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PRODUCTION OF CELLULASES AND XYLANASE FROM *BACILLUS SUBTILIS* MU S1 ISOLATED FROM PROTECTED AREAS OF MUNNAR WILDLIFE DIVISION

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ABSTRACT

The importance of cellulase and xylanase has increased in the current scenario due to their immense biotechnological applications, particularly in biofuel industries. Here, twenty four cellulolytic bacteria were isolated from soils of protected areas under Munnar Wildlife Division, after screening on carboxy methyl cellulose (CMC) agar plates. The isolate with highest zone of clearance (23 mm) was selected and its xylanolytic capability was confirmed by presence of zone on beechwood xylan agar plate. This Gram positive, spore forming bacterium was identified as *Bacillus subtilis* based on biochemical characteristics and 16S rRNA gene sequence analysis. The strain has been designated as MU S1 and the gene sequence deposited in Genbank with accession number KT715518. *B. subtilis* MU S1 showed elevated growth at 40 °C under agitation. The cellulases and xylanase activities were assayed under the optimum growth condition at every 12 h interval, upto 48 h. The maximum cellulolytic activities obtained in CMC media were endoglucanase (0.192 U/ml), exoglucanase (0.149 U/ml), FPase (0.06 U/ml) at 24 h and β-glucosidase (0.157 U/ml) at 48 h incubation. The highest xylanase acitivity (0.28 U/ml) was observed at 36 h of incubation in media supplemented with beechwood xylan. The potential of *B. subtilis* MU S1 to produce multiple enzymes makes it a promising nominee for bioconversions and other industrial applications.

Keywords: Cellulases, xylanase, 16S rRNA gene, *Bacillus subtilis,* Munnar Wildlife Division

INTRODUCTION

The growing concerns about the paucity of fossil fuels, rising cost and air pollution by incomplete combustion of fossil fuels, has resulted in utilization of lignocellulosic biomass as an alternative source of energy **(Gaur and Tiwari, 2015)**. Lignocellulosic biomass is a renewable and inexpensive energy source abundantly available in the environment as forestry, agricultural and agro-industrial wastes. The use of these wastes is however limited by the lack of cost effective hydrolytic enzymes. Significant research, therefore, have been directed towards identification of efficient enzyme producers and designing enzyme cocktail for lignocellulose hydrolysis besides those aimed at metabolic and genetic engineering of existing organisms **(Mohanram *et al.*, 2013)**.

Lignocellulose is mainly composed of three groups of polymers, namely cellulose, hemicellulose, and lignin. Cellulose and hemicellulose are sugar rich fractions of interest for use in fermentation processes, and enzymatic hydrolysis is the most powerful alternative process for the saccharification of these polymers **(Sarkar *et al.*, 2012)** .Cellulose, an unbranched homopolymer of β-1, 4 linked D-glucose molecules, is the most dominant component of lignocelluloses. The complete degradation of cellulose involves the synergistic action of three hydrolases. First, endo-β-1, 4-glucanases [EC 3. 2. 1. 4] nicks the internal cellulose chain, after which exo-β-1,4-glucanases or cellobiohydrolases [EC 3. 2. 1. 91] attacks the ends of the crystalline structure and releases cellobiose processively or nonprocessively and finally, β-1,4-glucosidases [EC 3. 2. 1. 21] cuts cellobiose and cellooligosaccharide to produce glucose **(Bhat and Bhat, 1997)**. Hemicellulose, the second most abundant constituent of lignocellulosic biomass, includes xylan, galactan, mannan, arabinan, and uronic acids **(Dodd and Cann, 2009)**. Xylan, containing β -1, 4-linked D-xylopyranose residues, is the most abundant of the hemicelluloses. Endo-1, 4-β-xylanase [EC 3.2.1.8] is the enzyme that cleaves the glycosidic bonds in the xylan backbone, bringing about a reduction in the degree of polymerization of the substrate **(Reilly, 1981; Puls and Poutanen, 1989)**.

Cellulolytic and xylanolytic enzymes are abundantly produced by bacteria, fungi, actinomycetes, protozoa, algae, gastropods, arthropods, nematodes, and so forth **(Kuhad *et al.*, 2011; Beg *et al.*, 2001)**. Generally fungi are better enzyme producers than bacteria but still active research on bacterial enzymes is continuing, because bacteria have a higher growth rate than fungi, leading to greater production of enzymes. Moreover, bacterial enzymes are more efficient biocatalysts and product recovery is much simpler than in case of fungi **(Maki *et al.*, 2009)**.

Lignocellulolytic enzyme producers have been isolated and characterized from various sources like forest soil **(Woo *et al.*,2014)**, hot springs **(Tamariz-Angeles *et al.*, 2014)**, composts **(Fathallh Eida *et al.*, 2012)**, dairy manure soil, **(Devi and Kumar, 2012)** and termites **(Dheeran *et al.*, 2012; Sreena *et al.*, 2015)**. Soil is a rich source of microorganisms harbouring industrially important enzymes. It has a wide array of aerobic lignocellulolytic bacteria, belonging to various phyla including *Firmicutes*, *Actinobacteria*, *Bacteroidetes,* and *Proteobacteria.* Among them the members of the genus *Bacillus*, *Cellulomonas*, *Streptomyces*, *Cytophaga*, *Cellvibrio,* and *Pseudomonas* have been characterized **(Lynd *et al.*, 2002)**.

Cellulases and xylanases have demonstrated their biotechnological potential in various industries including paper and pulp, agriculture, food, animal feed, brewing and wine making, biomass refining, textile and laundry **(Bhat, 2000)**. However, the most significant application of the enzymes is in the bioconversion of lignocellulosic biomass into fermentable sugars that may be used by yeasts to produce ethanol **(Kuhad and Singh, 1993; Zaldivar *et al.*, 2001)**.

The rising demand for new and competent enzymes has led to the bioprospecting of enzyme producers from less studied environments. The protected areas that come under the jurisdiction of Munnar Wildlife Division are diversity hotspots but little studied and exploited. Therefore, in the present study, we investigated the forest soils from this area as potential source for the isolation of cellulolytic bacteria. The selected isolate was identified and its ability to degrade CMC and xylan by producing multiple enzymes was studied.

MATERIALS AND METHODS

Sample collection

Soil samples were collected in sterile containers from different locations of protected areas under the Munnar Wildlife Division. The protected areas under the jurisdiction of Munnar Wildlife Division are Eravikulam National Park, Chinnar Wildlife Sanctuary and Shola National Parks which include Mathikettan Shola , Anamudi Shola , Pambadum Shola , and the Kurinjimala Sanctuary. The samples were stored at 4 °C until use.

Enrichment and isolation of cellulase producers

One gram soil sample was aseptically transferred to 100 ml of sterile CMC (carboxy methyl cellulose) broth and incubated at 37 °C for 5 days for enrichment of cellulase producing bacteria. The CMC broth contained the following components in g/L: CMC (10.0), NaCl (6.0), $(NH_4)_2SO_4$ (1.0), KH_2PO_4 (0.5), K_2HPO_4 (0.5), $MgSO_4$ (0.1), $CaCl_2$ (0.1), $NaNO_3$ (0.1) and Yeast extract (1.0). The pH of the medium was adjusted to 7.0. A loopful of the sample from the enriched culture was streaked on CMC agar plates and incubated for about 24-48 h at 37 °C. Isolated colonies were purified by re-streaking on CMC agar plates.

Qualitative screening of cellulase producers

Cellulase production by the isolated colonies were detected by flooding the streaked plates with congo red solution (1 mg/ml in water) for 15 min, and then de-staining with 1M NaCl solution for 10-15 min. Potential cellulase producers were selected based on zone of clearance diameter. The one with the highest zone was chosen and studied further. The xylanase producing ability of the isolate was also determined in the similar way using xylan agar plate, which was prepared by replacing CMC in the isolation media with beechwood xylan. Pure culture of the isolate was prepared and stored at 4°C.

Morphological and biochemical characterization

The selected isolate was subjected to morphological and biochemical tests and identified up to genus level according to Bergey's Manual of Determinative Bacteriology **(Bergey *et al.*, 1957)**. For species identification 16S rRNA gene was analyzed.

Molecular characterization and phylogenetic analysis

Pure genomic DNA was isolated using XcelGen Bacterial gDNA kit (Cat No: *XG2411-01) f*ollowing manufacturer's protocol. The 16S rRNA gene was amplified from genomic DNA by polymerase chain reaction with two primers: 8F: 5'-AGAGTTTGATCCTGGCTCAG-3' and 1492R: 5′-ACG GCTACCTTGTTACGACTT-3′. The PCR reaction began with initial denaturation at 95 °C for 2 min, followed by 30 amplification cycles of: primary denaturation at 94 °C for 30 s, primer annealing at 52 °C for 30 s and extension at 72 °C for 90 s. The final extension was performed at 72 °C for 10 min and the product was analyzed using agarose gel electrophoresis to confirm the targeted gene amplification **(Singh *et al.*, 2013)**.

