presence of different DEPS concentrations was carried out. Data shown in Figure 1 indicated that the agro-industrial date palm syrup or DEPS can be used as a suitable renewable carbon source during PHB production. Maximum PHB production was recorded at the end of log phase and approximately 1.65 g/L of PHB was accumulated after 48 h during cultivation on modified E2 medium supplemented with 2 g/L of DEPS-I or date syrup. Furthermore, lower concentration of DEPS-I enhanced PHB accumulation (average of 42% CDW as well as 52.65% CDW of PHB accumulated after 24 and 48 h at concentration of 2 and 2.5 (w/v) of DEPS-I, respectively). **Omar *et al.* (2001)** successfully used date palm syrup or DEPS as carbon source during PHB production. **Page (1992)** reported that the polymer production greatly enhanced in presence of unrefined sugars as well as complex nitrogen sources. It is assumed that various sugars namely; sucrose, glucose and fructose in date syrup or DEPs can be used as a carbon source by the *B. megaterium* SW1-2 cells during PHB production and may contribute to enhanced production. As previously recorded, glucose or sucrose can be used as carbon source during biopolymer production by many bacteria **(Zhang *et al.*, 1994, Valappil *et al.*, 2007; Adwitiya *et al.*, 2009; Reddy *et al.*, 2009, Wang, 2011, Belal, 2013)**, supporting this conclusion.

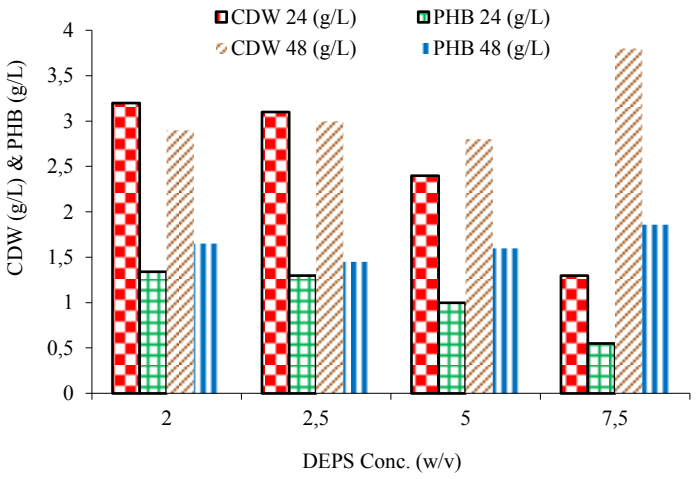


Figure 1 Effect of different date palm syrup or DEPS on growth and PHB production by *B. megaterium* SW1-2.

PHB accumulation by *Bacillus megaterium* SW1-2 cultivated on Molasses

In a trial to use other low-cost substrates for PHB production, *B. megaterium* SW1-2 allowed to grow on sugar cane molasses. Results revealed that *B. megaterium* SW1-2 showed clear growth and PHB accumulation during cultivation on modified E2 medium supplemented with sugar cane molasses as a sole carbon source (Figure 2). It revealed that 5 g/L of sugar cane molasses was the optimal concnetration for PHB production by *B. megaterium* cells. Approximately, 29.24% and 26.1% CDW of PHB accumulated after 24 and 48 h, respectively. Lower concentrations of molasses resulted in approximately 50% reduction in PHB, while clearly enhanced cell biomass. In concordance with our results, **Beaulieu *et al.* (1995)** used cane molasses together with ammonium salt for growth of *Alcaligenes eutrophaus* and PHB production. Recombinant *Escherichia coli* strain (HMS174/pTZ18u-PHB) capable of PHB production was reported by **Liu *et al.* (1998)**. **Belal (2013)** successfully used simple carbon sources as well as molasses as a source of carbon for PHB production. It is assumed that higher concentrations of sugars in cane molasses namely; sucrose and fructose contribute to enhanced production of PHB in *B. megaterium* SW1-2.

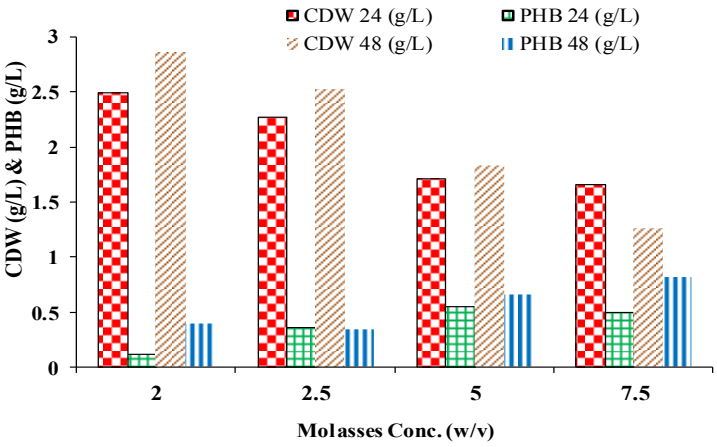


Figure 2 Effect of different sugar cane molasses on growth and PHB production by *B. megaterium* SW1-2.