The amplified product was excised from the gel and purified using XcelGen DNA Gel/PCR Purification Mini Kit (Cat No: XG3511-01/3514). The amplicon was sequenced using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA) on ABI 3730xl Genetic Analyzer (Applied Biosystems) following manufacturer's instructions. The consensus sequence generated from forward and reverse sequence data using aligner software was used to carry out BLAST with the nr database of NCBI genbank. Based on maximum identity score, best twenty sequences with 99 % to 100 % similarity were selected and a phylogenetic tree was constructed with the candidate strain. Multiple sequence alignment was performed using CLUSTAL W **(Thompson *et al.*, 1994)** and evolutionary history inferred by the neighbor-joining method **(Saitou and Nei, 1987)**. The evolutionary distances were computed using Kimura 2-parameter method **(Kimura, 1980)** and phylogenetic analysis was carried out with MEGA 5 **(Tamura *et al.*, 2011)**. Bootstrap resampling analysis for 1000 replicates was performed to estimate the confidence of tree topologies **(Felsenstein, 1985)** and the tree generated was visualized using FigTree v1.4.2 **(Rambaut A, 2014)**.

Effect of physical parameters on growth

The growth condition of the isolate was optimized by inoculating it in isolation media and incubating at different temperatures (30 °C, 40 °C and 50 °C) under static and shaker condition (150 rpm). The culture was withdrawn after 24 h and analyzed for bacterial growth by measuring the absorbance at 650 nm.

Enzyme activity assay

The crude enzyme for quantitative assay was prepared by inoculating 50 ml of the production media with 1 % starter culture and incubating at 40 °C for 48 h in an orbital shaker (150 rpm). CMC and beechwood xylan were used as carbon source in the production media for cellulase and xylanase production respectively. The samples were collected at every 12 h interval and centrifuged at 10,000 g for 10 min to separate the bacterial cells. The supernatants were used as crude enzyme for quantitative assay.

Endoglucanase (CMCase) assay was performed by incubating 1 ml of crude enzyme with equal volume of 1 % CMC substrate in 0.05 M sodium citrate buffer (pH 5.0) for 30 min at 40 °C. Exoglucanase (avicelase) and β-glucosidase (cellobiase) activity assays were conducted similarly with 1 % avicel and cellobiose as substrate respectively. Filter paper activity (FPase) assay for total cellulases was carried out using 50 mg strip of Whattman filter paper No. 1 (1 x 6 cm) under the same conditions with incubation time of 1 h **(Ghose, 1987)**. Cellulase activities were calculated by measuring the amount of reducing sugars released by dinitrosalicylic acid (DNS) method **(Miller, 1959)**. All experiments were performed in duplicates and the average was used to assess enzymatic activities of the test isolate. The enzyme activity was determined using calibration curve of glucose. One unit of cellulase activity is defined as the amount of enzyme required to liberate 1µmol of glucose per minute under the assay conditions.

Xylanase activity was assayed using 1 % beechwood xylan with modifications to the protocol of **Bailey *et al.* (1992)**. The substrate was dissolved in 0.05 M sodium citrate buffer (pH 5.0) and incubated with enzyme for 30 min at 40 °C. The released reducing sugar was determined as previously mentioned. Xylose was used as standard to prepare calibration curve. One unit of xylanase activity is defined as the amount of enzyme required to liberate 1 µmol of xylose per minute under the assay conditions.

RESULTS AND DISCUSSION

Isolation and screening of enzyme producers

Soil and the litter horizon contain the largest pool of organic C in the terrestrial biosphere and hence extracellular enzyme producers thrive in these environments **(Magnani *et al.*, 2007)**. According to the studies of **Hatami *et al.* (2008)** total number of bacteria and the number and percentage of cellulolytic bacteria in forest soil samples are more than farming soil. In this study protected areas under Munnar Wildlife Division were explored for novel cellulase producers as there are few reports on isolation of cellulose degraders from this area.

Soil samples were collected from different forests and screened for cellulase producing bacteria, after enrichment in CMC broth. The samples were rich source of enzyme producers. Although many bacteria were isolated from CMC enriched media, not all of them showed cellulase activity on plate assay. Twenty four colonies displayed visible zone of clearance. Among these, the strain isolated from Eravikulam National Park showed the highest zone (23 mm) and was selected for further studies. The xylanolytic potential of the strain was tested and confirmed by the presence of zone on xylan supplemented media. The isolate was designated as MU S1.

Identification of strain MU S1

The colonies of MU S1 on nutrient agar plates were irregular, flat and opaque. The isolate was characterized as Gram positive, spore forming motile rod. It gave positive test for Voges-Proskauer, citrate, catalase, oxidase, nitrate reduction and starch hydrolysis whereas negative for indole, methyl red and urease. The

bacterium could ferment glucose, sucrose and mannitol but not lactose. These results indicated that the strain belonged to genus *Bacillus*. The predominance of genus *Bacillus* in soil and waste with high cellulose content has been widely reported **(Ulrich and Wirth, 1999; Pourcher *et al.*, 2001)**. *Bacillus* strains are well-known for their spore forming ability and production of secondary metabolites like antibiotics. These strategies give them an additional advantage over competitors under conditions of slow growth on cellulosic substrates **(Lynd *et al.*, 2002)**.

In order to reveal the true identity of strain MU S1, molecular and phylogenetic analysis were carried out. The 16S rRNA gene was amplified and sequenced. The consensus sequence of 1362 bp generated from forward and reverse sequence data was used for BLAST analysis. The BLAST results revealed 99 % similarity with *Bacillus subtilis* strain BCX-1 (KM378567.1). A phenogram reflecting the relationship among the strain and candidate sequence of related strains obtained from NCBI database are presented in Figure 1. The phylogenetic analysis displayed close similarity with *Bacillus* sp. JBP-21 (KM675950.1) and *Bacillus subtilis* strain LD181 (KJ564129.1). From results of biochemical and molecular analysis the isolate was affirmed to belong to *B. subtilis*. The sequence was deposited in Genbank database with accession number KT715518. *Bacillus subtilis* strains with cellulolytic and xylanolytic potentials have been isolated from variety of sources like agricultural soil **(Kim *et al.*, 2012)**, water and soil of the Amazon region **(Heck *et al.*, 2002)**, termites **(Tarayre *et al.*, 2014)**, snail **(Dar *et al.*, 2015)** etc.

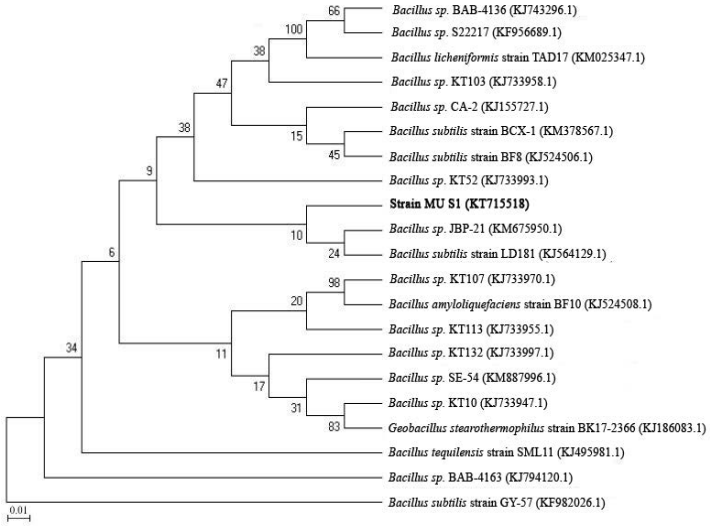

Figure 1 Phylogenetic tree based on 16S rRNA gene sequence, showing relationship between the isolate MU S1 and closely related strains.

Optimization of growth conditions

Generally, it is found that cell growth and enzyme production are highly correlated **(Seo *et al.*, 2013; Singh *et al.*, 2013; Sizova *et al.*, 2011).** Physical parameters like temperature and aeration are known to have a significant influence on enzyme production and are critical parameters essential for the success of a fermentation reaction. To find the optimum growth conditions of *Bacillus subtilis* MU S1, it was grown at different temperatures under static and shaking (150 rpm) conditions (Figure 2). Even though the cultures showed growth at 30 °C, 40 °C and 50 °C the optimum growth was at 40 °C. This was in accordance with **Sethi *et al.* (2013)** who recorded an optimum growth at 40 °C for *Bacillus sp*. **Jansová *et al.* (1993)** recorded maximum specific growth rate of *Bacillus subtilis* 115 in the temperature range of 45–48 °C. Temperature was found to regulate enzyme synthesis at mRNA transcription and probably translation levels **(Gayda *et al.*, 1985; Kuriki, 1987)**. It also influences extracellular enzymes secretion, possibly by changing the physical properties of the cell membrane **(Yatvin, 1987)**.

As with temperature, microorganisms also vary in their oxygen requirement. Oxygen acts as a terminal electron acceptor for oxidative reactions to provide energy for all the cellular activities. It was found that the strain MU S1 favored agitation at all temperatures. Agitation is reported to increase the rate of oxygen and nutrient transfer from the liquid medium to the cells. It also prevents bacterial clumps or biofilm formation, ensuring prolific bacterial reproduction **(Brown *et al.*, 1987)**. The result was in agreement with the studies of **Deka *et al.* (2013)** and **Sanghi *et al.* (2009)** who recorded a positive effect of agitation on growth and enzyme production by *Bacillus subtilis*.

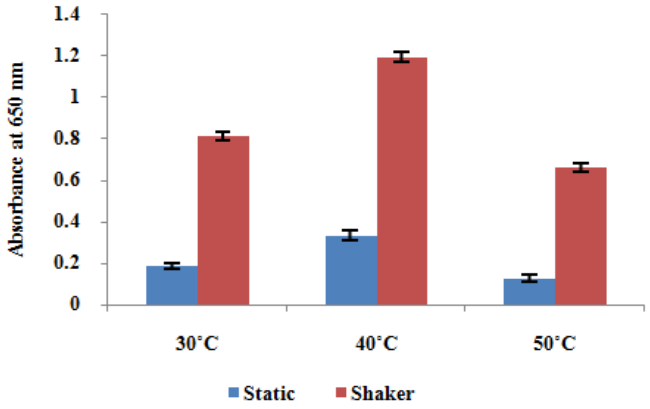

Figure 2 Growth profile of *B. subtilis* strain MU S1 under different conditions after 24 h incubation. Error bars indicate standard deviation of duplicate observations.