FT-IR spectroscopy analysis

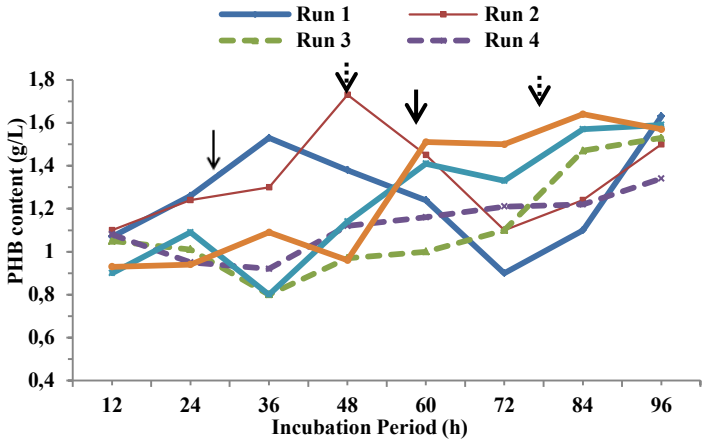
FT-IR Spectroscopic analysis of the polymer extracted from *B. megaterium* cells grown on modified E2 medium with date syrup or DEPS-I as a sole carbon source revealed a clear C–H and carbonyl stretching bands highly characteristic to PHB. Two absorption bands occurring at 2921.36 and 2859.92 cm^{-1} indicated the presence of aliphatic group $-CH_3$ and $-CH_2$. The two other absorption bands at 1718.26 and 1267 cm^{-1} in the PHB sample conforming the presence of C=O and C–O stretching groups and were identical to PHB from some bacilli **(Hong *et al.*, 1999, Pandian *et al.*, 2010; Valappil *et al.*, 2007)**.

H^1NMR and C^{13}NMR spectroscopy

Furthermore, analysis of extracted polymer by H^1NMR revealed three groups of distinctive signals of the PHB polymer. A doublet at 1.22 and 1.25 ppm represent methyl group ($-CH_3$) coupled to one proton and 2.28 ppm resulted from methylene group ($-CH_2$) adjacent to an asymmetric carbon atom. The third signal was at 5.2 ppm attributed to a methyne group (-CH). Furthermore, C^{13}NMR analysis was used to determine the structure of the isolated polymer from *B. megaterium* SW1-2 grown on the modified E2 medium. Four narrow lines appeared which were identical to the C^{13}NMR spectra of PHB **(Doi and Abe, 1990)**. The four peaks assigned for methyl (CH_3; 21.2 ppm), methylene (CH_2; 42.7 ppm), methine (CH; 68.5 ppm) and carbonyl (C=O; 169.7 ppm) carbon resonance of PHB **(Doi and Abe, 1990)**. Analysis collectively confirmed the molecular composition of the polymer to be PHB.

Fed-batch cultivation and application of different pulsed feeding strategies

Since PHB is a carbon-based biopolymer, it depends mainly on the nature and concentration of carbon source during synthesis and accumulation. Unfortunately, longer incubation period during batch cultivation leads to nutrient limitations especially for C-source, hence fed-batch cultivation may provide promising solution. In this study commercial date syrup or DEPS-I was added in two pulsed feeding experiment. Results presented in figure 3 indicated that fed-batch cultivation can be used as successful strategy to optimize PHB production by *Bacillus megaterium* SW1-2 especially when using 48 h (runs 6 and 4) and 84 h (runs 2 and 4) pulsed feeding process. Modified E2 medium was preferred for PHB accumulation, recording total amount of 10.11 and 11.34% CDW of PHB for run1 and 2 after 96 h cultivation, respectively. On the other hand, the lowest amount of PHB accumulated, 8.93% and 9% CDW of PHB during use of complex nutrient broth medium and applying the two different pulsed feeding processes run 3 and run 4, respectively. Surprisingly, there was general trend of increase in PHB production after feeding process and the total amount of PHB after fed-batch cultivation for 96 h recorded 6.86-fold increase in comparison to batch cultivation. **Albuquerque *et al.* (2007)** developed several strategies for polyhydroxyalkanoate (PHA) production from sugar cane molasses. They reported that beet molasses successfully replaced glucose as sole carbon source to produce poly-b-hydroxybutyrate by a recombinant *Escherichia coli* strain (HMS174/pTZ18u-PHB). Also, the positive impact of fed-batch cultivation on PHB production was reported by **Hameih *et al.*, (2013).**