Enzyme activity assay

The cellulolytic and xylanolytic potential of the strain was examined after inoculating in production media and incubating at 40 °C in an orbital shaker. The activity was assayed at 12 h intervals and the results were recorded. It was observed that strain MU S1 very effectively utilized CMC and beechwood xylan for growth by secreting multiple enzymes (endoglucanase, exoglucanase, β-glucosidase, FPase and xylanase) extracellularly.

Figure 3 shows cellulolytic activity profile obtained during incubation of *Bacillus subtilis* MU S1 in CMC broth for 48 h. Endoglucanase and exoglucanase activity reached a peak after 24 h incubation and decreased thereafter. β- glucosidase activity which was an exception to this case was found to rise even after 24 h. Similar incubation time was reported in *Bacillus subtilis* AS3 **(Deka *et al.*, 2011)**. The main reason for decrease of cellulolytic acitivity after 24 h could be catabolite repression of cellulase genes in the presence of glucose, which is the major end product of cellulose digestion. Another reason for the decrease could be inhibition of endoglucanases and exoglucanases by cellobiose. Also, rapid consumption of cellobiose by β- glucosidase leads to glucose accumulation, causing β- glucosidase product inhibition.

Bacillus subtilis MU S1 exhibited higher endoglucanase activity (0.192 U/ml) compared to other cellulolytic enzymes. This is in agreement with previous studies which also recorded a CMCase activity greater than exoglucanase, β-glucosidase and FPase **(Pason *et al.*, 2006; Saratale *et al.*, 2012**). The endoglucanase activity obtained in the present study was higher than that exhibited by some known natural isolates, for example, *Bacillus subtilis* AS3 **(Deka *et al.*, 2011)** isolated from cowdung (0.07 U/ml); *Bacillus amyloliquefaciens* SS35 **(Singh *et al.*, 2013)** isolated from rhinoceros dung (0.079U/ml); *Brevibacillus* sp. DUSELG12 and *Geobacillus* sp. DUSELR7 **(Rastogi *et al.*, 2009)** isolated from gold mine (0.02 U/ml and 0.058 U/ml respectively).

Exoglucanase activity measured using avicel as substrate was found to reach maximum (0.149 U/ml) after 24 h incubation. Similar activity (0.18 U/ml) was observed by *Bacillus licheniformis* NLRI X-33 isolated from cow feces **(Kim *et al.*, 2004)**. Generally, avicelase activity is found to be lower than CMCase and β-glucosidase (**Kumar and Parikh, 2015; Waghmare *et al.*, 2014)**. In a study carried out by **Soares *et al.* (2012)** 46.9 % of the isolates displayed the presence of endoglucolytic activity, 9.1 % showed exoglucolytic activity, while only a minority (4.72 %) could degrade both the substrates on plate assay.

β -glucosidase (cellobiase) activity of the strain increased beyond 24 h and then remained almost constant upto 48 h. The highest activity obtained was 0.157 U/ml. Comparable cellobiase activity has been observed in different *Bacillus sp*. **(Li *et al.*, 2012; Seo *et al.*, 2014)**. Our results show some contrast to earlier findings **(Kim *et al.*, 2012)** in which *Bacillus subtilis* strains were unable to secrete cellobiase, although its presence was detected in cell debris indicating its membrane association. **Kim *et al.* (2004)** also reported lack of extracellular β-glucosidase in *Bacillus licheniformis* NLRI X-33.

FPase (total cellulase) assay performed using filter paper as substrate, measures the ability of crude enzyme to act on both amorphous and crystalline cellulose. Since degradation of filter paper requires the concerted action of endo and exo cellulases, this assay is the best measure of total cellulase activity. The isolate MU S1 displayed highest FPase activity of 0.06 U/ml after 24 h incubation. Comparable activities were obtained by *Brevibacillus* sp. DUSELG12 and *Geobacillus* sp. DUSELR7 studied by **Rastogi *et al.* (2009)**. Whereas **Ariffin *et al.* (2006)** recorded slightly lower FPase activity (0.011 U/ml) by *Bacillus pumilus EB3*.

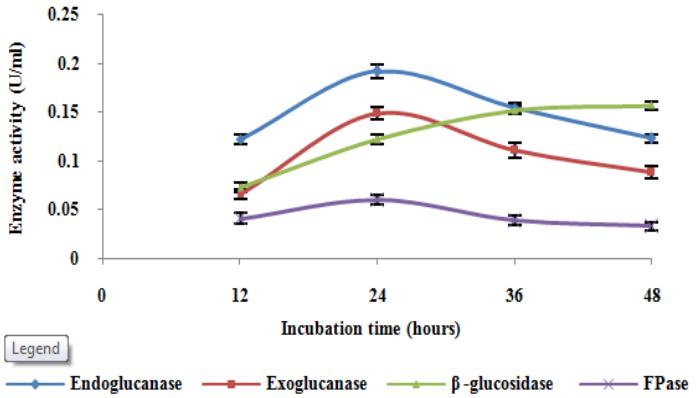

Figure 3 Cellulase activity profile of *Bacillus subtilis* MU S1.Error bars indicate standard deviation of duplicate observations.

Figure 4 illustrates the xylanase activity profile of *B. subtilis* MU S1 during 48 h incubation in production media with beechwood xylan as carbon source. Xylanase activity was found to reach a peak at 36 h with activity of 0.28 U/ml and decreased subsequently. In congruence with our findings, many previous studies have also indicated xylanase activity from cellulolytic *Bacillus sp.* As in many studies cited earlier, **(Heck *et al.*, 2002; Pajni *et al.*, 1989; Ali *et al.*, 2013)** xylanase activity of strain MU S1 was found to be higher than the corresponding CMCase activity. However, results with greater CMCase activities have also been documented **(Seo *et al.*, 2013)**.

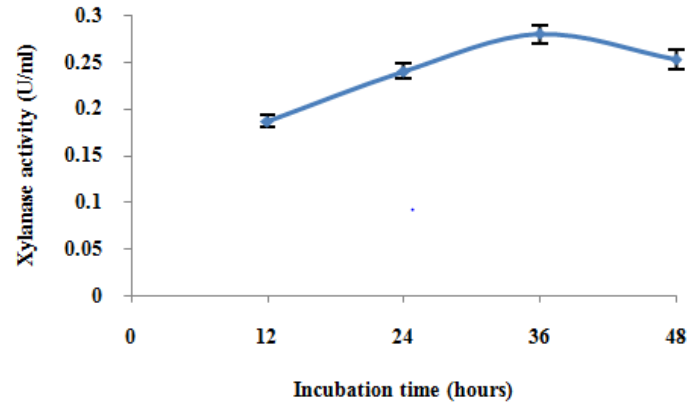

Figure 4 Xylanase activity profile of *Bacillus subtilis* MU S1. Error bars indicate standard deviation of duplicate observations.

CONCLUSION

The ever-increasing demand for highly competent enzymes has accelerated the search for novel enzyme producers from unexplored environments. In the present investigation we were successful in isolating twenty four cellulose degrading bacteria from the protected forests of Munnar Wildlife Division. These unexplored areas were definitely an affluent source of cellulase producers. The best bacterial isolate selected based on zone diameter, was characterized and identified as *Bacillus subtilis.* This isolate designated as *Bacillus subtilis* MU S1(accession no. KT715518) displayed elevated growth at 40 °C under agitation condition. The strain produced promising levels of cellulases (endoglucanase, exoglucanase, β-glucosidase, FPase) and xylanase. The ability of the isolate MU S1 to produce multiple enzymes makes it a prospective candidate for a variety of industrial applications mainly biofuel industry. The efficiency of the isolate can be further improved by media optimization and genetic modifications.

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BOX-BEHNKEN EXPERIMENTAL DESIGN MEDIATED OPTIMIZATION OF AQUEOUS METHYLPARATHION BIODEGRADATION BY *Pseudomonas aeruginosa* mpd STRAIN

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ABSTRACT

Biotreatment of methylparathion was studied in aqueous mineral salts medium containing bacterial culture to demonstrate the potential of the novel strain of *Pseudomonas aeruginosa* mpd. A statistical Box–Behnken Design (BBD) of experiments was performed to evaluate the effects of individual operating variables and their interactions on the methylparathion removal with initial concentration of 1000 mg l^{-1} as fixed input parameter. The temperature (X_1), pH (X_2), reaction time (X_3) and agitation (X_4) were used as design factors. The result was shown that experimental data fitted with the polynomial model. Analysis of variance showed a high coefficient of determination value 0.9. The optimum biodegradation of MP in terms MP removal (Y_1), COD removal (Y_2) and TOC removal (Y_3) were found to be 95.2 %, 82 % and 61.2 % respectively. The maximum growth (Y_4) was 2.18 optical density (OD). The optimum biodegradation correspond to the factors combination of middle level of X_1 (33 °C), X_2 (7.0), X_4 (150 rpm) and the highest level of X_3 (96h). MP removal and its residues were detected using spectral analysis. The study demonstrates the optimum MP biodegradation potential of this strain could use MP as the sole Carbon/Phosphate source. BBD confirmed to be dependable in developing the model, optimizing factors and analyzing interaction effects. Data from this study will be helpful in the design of small-scale field experiments and subsequently an in situ methylparathion biotreatment system for field application.