Legend:

- Runs 1,3,5 = Feeding after 24 and 60 h & Runs 2,4,6 = Feeding after 48 and 84 h
- Runs 1,2 = MSM & Runs 3,4 = NB (conc.= 50%) & Runs 5,6 = MSM+NB (50% NB+50% MSM)
- Arrows, pulsed feeding: straight = 24 and 60 h & dashed = 48 and 84 h

Figure 3 Strategies for fed-batch cultivation and PHB production by *B. megaterium* SW1-2 using two pulsed feeding processes.

Evaluation of factors affecting PHB production

Optimization of PHB production by application of Plackett-Burman design

For optimization studies a series of 14 experiments, with two central points (3 and 13) having the same concentrations used in basal medium, were carried out to screen for the most significant variables affecting PHB production. As presented in Table 1, ten crucial variables; covering biochemical and cultivation parameters were prescribed into 2 levels, coded -1, and +1. The design of the optimization experiment is given in Table 2 together with the experimental results (% CDW). Regression analysis was performed to fit the response function (PHB production) with the experimental data. Analysis of variance indicated that PHB production can be well described by a polynomial model with a relatively high coefficient of determination ($R^2 = 0.93$).

$$Y_{\%\ yield} = 27.99 - 1.29X_1 + 7.34X_2 + 2.33X_3 - 1.51X_4 - 1.01X_5 + 2.96X_6 + 9.56X_7 + 0.77X_8 - 5.78X_9 - 5.69X_{10}$$

One of the advantages of the Plackett-Burman design is to rank the effect of different variables on the measured response independent on its nature (either nutritional or physical factor) or sign (contributes positively or negatively) as in (Figure 4A). Ranking of factor estimates in a Pareto chart (Figure 4B) benefits in displays the magnitude of each factor estimate and is a convenient way to view the results of Plackett-Burman design **(Strobel and Sullivan, 1999)**. It can be seen that, among those variables DEPS-II and inoculum concentration followed by KH_2PO_4 and $(NH_4)_2SO_4$ found to be the most significant variables that encourage PHB production. Interestingly, **Beaulieu *et al.* (1995)** reported that the best growth and PHB production were obtained with ammonium sulfate and sugar cane molasses as the growth activator. Indeed, DEPS-II or date syrup-II (Fresh syrup) is more significant than DEPS-I (commercial syrup) as indicated by the lower *p*-value (0.05) (Table 3).

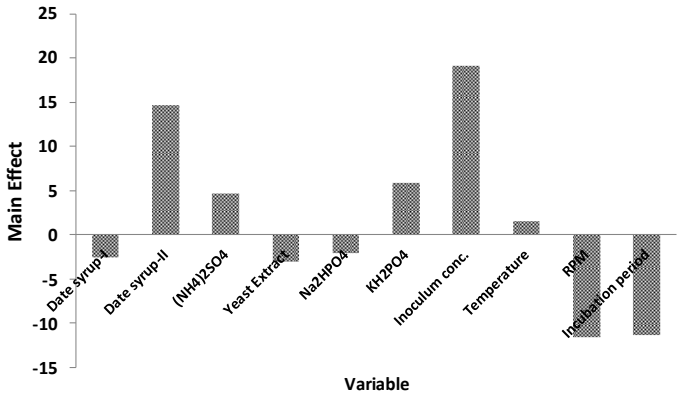


Figure 4A Effect of different environmental conditions on PHB production from *Bacillus megaterium* SW1-2.

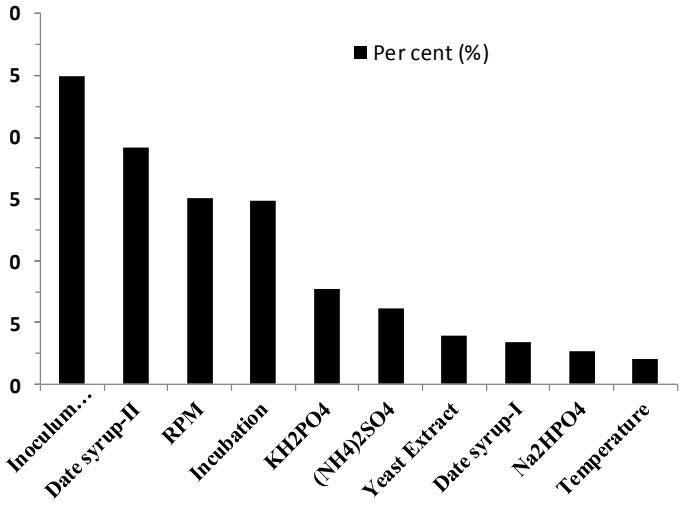


Figure 4B Pareto plot for Plackett-Burman parameter estimates of PHB production by *Bacillus megaterium* SW1-2.