Keywords: Wastewater Biotreatment. Design Optimization. *Pseudomonas aeruginosa*. Biodegradation. *O,O*-dimethyl -*O*-4-nitrophenylphosphorothioate

INTRODUCTION

Continuous and excessive use of organophosphorus (OP) compounds has led to the contamination of several ecosystems in different parts of the world (**Cisar and Snyder, 2000; Tse *et al.*, 2004**). Thiophosphoric acid esters, such as parathion, methylparathion (MP) and tetrachlorvinphos, are hazardous pollutants and their accumulation in the environment is a recognized ecological threat (**Kaloyanova and Tarkowski, 1981**). Methods for their enhanced degradation are an urgent task of contemporary chemical technology and biotechnology. Its widespread use has caused environmental concern due to its frequent leakage into surface and ground waters. The drinking water directive (Council directive 98/ 83/ EC) sets an allowed contaminant level of 0.1 mg/L for a single pesticide and 0.5 mg/L for the total sum of pesticides. Industries manufacturing pesticides release wastewater in water bodies or land. Although industries treat their wastewater by activated sludge process, no attention is paid to remove the specific pesticides or their metabolites which exert toxicity at very low concentrations. Therefore, there is a need for economically dependable methods for organophosphorus (OPs) detoxification from the environment. To date, bacterial transformations have been the main focus in research on organophosphate pesticide degradation. *Pseudomonas aeruginosa*, *Clavibacter michiganense* (**Subhas and Dileep, 2003**), *Arthrobacter atrocyaneus*, *Bacillus megaterium* and *Pseudomonas mendocina* (**Bhadbhade *et al.*, 2002**), *Agrobacterium radiobacter* (**Horne *et al.*, 2002**), and other *Pseudomonas* species (**Ramanathan and Lalithakumari, 1999**) have been reported to degrade OP in solutions and soils. Use of specific microorganism adapted to the pesticides, in treatment of industrial effluents is not in practice (**Kanekar *et al.*, 2004**). Therefore, research should be concentrated to develop economical but effective microbial processes for the treatment of industrial effluents containing pesticides and take them to field. The aim of this research was to optimize the process variables for the biodegradation potential of the *Pseudomonas aeruginosa* mpd novel strain using response surface methodology (RSM).

MATERIAL AND METHODS

Bacterial culture conditions

A potential bacterial strain (*Pseudomonas aeruginosa* mpd) was isolated from pesticide exposed agricultural soil. The initial enrichment cultures were established in a synthetic wastewater containing mineral salts medium amended with the methylparathion (Devithion™ 50% EC) as the sole source of carbon and energy. The concentration of methylparathion used was 0.1%, pH was adjusted using 1N NaOH and 1N HCl (ELICO - L1127, India). The methylparathion contaminated synthetic wastewater was neutral pH and the mean value of methylparathion (MP) content was 1000 mg/L, chemical oxygen demand (COD) was 41950 mg/L and total organic carbon (TOC) was 10459 mg/L. The synthetic wastewater containing higher concentration of methylparathion with maximum level of 1000 mg/L was used in the present study. Stock solution of pure methylparathion (98.5%) was prepared by dissolving 1g in 100 mL methanol, made up to 1000 mL of distilled water and was used as a reference for instrumental analysis. It was reported that the pesticide pollution due to wastewater released from formulating or manufacturing pesticide industry were up to 1000 mgL^{-1} (**Chiron *et al.*, 1997**). Therefore in this research, synthetic wastewater containing methylparathion with maximum concentration of 1000 mgL^{-1} was used.

Organisms were subsequently grown on nutrient agar medium plates to obtain single colonies. A pure culture of methylparathion-degrading *Pseudomonas aeruginosa* was isolated by series of replating on MSM with methylparathion agar plates. Minimum inhibitory concentration (MIC) test with plate screening method was carried out to screen methylparathion resistant bacteria using methylparathion MSM with methylparathion agar plates. Based on the MIC test, the five potential bacterial cultures isolated were identified based on their morphological characters and biochemical tests as given in Bergey's Manual of Determinative Bacteriology (**Holt *et al.*, 2000**). For degradation studies, *Pseudomonas aeruginosa* was inoculated into sterile shake-bottles containing 250

mL of MSM, 0.1% (w/v) methylparathion and incubated under aerobic conditions on a shaker (150 rpm) for 96 h. The other parameters, i.e., pH value, culture temperature, time and agitation, were part of the experimental design. All experiments were performed in triplicate, and the results are expressed as an average of three replicates.

Optimization of methylparathion degrading condition by *Pseudomonas aeruginosa* mpd

In order to study the effect of variables on the degradation of methylparathion by biotreatment using potential bacterial strain, the process variables include pH, temperature time and agitation were optimized. Experimental design was set using the variables such as pH, temperature, time and agitation. The synthetic wastewater which consists of mineral salts medium amended with the methylparathion was set at various temperature (25-40°C), pH (5-9), time (24-168 h) and agitation (120-180 rpm) for analysis. The concentration of methylparathion used was 1000 mgL^{-1}. The pH was adjusted using 1N NaOH and 1N HCl with the help of pH meter (ELICO - L1127, India). During this process, estimation of various parameters such as residual methylparathion, COD removal, TOC removal and pH were analysed to measure the degradability of methylparathion. Response surface methodology (RSM) based on the Box-Behnken design of experiment was used to optimize these parameters and their interaction which significantly influenced methylparathion biodegradation.

Box–Behnken Experimental Design (BBD) of methylparathion bioremoval using RSM

A standard RSM design called Box-Behnken's Design (BBD) for biotreatment process was adopted to study the influence of variables for the removal of aqueous methylparathion. The method can reduce the number of experimental trials needed to evaluate multiple parameters and their interactions and for finding the most suitable condition and prediction of response (**Box and Behnken, 1960; Myers and Montgomery, 2002**). Among all the RSM designs, BBD requires fewer runs than the others, e.g., 29 runs for a 4-factor experimental design. By careful design and analysis of experiments, Box-Behnken design allows calculations of the response function at intermediate levels which were not experimentally studied and shows the direction if one wishes to change the input levels to determine the effects on the response (**Hamed and Sakr, 2001, Martínez-Toledo and Rodríguez-Vázquez, 2011**).

Table 1 The levels of variables in Box-Behnken statistical experiment design

Variable	Name	Coded level		
		-1	0	+1
X_1:A	Temperature (°C)	25	32.5	40
X_2:B	pH	5	7	9
X_3:C	Time (h)	24	96	168
X_4:D	Agitation (rpm)	120	150	180

The relation between the code values and none code values were:
$X_1 = (A- 32.5)/7.5$, $X_2 = (B - 7)/2$, $X_3 = (C - 96)/72$, $X_4 = (D - 150)/30$.

Table 2 Experimental design with coded and actual values

Run	Temp (°C)	pH	Time (h)	Agitation (rpm)	Temp (°C)	pH	Time (h)	Agitation (rpm)
	Coded Values				Actual Values			
1	1	0	0	-1	40	7	96	120
2	0	1	1	0	32.5	9	168	150
3	0	0	-1	-1	32.5	7	24	120
4	-1	0	0	-1	25	7	96	120
5	1	0	1	0	40	7	168	150
6	1	1	0	0	40	9	96	150
7	-1	0	1	0	25	7	168	150
8	0	0	1	1	32.5	7	168	180
9	-1	0	-1	0	25	7	24	150
10	1	0	-1	0	40	7	24	150
11	0	-1	0	-1	32.5	5	96	120
12	0	0	1	-1	32.5	7	168	120
13	0	0	0	0	32.5	7	96	150
14	-1	0	0	1	25	7	96	180
15	0	1	-1	0	32.5	9	24	150
16	0	0	0	0	32.5	7	96	150
17	0	1	0	1	32.5	9	96	180
18	0	-1	-1	0	32.5	5	24	150
19	0	1	0	-1	32.5	9	96	120
20	0	-1	1	0	32.5	5	168	150
21	1	0	0	1	40	7	96	180
22	0	-1	0	1	32.5	5	96	180
23	0	0	0	0	32.5	7	96	150
24	0	0	0	0	32.5	7	96	150
25	-1	1	0	0	25	9	96	150
26	-1	-1	0	0	25	5	96	150
27	0	0	0	0	32.5	7	96	150
28	1	-1	0	0	40	5	96	150
29	0	0	-1	1	32.5	7	24	180

Response surface methodology (RSM) based on the BBD of experiment was used to optimize the variables and their interaction which significantly influenced methylparathion biodegradation by the individual strains of *Pseudomonas aeruginosa* mpd. A four-factor, three-level Box-Behnken design was used in the biotreatment process. The Box-Behnken design is an independent, rotatable quadratic design with no embedded factorial or fractional factorial points where the variable combinations are at the mid-points of the edges of the variable space and at the center. Among all statistical experiment designs, Box-Behnken design requires fewer runs than the others, e.g., 29 runs for a 4-factor experimental design. The low, middle and high levels of each variable were designated as −1, 0, and +1 respectively, as given in Table 1. For this biotreatment process, the variables and their values in brackets were three levels include temperature (25-40°C), pH (5-9), time (24-168 h) and agitation (120-180 rpm), at constant methylparathion concentration 1000 mgL^{-1}(0.1%). This also enabled the identification of significant effects of interactions for the batch studies. This also enabled the identification of significant effects of interactions for the batch studies. In system involving four significant independent variables X_1, X_2, X_3, and X_4, the mathematical relationship of the response of these variables can be

approximated by quadratic (second degree) polynomial equation (**Box and Behnken, 1960**). A total of 29 experiments were carried out. The design consists of three replicated center points, and a set of six points lying at the midpoints of each edge of the multidimensional cube (Table 2). Response functions, describing variations of dependent factors (*Y*) (methylparathion removal, COD removal, TOC removal, bacterial growth for bacterial with the independent variables (X_i) (temperature, pH, time and agitation) can be written as follows (Eq.1):

$$Y = b_0 + \sum b_i x_i + \sum_{\dots} b_{ij} x_i x_j + \sum b_{ii} x^2_{ii} \quad \dots (1)$$

Linear Interaction Square

Where, Y is the predicted response in percentage of methylparathion removal, COD removal, TOC removal and bacterial growth in terms of optical density, b_o is the offset term and b_i is the linear effect while b_{ii} and b_{ij} are the square and the interaction effects, respectively. Experimental data points used in Box-Behnken statistical experiment design are presented in Table 1. The response function coefficients were determined by regression using the experimental data and the Stat-Ease Design Expert 8.0.4 program.