Table 3 Statistical analysis of Plackett-Burman design showing coefficient values, t- and P-values for each variable.

Variable	Coefficient	*t Stat*	*P-value*
Date syrup-I	-1.29	-0.57	0.61
Date syrup-II	7.34	3.26	0.05
$(NH_4)_2SO_4$	2.33	1.03	0.38
Yeast Extract	-1.51	-0.67	0.55
Na_2HPO_4	-1.01	-0.45	0.68
KH_2PO_4	2.96	1.31	0.28
Inoculum conc.	9.56	4.24	0.02
Temperature	0.77	0.34	0.75
RPM	-5.78	-2.56	0.08
Incubation period	-5.69	-2.52	0.09

Interestingly, cane molasses contains many trace elements and vitamins such as thiamine, riboflavin, pyridoxine, and niacinamide **(Crueger and Crueger, 1984)** thus can be used as growth activators and help in enhancement of PHB production **(Beaulieu *et al.*, 1995)**. On the other hand, agitation (RPM) and incubation period have highly negative effect on PHB production. While, other factors namely; DEPS-I, yeast extract and Na_2HPO_4 still showed negative effect. Interestingly, the presence of growth activators in fresh DEPS-II as well as the absence of any preservative materials may explain its positive effect in comparison with the commercial DEPS-I. Furthermore, higher content of growth activators in fresh date syrup or DEPS-II makes it suitable substitutes for yeast extract that showed negative effect.

Results revealed that PHB production yield by *B. megaterium* SW1-2 was 53.6% CDW when cultivated on optimized medium developed by Plackett-Burman. Therefore, the statistical experimental design proved to be a powerful and useful tool for enhancing PHB production and confirm the necessity of the optimization process. In concordance with the obtained results in this work, enhanced PHB production by *Lactobacillus acidophilus* using statistical experimental design was reported by **Hamieh *et al.*, (2013)**. Interestingly, results reported in this study represent the first investigation on optimization of PHB production by application of statistical experimental design using date syrup or DEPS.

CONCLUSION

One of the most crucial variables affecting PHB production-economy is the nature of carbon source. *B. megaterium* SW1-2 stain exhibited nutritional versality in terms of varied growth and PHB accumulation during cultivatrion on different concentrations of the carbon-based agro-industrial wasts namely; date palm syrup (DEPS) or sugar cane molasses. PHB was successfully synthesized and intracellular accumulated in *B. megaterium* SW1-2 in presence of any of the used agro-industrial wastes as proven by FT-IR and NMR spectroscopy. Application of six different fed-batch cultivation strategies provides promising solution for nutritional limitation problems and clearly showed improved PHB production. Fed-batch cultivation recorded 6.87-fold increase in total amount of PHB produced after 96 h as compared with the amount produced under batch

cultivation. Experimental design namely; Plackett-Burman proved to be a powerful and useful tool for enhancing PHB production (53.6% CDW). The use of cheaper carbon sources such as date syrup (DEPS) or sugar cane molasses rather than glucose or sucrose greatly lower process economy and increase promises for biotechnological production of PHB biopolymer on industrial scale.

Acknowledgement: Authors are grateful for the financial support of this work by the Deanship of Scientific Research, Dammam University (Grant Application No-2012052).

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JMBFS
Journal of Microbiology, Biotechnology and Food Sciences
International peer-reviewed scientific online journal
Published by Faculty of Biotechnology and Food Sciences

APPLICATION OF RYE SSR MARKERS FOR DETECTION OF GENETIC DIVERSITY IN TRITICALE

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doi: 10.15414/jmbfs.2016.5.6.623-626

ARTICLE INFO

Received 24. 2. 2016
Revised 6. 4. 2016
Accepted 14. 4. 2016
Published 1. 6. 2016