The response functions for percentage of methylparathion removal, COD removal, TOC removal and bacterial growth in terms of optical density were approximated by the standard quadratic polynomial equation as presented in Eq. 2.

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_4 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{44}X_4^2 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{14}X_1X_4 + b_{23}X_2X_3 + b_{24}X_2X_4 + b_{34}X_3X_4 \quad \dots (2)$$

Where *Y* is the predicted response, i.e. the methylparathion removal; X_1, X_2, X_3 and X_4 are the coded levels of the independent factors: temperature, pH, time and agitation. The regression coefficients are: b_0 – the intercept term; b_1, b_2, b_3 and b_4 – the linear coefficients; b_{12}, b_{13}, b_{14}, b_{23} b_{24} b_{34} – the interaction coefficients and b_{11}, b_{22}, b_{33}, b_{44} – the quadratic coefficients. The model evaluates the effect of each independent factor on the response.

The normal practice is to test within the feasible range, so that the variation in the process does not mask the factor effect. A total of 29 trials were necessary to estimate the coefficients of the model using multiple linear regressions. Hence, about 29 treatments were conducted in the present study and analysis the variance. The data obtained from 29 experiments, were used to find out the optimum point of the process parameters using Box-Behnken Design in Response surface methodology. All the data were treated with the aid of Design Expert by Stat Ease Inc, Minneapolis (Design Expert. 8.0.4). For this bacterial biotreatment process, the methylparathion removal conditions are presented in Table 2, according to the experimental design (Table 1).

Preparation of Sample for Residual Analysis

The biotreated samples were centrifuged at 10,000 rpm for 15 min using high-speed refrigerator centrifuge (CR22GII- Hitachi, Japan). The centrifuged samples were filtered through 0.2 µm sterile syringe nylon filters and then used for analysis of residual methylparathion and intermediate products using HPLC.

Methylparathion determination

The methylparathion removal efficiency of the bacterial biotreatment process was analyzed in terms of COD, TOC and residual methylparathion concentration of the wastewater before and after the treatment process. The samples were withdrawn at different time intervals after biotreatment from 0 to 168 h were analyzed for COD, TOC as per standard procedure laid down in APHA (1998). All experiments were performed in triplicates. The pH of the treated wastewater was adjusted and monitored using pH meter (ELICO - L1127, India). The residual methylparathion was analysed using UV-Vis spectrophotometer (Shimadzu - UV- 3600, Japan), HPLC (Shimadzu, SPD-20A, Japan) and GC-MS (Perkin Elmer-Clarus 600, Germany).

Estimation of Growth

Growth in terms of optical density (Bacteria) was estimated. The increase in growth of bacteria for every 24 h was monitored by measuring optical density (OD) at 600 nm on a UV-Visible Spectrophotometer (Shimadzu - UV- 3600, Japan).

Spectral Analysis

Degradation of methyl parathion and subsequent formation and eventual disappearance of intermediate products in the reaction mixture as a function of pH and time was monitored using UV-Vis spectroscopy. The filtered samples were scanned using UV-Vis-NIR Spectrophotometer (Shimadzu - UV- 3600, Japan), at 277nm. The centrifuged and filtered samples were analyzed for residual methylparathion using HPLC, (Shimadzu, Japan) on a reverse phase C18 column [(250 x 4.60 mm) (Desc. Luna 5µ C18 (20)-100A Phenomenex)], at a flow rate of 1.5 mL min^{-1}. Mobile phases consisted of solution A (HPLC grade water) and solution B (HPLC grade methanol) in the ratio of 1:4 respectively. The isocratic gradient mode with pressure limit of 20 MPa and the total run time for 20 min. The sample was injected at a rate of 20 µL and was detected at 277nm using UV detector (SPD-20A, Japan). Under the conditions described above, the retention time (RT) of methylparathion standard was 3.3 min.

RESULTS AND DISCUSSION

Box-Behnken Experimental design and statistical analysis of methylparathion biotreatment

Box-behnken statistical experimental design was used to investigate the effects of the three independent variables on the response function and to determine the optimal conditions for maximizing the removal of methylparathion, COD, TOC and growth of bacteria. The optimization procedure involves studying the response of the statistically designed combinations, estimating the coefficients by fitting the experimental data to the response functions, predicting the response of the fitted model and checking the adequacy of the model. The independent variables were the temperature (X_1), pH (X_2), time (X_3) and agitation (X_4). The low, center (middle) and high levels of each variable are designated as -1, 0, and +1, respectively as shown in Table 1. The response functions are the methylparathion removal (Y_1), COD removal (Y_2), TOC removal (Y_3) and growth of bacteria (Y_4). The experimental values and predicted values are presented in Table 2. The center point (0, 0, 0, 0, 0) was repeated five times and the same results were obtained indicating the reproducibility of the data. Observed and predicted removal (%) for methylparathion, COD, TOC and bacterial growth (OD) are compared in Table 3. Yuan et al., (2006) reported optimization of a medium for enhancing nicotine biodegradation by Ochrobactrum intermedium DN2 by using RSM. Furthermore **Usharani *et al.*, (2013)** reported optimization of Phosphate removal by bacterial consortium in batch scale process using response surface methodology. Results obtained during the present study showed the importance of using RSM based on the BBD of experiment for the optimization of aqueous methylparathion biotreatment and degradation by potential microbial strains.

Analysis of Variance

The data obtained from the experiments were used for the analysis of variance. Table 4 and 5, shows the ANOVA results of the model of response surface showing the removal of methylparathion, COD and TOC by *Pseudomonas aeruginosa* mpd and its growth in terms of optical density as a function of temperature, pH, time and agitation. The model F-value obtained (6903.04, 3961.11, 4683.83 and 4.55) from each source implied the respective model was significant for the removal of methylparathion, COD, TOC and bacterial growth in terms of optical density. The 'P' value lower than 0.01% (or 0.0001) indicates that the respective model is considered to be statistically significant (**Montgomery 1991, 2004**). In Table 5, the "lack of fit F- value" of 1.45 for methylparathion removal, 0.36 for COD removal, 2.90 for TOC removal and 1.5 for growth in OD implies that the lack of fit phenomenon is not important relatively to pure error, indicating the suggested model is well fitted to the observed methylparathion removal, COD removal, TOC removal and growth of *Pseudomonas* sp in OD. Figure 1, show their actual and predicted plot for (a) methylparathion removal, (b) COD removal, (c) TOC removal and (d) growth. The actual values are the measured response data for particular run and the predicted values are the results generated using the approximating functions. It was found that the removal of methylparathion, COD, TOC and bacterial growth (OD) which measured the signal to noise ratio was greater than 4, reaching the ratio of 135.92, 69.66, 43.75 and 30.60, respectively indicates an adequate signal. This indicates the model is adequate to be used to navigate the design space.

The Regression Model Coefficients

The application of RSM offers an empirical relationship between the response function and the independent variables. The mathematical relationship between the response function (Y) and the independent variables (X) can be approximated by a quadratic polynomial equation as given in Eq. 2. By applying multiple regression analysis of the experimental data, the experimental results were fitted with a second-order polynomial equation. Thus, mathematical regression models for methylparathion removal using the coded factors are given in Eqs. (1) – (4).

$$Y_1 = 95.2 + 4.67X_1 + 2.75X_2 + 5.75X_3 - 2.67X_4 - 20.81X_1^2 - 18.18X_2^2 - 19.18X_3^2 - 14.31X_4^2 - 0.50X_1X_2 + 4.25X_1X_3 - 7.75X_1X_4 - 4.50X_2X_3 + 2.25X_2X_4 - 3.0X_3X_4 \quad \dots (1)$$

$$Y_2 = 82.0 - 0.757X_1 + 2.5X_2 + 1.33X_3 + 2.58X_4 - 13.25X_1^2 - 11.87X_2^2 - 15.62X_3^2 - 17.50X_4^2 - 1.50X_1X_2 + 1.0X_1X_3 - 4.25X_1X_4 + 1.0X_2X_3 + 1.0\ X_2X_4 + 2.50X_3X_4 \quad \ldots (2)$$

$$Y_3 = 61.2 - 1.257X_1 + 2.58X_2 + 0.92X_3 + 1.75X_4 - 15.98X_1^2 - 12.73X_2^2 - 15.47X_3^2 - 19.22X_4^2 - 0.25X_1X_2 + 2.25X_1X_3 - 4.75X_1X_4 + 4.25X_2X_3 + 2.25\ X_2X_4 + 1.25X_3X_4 \quad \ldots (3)$$

$$Y_4 = 2.18 - 0.075X_1 + 0.13X_2 + 0.13X_3 + 0.12X_4 - 0.38X_1^2 - 0.44X_2^2 - 0.38X_3^2 - 0.56X_4^2 - 0.025X_1X_2 - 0.025X_1X_3 - 0.17X_1X_4 + 0.25X_2X_3 + 0.13\ X_2X_4 + 0.15X_3X_4 \quad \ldots (4)$$

Where Y_1 (% methylparathion removal), Y_2 (% COD removal), Y_3 (% TOC removal) and Y_4 (bacterial growth in OD) is the predicted responses where as the X_1 (temperature), X_2 (initial pH), X_3 (time) and X_4 (agitation) are the coded variables.