Regular article

OPEN ACCESS

ABSTRACT

Present study aims to testify usefulness of particular rye SSR markers for the detection of genetic diversity degree in the set of 20 triticale cultivars coming from different European countries. For this purpose, a set of six rye SSR markers were used. The set of six polymorphic markers provided 22 alleles with an average frequency of 3.67 alleles per locus. The number of alleles ranged between 2 (*SCM43*) and 5 (*SCM28, SCM86*). Resulting from the number and frequency of alleles diversity index (DI), polymorphic information content (PIC) and probability of identity (PI) were calculated. An average value of PIC for 6 SSR markers was 0.505, the highest value was calculated for rye SSR marker *SCM86* (0.706). Based on UPGMA algorithm, a dendrogram was constructed. In dendrogram cultivars were divided into two main clusters. The first cluster contained two cultivars, Russian cultivar Greneder and Slovak cultivar Largus, and second included 18 cultivars. Genetically the closest were two Greek cultivars (Niobi and Thisbi) and were close to other Greek cultivar Vrodi. It was possible to separate triticale cultivars of spring and winter form in dendrogram. Results showed the utility of rye microsatellite markers for estimation of genetic diversity of European triticale genotypes leading to genotype identification.

Keywords: Triticale, microsatellites, polymorphism, dendrogram

INTRODUCTION

Triticale (x *Triticosecale* Witt.) is the intergeneric hybrid between wheat and rye, has gained considerable importance in recent years in Europe **(Tams *et al.*, 2004)**. It combines desirable traits of both parents **(Salmanovicz *et al.*, 2013)**. It is a crop with broad genetic potential and is widely adapted to abiotic stress conditions such as aluminium toxicity, drought, salinity, acidic or waterlogged soils **(Kuleung *et al.*, 2004)**. Triticale is also resistant to different diseases of wheat **(Leonova *et al.*, 2005)**. Triticale constitutes a valuable genetic resource for transferring genes of interest from rye into wheat, particularly those related to biotic and abiotic stresses **(Vaillancourt *et al.*, 2007)**. Knowledge of germplasm diversity has a significant impact on the improvement of crop plants. Molecular markers can provide an effective tool for efficient selection of desired agronomic traits because they are based on the plant genotypes and thus, are independent of environmental variation. Nowadays, several molecular markers are developed, of which simple sequence repeats (SSRs) or microsatellites are the most widely used types **(Benor *et al.*, 2008)**. SSR markers were successfully used for many purposes, such as mapping (Tams *et al.*, 2004; Bickel *et al.*, 2011), testing the authenticity of genetic stocks **(Pestsova *et al.*, 2000)** and tagging resistance genes **(Peng *et al.*, 1999)**. Simple sequence repeat (SSR) markers show a relatively good transferability between closely related species **(Botes & Bitalo, 2013)** and they are one of the most promising molecular marker types to identify or differentiate genotypes within a species **(Salem *et al.*, 2008)**. They were sucessufully used in many plant species, e.g. wheat **(Röder *et al.*, 1995; Huang *et al.*, 2002, Gregáňová *et al.*, 2005, Hudcovicová *et al.*, 2013)**, rye **(Khlestkina *et al.*, 2004, Balážová *et al.*, 2016)**, triticale **(Kuleung *et al.* 2004; Tams *et al.*, 2004, da Costa *et al.*, 2007; Odrouškova & Vyhnanek, 2013, Balážová *et al.*, 2015)**, rice **(Jiang *et al*, 2010)**, maize **(Ignjatovic-Micic et al., 2015)**, and flax **(Bickel *et al.*,2011).**

The aim of this study was to testify usefulness and transferability of six rye SSR markers to triticale (x *Triticosecale* Witt.) and detect a genetic variability among a set of twenty European cultivars.

MATERIAL AND METHODS

Plant material and DNA extraction

Twenty European triticale cultivars (x *Triticosecale* Witt.) were used for analysis (Table 1). Cultivars were provided by Gene Bank of Slovak Republic in Plant Production Research Center Piešťany, Bratislavská 122, 921 68 Piešťany, Slovakia (12 winter triticale and 8 spring triticale). Samples originated from 6 countries: i.e. Germany (6), Spain (4), Slovakia (4), Greece (4), Czech Republic (1), Russia (1).

DNA isolation

DNA was isolated from 100 mg freshly-collected leaf tissue according to GeneJET™ protocol (Fermentas, USA). The concentration and quality of DNA was checked up on 1.0 % agarose gel stained by ethidium bromide and detecting by comparing to λ-DNA with known concentration.