Table 3 The observed (experimental) values and model response (predicted) values obtained from combination of process variables

Run	Y_1		Y_2		Y_3		Y_4	
Run	MP Removal (%)		COD Removal (%)		TOC Removal (%)		Growth-MP^+ (OD)	
	EV	PV	EV	PV	EV	PV	EV	PV
1	75	75.17	52	52.17	28	27.75	1.2	2.352
2	62	61.84	59	59.34	41	40.75	1.8	1.871
3	55	55.63	48	47.47	24	25.09	1.2	2.277
4	51	50.35	45	45.17	21	20.75	1	2.152
5	70	69.88	55	54.71	32	31.67	1.5	1.451
6	63	63.13	57	57.13	33	33.57	1.4	1.396
7	52	52.06	54	54.21	30	29.67	1.7	1.651
8	62	61.81	55	55.29	32	30.43	1.6	2.759
9	49	49.06	53	53.55	32	32.33	1.3	1.351
10	50	49.88	50	50.05	25	25.33	1.2	1.251
11	65	60.38	48	48.55	28	27.17	1	2.181
12	73	73.13	45	45.13	23	24.43	1.1	2.227
13	95	95.2	81	82	60	61.2	2.2	2.18
14	61	60.53	59	58.83	33	33.75	1.6	2.734
15	60	59.34	54	54.68	31	30.41	1.1	1.121
16	95	95.2	82	82	61	61.2	2.1	2.18
17	65	60.56	59	58.71	35	35.83	1.6	2.679
18	45	44.84	52	51.68	33	33.75	1.4	1.355
19	66	70.38	52	51.55	29	27.83	1.1	2.197
20	65	65.34	53	52.34	26	27.09	1.1	1.105
21	54	54.35	49	48.83	21	21.75	1.1	2.234
22	55	59.56	51	51.71	25	26.17	1	2.163
23	95	95.2	83	82	62	61.2	2.2	2.18
24	96	95.2	83	82	62	61.2	2.2	2.18
25	54	54.81	62	61.63	36	36.57	1.6	1.596
26	48	48.31	54	53.63	32	30.91	1.3	1.28
27	95	95.2	81	82	61	61.2	2.2	2.18
28	59	58.63	55	55.13	30	28.91	1.2	1.18
29	56	56.31	48	47.63	28	26.09	1.1	2.209

Experimental values - EV; Predicted values - PV

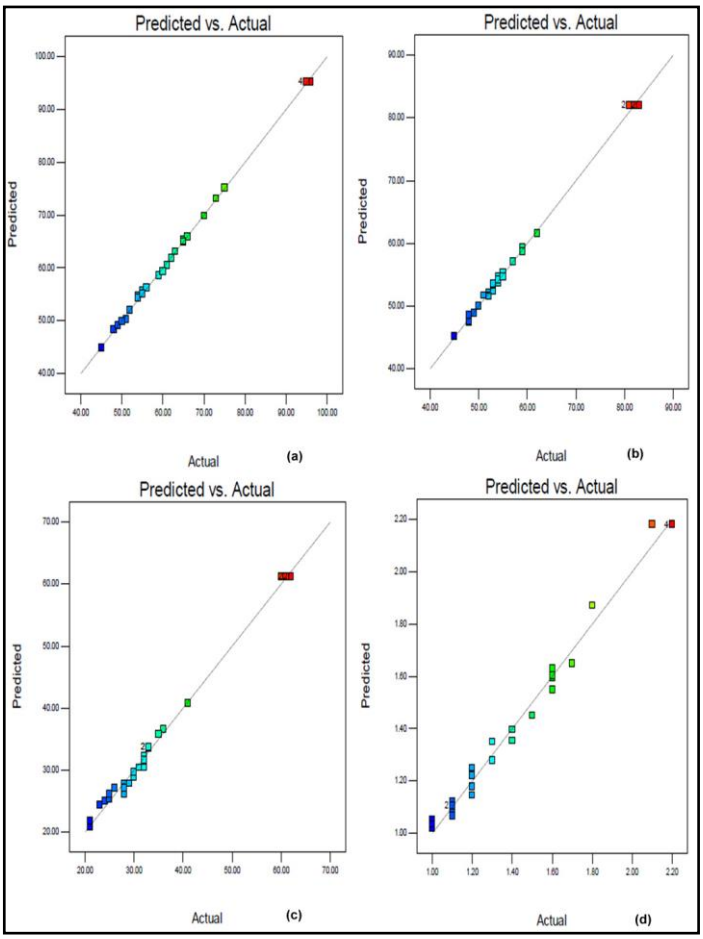

Figure 1 The actual and predicted plot for (a) Methylparathion removal (%), (b) COD removal (%), (c) TOC removal (%) and (d) growth (OD)

Analysis of optimized process variables by response surface plots

The optimum values of the selected variables were obtained by solving their regression equation and analyzing response surface contour plots. Response Surface plots as a function of four factor at a time maintaining all other factors at a fixed level (zero for instance) are more helpful in understanding both the main and interaction effects of the four factors. The plots can be easily obtained by calculating the data from the model. The values were taken by one factor, where the second varies with constant of a given Y-values. The yield values of the different concentrations and range of the variable can also be predicted from respective response surface plots. The coordinates of the central point within the highest optimum concentration of the respective components. Figures 2 to 5 show their response surface obtained as a function of temperature, pH, time and agitation against methylparathion removal, COD removal, TOC removal and growth of bacteria in terms of optical density.

Optimum values and validation of the model

The methylparathion removal by *Pseudomonas aeruginosa* mpd was predominantly influenced by the combined effects of the environmental factors include temperature, pH, incubation period (time) and agitation. The point prediction from the analysis of variables for the response surface model showed the maximum methylparathion removal (95.2 %), COD removal (82 %), TOC removal (61.2 %) and growth (2.18 OD) by *Pseudomonas aeruginosa* (mpd) in synthetic wastewater containing 1000 mgL^{-1} of methylparathion at optimum conditions of pH (7), temperature (32.5 ^{o}C) and agitation at 150 rpm for 96 h of incubation period. As can be seen, there is not much difference between the experimental values and model response values obtained. This confirmed that RSM could be effectively used to predict the removal performance of methylparathion from wastewater by potential bacterial strain (*Pseudomonas aeruginosa* mpd).

The maximum experimental response for methylparathion removal was 95 % whereas the predicted value was 95.2 % indicating a strong agreement between them. The optimum values of the tested variables are at pH 7, 32.5^{o}C temperature and agitation at 150 rpm for 96 h of incubation time as shown in perturbation graph (Figure 6.). The model was also validated by conducting the experiments under the optimized conditions, which resulted in the methylparathion removal of 96 % (Predicted response 95.2 %), thus proving the validity of the model.

The temperature is the most suitable variable for the growth of the isolates as well as the methylparathion (MP) removal which was found to be growth related processes. Temperature is another abiotic factor that influences the rate and extent of bioremediation since it affects microbial activity with rates of metabolic reactions generally increasing with increasing temperature (**Baker, 1994 and Hong *et al.*, 2007**). Shake culture or aerated culture conditions are better for the growth and removal of methylparathion. The rise in temperature of the synthetic wastewater medium may accelerate the chemical reactions, reduces solubility of gases, amplifies taste and odour and elevates metabolic activity of organisms. This in turns reduce the organic loads in terms of COD and TOC in the wastewater. The decrease in COD and TOC may increase the biodegradation of methylparathion. This may be the cause for the increase in the biodegradability of methylparathion from the medium. So, the organic loads in terms of these parameters may increase the removal of methylparathion from aqueous solution. It was noted that the removal of organic load in terms of COD was proportional to the disappearance of cypermethrin (**Jilani and Khan, 2006**). Similar correlations were also observed by **Berchtold *et al.*, (1995).**

The optimum growth of the strain was found to be pH 7. The strain mpd can degrade methylparathion from pH7; this could perhaps be due to increased bioavailability of methylparathion and optimal biotic activity of cells in this pH. The pH from 5 to 8 suggested that dissipation of methylparathion was mediated by the cometabolic activities of the bacteria and also the rate of degradation of methylparathion was low in acidic but increased considerably with an increase in pH. **Brajesh *et al.*, (2004)** also reported similarly that the pH from 4.7 to 8.4 for chlorpyrifos by *Enterobacter* strain (B-14). The optimum conditions were more favorable for the growth of the bacteria. It may be either metabolize or co-metabolize the methylarathion in the medium as a nutrient or energy source for their growth and metabolism. The degradation of methylparathion supported cell growth, indicating that isolated strain could utilize methylparathion as a phosphorus source. The pH condition would be significance while emergent an effective remediation strategy.