PCR conditions

For analysis, six microsatellite primer pairs were chosen according to the literature **(Saal and Wricke, 1999)**. Used primers belong to rye-derived primers (*SCM*) localised on 2R, 5R and 6R chromosomes (Table 2). PCR amplification was performed in 20 µl volume containing PCR water, 5 x Green *GoTaq*® Flexi Buffer, 100 µM dNTP Mix, 0.3 µM primers (Forward and Reverse primer), 1.5 mM $MgCl_2$, 0.4 U *GoTaq*® *polymerase* (Promega, USA). PCR reactions were performed in a thermocykler (Bio-Rad, USA) in 0.2 ml tubes. The PCR program consisted of these steps: an initial denaturation (1 cycle): 2 min. at 93 ^{0}C, (29 cycles) denaturation: 1 min. 93 ^{0}C, annealing 2 min. with different temperature for each primer pair and extension 2 min. at 72 ^{0}C.

Table 1 List of the used triticale cultivars

No.	cultivar	country of origin	form
1	Flavius	Slovakia	winter
2	Largus	Slovakia	winter
3	Pletomax	Slovakia	winter
4	Kandar	Slovakia	winter
5	Amarillo 105	Germany	winter
6	Trizeps	Germany	winter
7	Mungis	Germany	winter
8	Trimmer	Germany	winter
9	Trigold	Germany	winter
10	Cosinus	Germany	winter
11	Senatrit	Spain	spring
12	Sierra de Villuercas	Spain	spring
13	Sierra de Almaraz	Spain	spring
14	Tentudia	Spain	spring
15	Kinerit	Czech Republik	winter
16	Vrito	Greece	spring
17	Niobi	Greece	spring
18	Vrodi	Greece	spring
19	Thisbi	Greece	spring
20	Greneder	Russia	winter

Electrophoresis and staining conditions

The PCR amplicons (5µl) were resolved by electrophoresis in 6.0 % denaturating polyacrylamide gel and run with 1.0 x TBE buffer. Electric voltage and time were different for each marker. The electric voltage ranged from 1800 to 2000 V and time of electrophoretic separation differs for each marker (3-4 hours) influenced by the predicted size of fragments. After electrophoresis, gels were fixed and stained with silver according to [22]. Final PCR amplicons were scanned in UVP PhotoDoc-t® camera system. The size of alleles was determined by comparing with 10 bp standard lenght marker (Invitrogen: 100-330 bp). Each band was treated as a single allele.

Data analysis

For statistical evaluation, all gels were scored in a binary matice on the base of presence (1) or absence (0) of particular allele. Information about presence of alleles and their frequency were used for calculation of Jaccard coefficient of genetic similarity by SPSS modul (SPSS inc., USA). Final dendrogram was constructed by hierarchic cluster analysis using UPGMA (Unweighted Pair Group Method using arithmetic Averages) algorithm by SPSS statistic programme version 17.

For the assessment of the polymorphism between triticale genotypes and usability of SSR markers for differentiation we used diversity index (DI) **(Weir, 1990**), the probability of identity (PI) (**Paetkau *et al.*, 1995**) and polymorphic information content (PIC) (**Weber, 1990**).

They were calculated according to formulas:

Diversity index (DI)

$$DI = 1 - \sum p_i^2$$

Probability of identity (PI)

$$PI = \sum p_i^4 + \sum_{i=1}^{i=n-1} \sum_{j=i+1}^{n} \left(2 p_i p_j\right)^2$$

Polymorphic information content (PIC)

$$PIC = 1 - \left(\sum_{i=1}^{n} p_i^2\right) - \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} 2 p_i^2 \cdot p_j^2$$

where pi and pj are frequencies of ith and jth allele of given genotype.

Table 2 Sequences and melting temperature value (TM) of six SSR primers

SSR marker	*Locus on chromosome*	*Sequences of primers 5´ → 3´*	*SSR repetition*	*Annealing temperature*
SCM28	6RL	5' CTGGTCCTGGTCTGGTGGGTC 3' CGCATCGGGTGTGTCGCATAC	$(GT)_{26}$	60°C
SCM43	2RS	5' CTAGGGGATTACAGGGAGGGCA 3' GTTCCCTTGTCCTACTCGTTACCG	$(GT)_{11}$	60°C
SCM86	6RL	5' CAGATAGATGGGTGTTGTGCG 3' CTCTTCTCGACATCCACACTCC	$(GT)_{20}$	60°C
SCM120	5RL	5' CATTGTTGCGAGTGTTGAAGC 3' TGTGCTGTCGTCGATGTTGTC	$(AC)_{10}$	60°C
SCM180	6RL	5' GTTTCGTCCCCGTTGCCATC 3' ACGTGTCGCTTTCCATTGCCC	$(GT)_6 (GA)_7$	60°C
SCM268	5RS	5' GCGCACCCCACACAACACG 3' GCGGTGGCGGTTGAGGAC	$(CA)_9$	65°C

RESULTS AND DISCUSSION

Six SSR markers were used in this study with the aim to detect genetic diversity in triticale (x *Triticosecale* Witt.) cultivars. This work focused on two principal goals: i.e. a) to testify the usefulness and efficiency of 6 rye SSR markers in close-related triticale and b) to find out a genetic background among triticale cultivars (bred in different European countries). Six SSR markers provided a polymorphic spectrum.