The optimum time for the incubation period was enhanced the growth of the bacteria and increase its metabolic activity. The log phase of the bacteria was extended and the secondary metabolites which include the release of the respective enzymes responsible for the hydrolysis of methylparathion degradation, or oxidation and reduction process may occur. This in turns may results in the higher reactivity of the pollutant and increases the degradation process. The optimum agitation observed was more encouraged for the growth of the bacteria by utilizing the nutrients from the uniformly distributed and suspended nutrients in the medium which may helps in oxidation process. It may be either metabolize or co-metabolize the methylarathion in the medium. Shake culture or aerated culture conditions are better for the growth and removal of methylparathion. Methylparathion removal under aerobic conditions suggesting that a constitutively expressed enzyme could be involved in the degradation. Repeated application of pesticides results in the enhanced ability of microbial population to degrade the pesticide. The study also suggests that methylparathion degrading bacterial culture should preferably be used for the management of methylparathion containing wastewater.

Table 4 ANOVA table for Y_1 (methylparathion removal in %), Y_2 (COD removal in %), Y_3 (TOC removal in %) and Y_4 (growth in optical density) responses

Source	DF	Y_1				Y_2				Y_3				Y_4			
		SS	MS	F	P	SS	MS	F	P	SS	MS	F	P	SS	MS	F	P
Model	14	6903	493	1857	< 0.0001	3961	282	522	< 0.0001	4683	334	202	< 0.0001	4.55	0.32	117	< 0.0001
A-X_1- Temp (^oC)	1	261	261	984	< 0.0001	6.7	6.7	12.4	0.0033	18.7	18.7	11.3	0.0046	0.06	0.06	24.3	0.0002
B-X_2- pH	1	90.7	90.7	341	< 0.0001	75	75	138	< 0.0001	80	80	48.4	< 0.0001	0.21	0.21	76.9	< 0.0001
C-X_3- Time (h)	1	396	396	1494	< 0.0001	21.3	21.3	39.3	< 0.0001	10	10	6.1	0.0270	0.18	0.18	67.5	< 0.0001
D- X_4- Agitation (rpm)	1	85.3	85.3	321	< 0.0001	80	80	147	< 0.0001	36.7	36.7	22.2	0.0003	0.16	0.16	58.8	< 0.0001
X_1 X_2	1	1	1	3.76	0.0727	9	9	16.6	0.0011	0.25	0.25	0.15	0.7032	0.00	0.00	0.90	0.3585
X_1 X_3	1	72.2	72.2	272	< 0.0001	4	4	7.38	0.0167	20.2	20.2	12.2	0.0035	0.00	0.00	0.90	0.3585
X_1 X_4	1	240	240	904	< 0.0001	72.2	72.2	133	< 0.0001	90.2	90.2	54.6	< 0.0001	0.12	0.12	44.1	< 0.0001
X_2 X_3	1	81	81	305	< 0.0001	4	4	7.38	0.0167	72.2	72.2	43.7	< 0.0001	0.25	0.25	90.1	< 0.0001
X_2 X_4	1	20.2	20.2	76.2	< 0.0001	4	4	7.38	0.0167	20.2	20.2	12.2	0.0035	0.06	0.06	22.5	0.0003
X_3 X_4	1	36	36	135	< 0.0001	25	25	46.1	< 0.0001	6.25	6.25	3.7	0.0722	0.09	0.09	32.4	< 0.0001
X_1^2	1	2808	2808	10579	< 0.0001	1138	1138	2102	< 0.0001	1655	1655	1001	< 0.0001	0.92	0.92	333	< 0.0001
X_2^2	1	2144	2144	8078	< 0.0001	914	914	1688	< 0.0001	1050	1050	635	< 0.0001	1.25	1.25	452	< 0.0001
X_3^2	1	2387	2387	8991	< 0.0001	1583	1583	2923	< 0.0001	1553	1553	940	< 0.0001	0.92	0.92	333	< 0.0001
X_4^2	1	1327	1327	5002	< 0.0001	1986	1986	3667	< 0.0001	2397	2397	1450	< 0.0001	2.07	2.07	746	< 0.0001
Residual	14	3.71	0.26			7.58	0.54			23.1	1.67			0.03	0.00		
Lack of Fit	10	2.91	0.29	1.458	0.3821	3.58	0.36	0.36	0.9143	20.3	2.0	2.9	0.157	0.03	0.00	1.54	0.3594
Pure Error	4	0.8	0.2			4	1			2.8	0.7			0.00	0.00		
Cor Total	28	6906				3968				4706				4.59			

Table 5 Analysis of variance (A NOVA) results of the model of methylparathion removal, COD and TOC removal by *Pseudomonas aeruginosa* mpd

Source	Sum of squares	Degree of freedom	Mean Square	F-value	Prob > F	Remarks
[a]**MP-Removal (%)**						
Model	6903	14	493.07	1857.3	<0.0001	**Significant**
Residual	3.716	14	0.2654			
Lack of fit	2.916	10	0.2916	1.4583	0.3821	Not- Significant
Pure error	0.8	4	0.2			
Cor Total	6906	28				
[b]**COD Removal (%)**						
Model	3961	14	282.94	522.3	<0.0001	**Significant**
Residual	7.58	14	0.54			
Lack of fit	3.58	10	0.36	0.36	0.9143	Not- Significant
Pure error	4.00	4	1.00			
Cor Total	3968	28				
[c]**TOC Removal (%)**						
Model	4683	14	334.55	202.47	<0.0001	**Significant**
Residual	23.13	14	1.6523			
Lack of fit	20.33	10	2.0333	2.9047	0.1579	Not- Significant
Pure error	2.8	4	0.7			
Cor Total	4706	28				
[d]**G-MP$^+$ (OD)**						
Model	4.553	14	0.3252	117.25	<0.0001	**Significant**
Residual	0.038	14	0.0027			
Lack of fit	0.030	10	0.0030	1.5416	0.3594	Not- Significant
Pure error	0.008	4	0.002			
Cor Total	4.592	28				

R-squared	Adj R-squared	Pred R-squared	Adequate precision
[a]R^2 = 0.9994;	R^2_{adj}= 0.9989;	R^2_{pred}= 0.9973	Adeq precision = 135.92
[b]R^2 = 0.9981;	R^2_{adj} = 0.9962;	R^2_{pred}= 0.9932	Adeq precision = 69.66
[c]R^2 = 0.9950;	R^2_{adj} = 0.9901;	R^2_{pred}= 0.9741	Adeq precision = 43.75
[d]R^2 = 0.9915;	R^2_{adj} = 0.9830;	R^2_{pred}= 0.9586	Adeq precision = 30.60

MP- *Methylparathion,* ***COD***- *Chemical oxygen demand,* ***TOC***- *Total organic carbon,*
G-MP$^+$ - *Growth in presence of methylparathion,* ***OD***- *Optical density*