A total of 22 alleles were detected (Table 3) with an average value of 3.67 alleles per locus. The number of alleles ranged from 2 (*SCM43*) to 5 (*SCM28, SCM86*). Very similar average number of detected alleles per locus was detected by authors **Vyhnánek *et al.* (2009)**, **Ondroušková & Vyhnánek (2013)** and **Kuleung *et al*. (2004). Ondroušková & Vyhnánek (2013)** used 25 SSR wheat and rye SSR markers for the study of genetic variability in 10 triticale cultivars. They detected 84 alleles (3.36 alleles per locus). **Vyhnánek *et al.* (2009)** studied 16 genotypes of triticale using 48 markers (27 wheat and 21 rye SSR markers) and detected 184 alleles with an average of 3.83 alleles per locus. **Kuleung *et al.* (2004)** detected on average 4.2 alleles per locus using 43 wheat and 14 rye SSR markers for the study of genetic diversity of 80 hexaploid accessions. Higher average number of alleles was detected by **Tams *et al*. (2004)** (6.8 alleles and per locus). This value is considerably higher compared to our results, but it could be caused by higher number of SSR markers. The highest number of alleles (5) was detected by SSR marker *SCM28* and *SCM86*. **Kuleung *et al*. (2006)** detected the same number of alleles by SSR marker *SCM268* (3) but lower number of alleles (3) compared to our results (4) by SSR marker *SCM120*.

As mentioned above, the usefulness of SSR markers is not influenced just by an overall number of detected alleles, but the most important factor is their distribution and frequencies. The way how to find out their effectiveness is to calculate Diversity index (DI), Polymorphic information content (PIC) and Probability of identity (PI) based on frequencies of alleles.

Diversity index (DI) for six rye SSR markers varied from 0.172 (*SCM43*) to 0.710 (*SCM28, SCM86*) with an average of 0.540. **Kuleung *et al*. (2006)** and **Tams *et al*. (2004)** calculated the same diversity index (0.54) for the study of genetic diversity of triticale cultivars using 57 SSR markers and 93 SSR markers, respectively. **Ondroušková & Vyhnánek (2013)** calculated similar average diversity index (0.55). Higher values of DI (0.65) were reported by **(Balážová *et al.*, 2015)** who used wheat genomic SSR markers for the study of genetic diversity of triticale cultivars.

Polymorphic information content (PIC) varied from 0.157 (*SCM43*) to 0.706 (*SCM86*) with an average of 0.505. An average value of PIC was similar compared to work **Ondroušková & Vyhnánek (2013)** who reported on average 0.5 and **Vyhnánek *et al.* (2009)** who calculated 0.48. Probabilities of identity (PI) ranged from 0.044 (*SCM86*) to 0.699 (*SCM43*) with an average of 0.264. **Korkovelos *et al.* (2008)** claimed that the more effective SSR in discriminating among genotypes is the one having the higher PIC and DI along with lower

PI values. According to this allegation, the most effective marker for the genetic diversity detection is *SCM86*. As appropriate markers, we also recommend *SCM28 and SCM180*, respectively. Only one marker (*SCM43*) reached considerably unfavourable results of DI, PIC, PI and number of alleles compared to average values of tested set.

Table 3 Statistical characteristics of used primers

SSR marker	Number of alleles	DI	PIC	PI
SCM28	5	0.710	0.689	0.074
SCM43	2	0.172	0.157	0.699
SCM86	5	0.710	0.706	0.044
SCM120	4	0.534	0.500	0.237
SCM180	3	0.607	0.532	0.222
SCM268	3	0.505	0.444	0.305
average	3.67	0.540	0.505	0.264

DI – Diversity index; PIC – Polymorphic information content; PI – Probabilities of identity

Resulting from number and frequencies of alleles, a UPGMA dendrogram based on Jaccard´s coefficient of similarity was constructed (Figure 1). Dendrogram revealed genetic relationships among analysed set of winter and spring triticale genotypes. Cultivars were divided into two main groups (1 and 2). First cluster (1) contained two cultivars, Slovak cultivar Largus and Russian cultivar Greneder. Second cluster of 18 cultivars was further subdivided into two subclusters (2a and 2b), subcluster 2a included 11 triticale cultivars and subcluster 2b contained 7 cultivars. All (4) Greek cultivars were included in subcluster 2b. Genetically the closest were two Greek cultivars, Niobi and Thisbi from subcluster 2b. We can consider their close genetic background. Another Greek cultivar Vrodi was also placed nearby Niobi and Thisbi. Most of cultivars from subcluster 2b are of spring form (85.7 %). Subclusters 1 and 2a comprised predominantly of winter triticale cultivars (84.6%).

Vyhnánek *et al.* (2009) tested set of 16 accessions using 48 SSR markers. One Russian genotype had been significantly differentiated from all set of accessions. **Da Costa *et al.* (2007)** divided 54 accessions of triticale based on wheat SSR markers into seven main groups using UPGMA dendrogram based on Jaccard's coefficient of similarity. Since most of the analyzed germplasm was derived from Mexican triticale, they detected a high similarity among triticale cultivars and moderate variability.

Kuleung *et al.* (2004) and **Tams *et al*. (2004)** reported that the diversity revealed by rye SSR markers in triticale was lower in comparison to wheat microsatellites. We can confirm this statement by our earlier study **(Balážová *et al.*, 2015)** where we have detected higher average values of PIC and DI of used wheat SSR markers to study genetic diversity of triticale cultivars compared to today´s study with rye SSR markers.

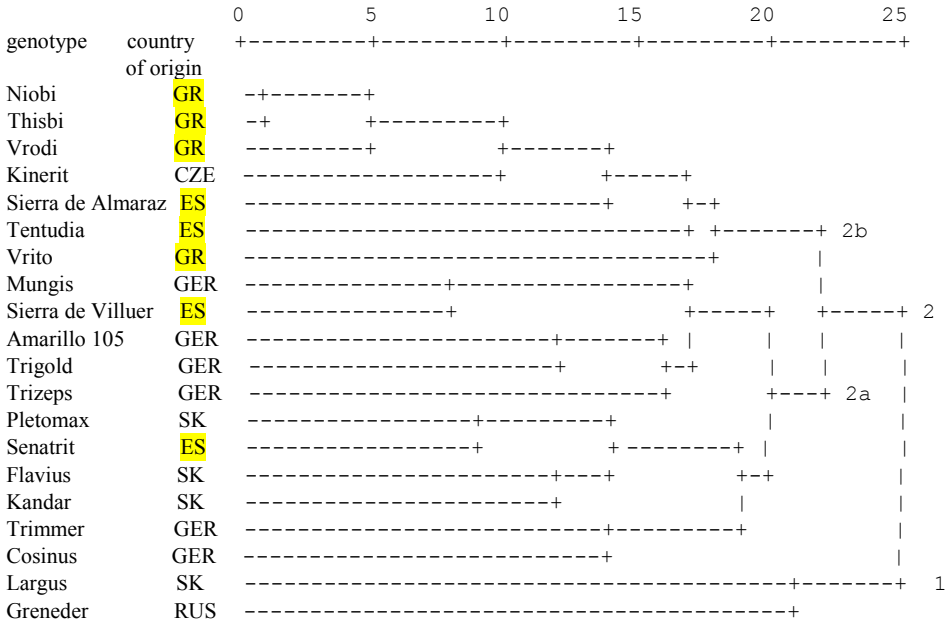

Figure 1 Dendrogram of 20 triticale genotypes based on data from six rye SSR markers. Yellow labelled cultivars are of spring form

CONCLUSION

A tested set of six rye SSR markers allowed significantly distinguish 18 out 20 cultivars. Only two Greek cultivars, Niobi and Thisbi, had not been separated from each other. In dendrogram triticale of spring form significantly separated from winter form triticale cultivars. An average value of PIC and DI indicate that used SSR markers are moderately polymorphic. We can recommend all rye SSR markers used for diversity analysis accept one marker (*SCM43*) which reached considerably unfavourable results of PIC and DI values. Anyway, used rye SSR markers represent an efficient and useful marker system for detection of genetic variability in closed-related triticale.

Acknowlegments: This work was funded by European Community under project No. 26220220180: Building Research Centre „AgroBioTech" (50%) and KEGA project No 021SPU-4/2015 (50%). This work originated also thanks to the support within Operational Programme Research and Development for the project: "Transfer, use and dissemination of research results of plant genetic resources for food and agriculture" (ITMS: 26220220058), cofinanced from the resources of the European Union Fund for Regional Development.

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