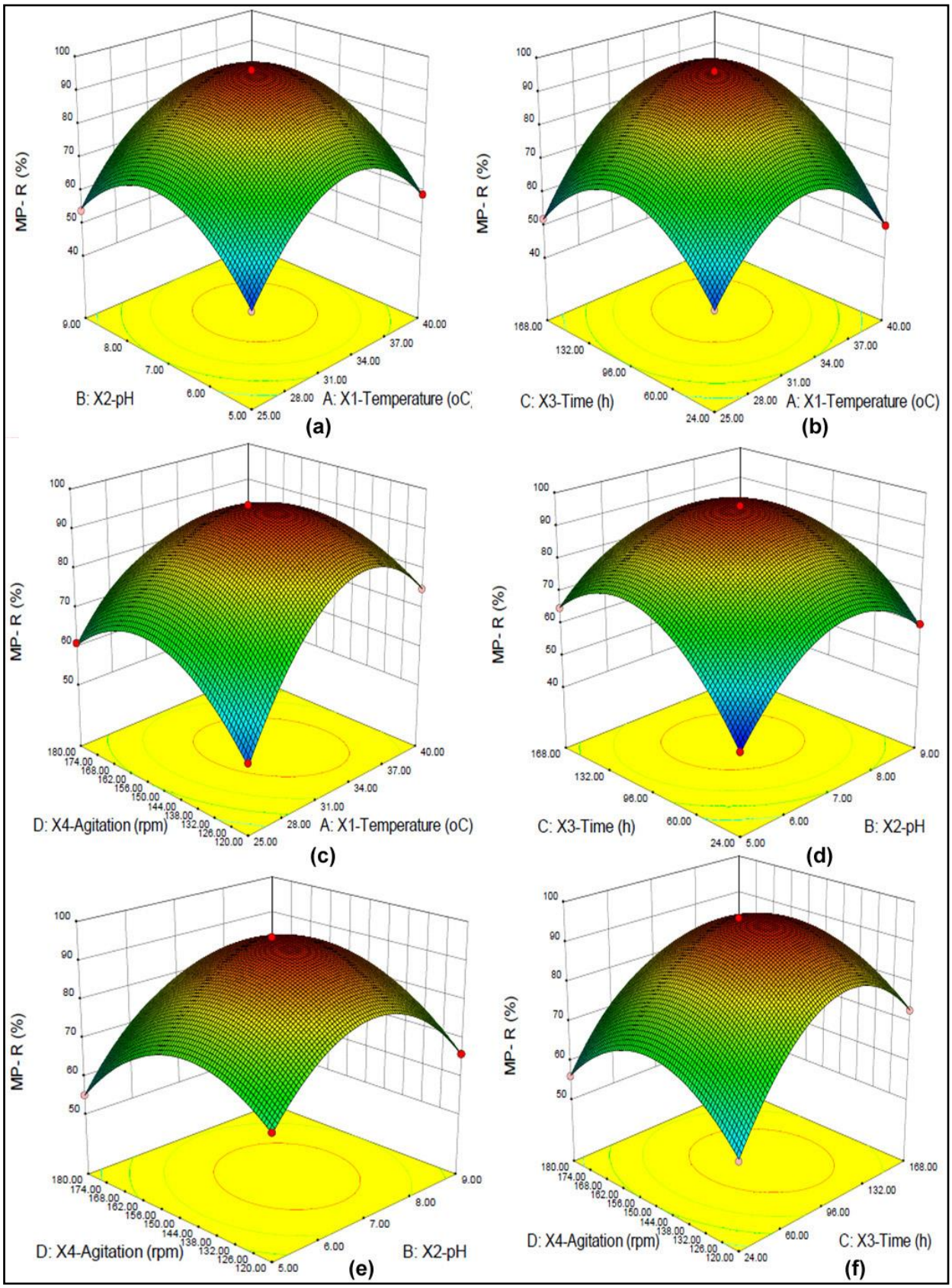

Figure 2a Response surface plot of the combined effects of pH, temperature, time and agitation on the percentage removal of Methylparathion by Pseudomonas aeruginosa mpd

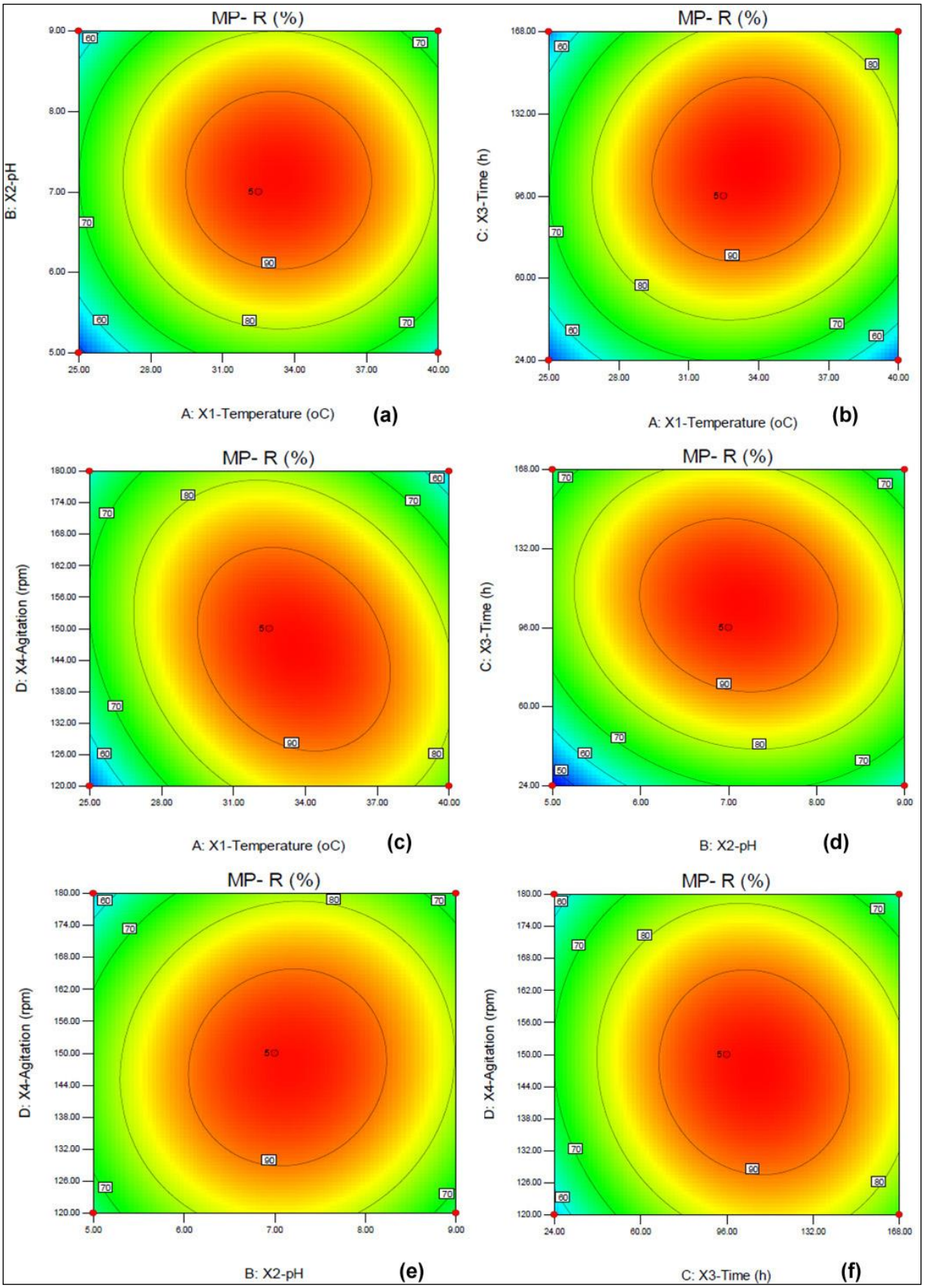

Figure 2b Contour surface plot of the combined effects of pH, temperature, time and agitation on the percentage removal of Methylparathion by *Pseudomonas aeruginosa* mpd

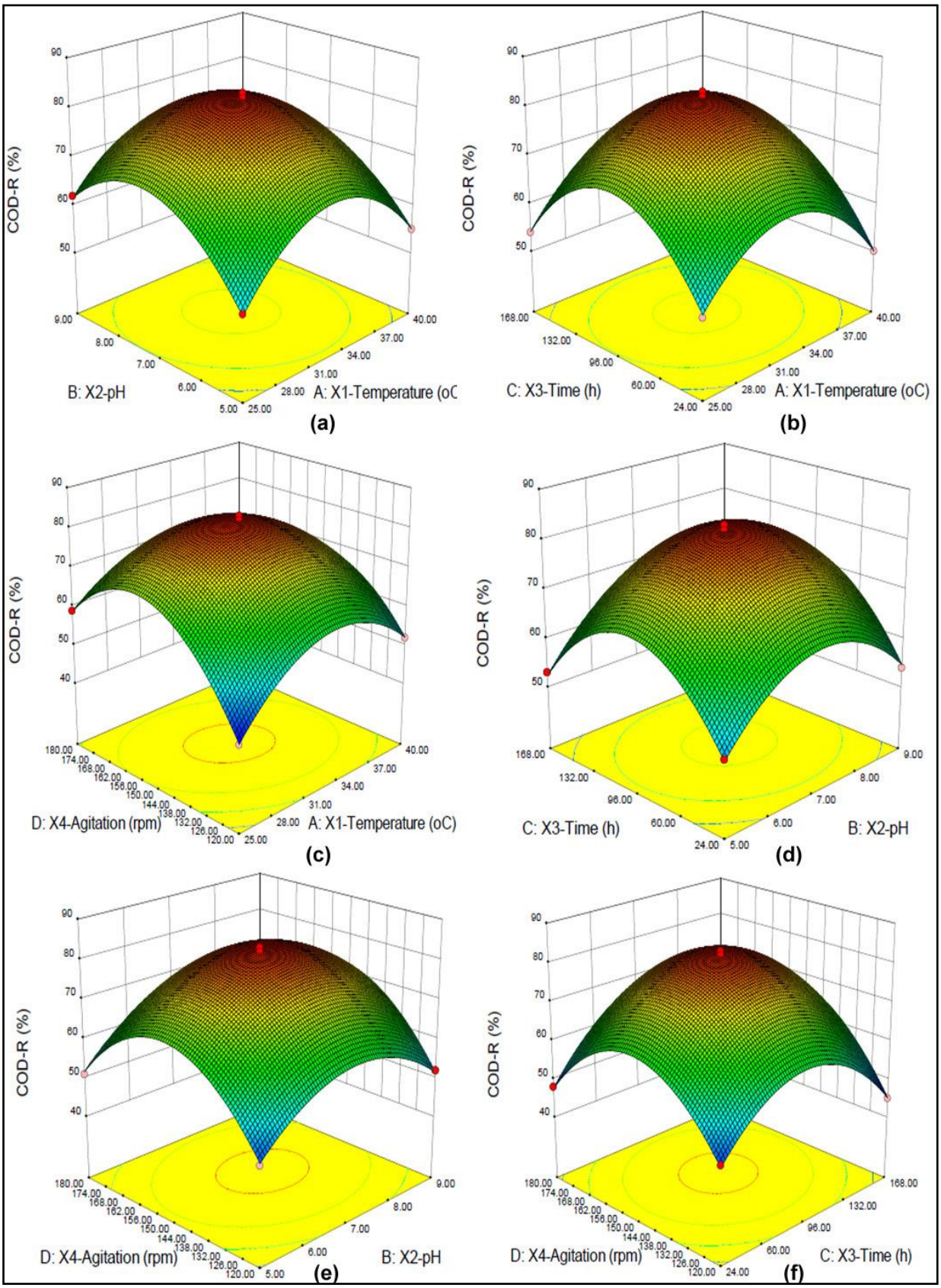

Figure 3 Response surface plot of the combined effects of pH, temperature, time and agitation on the percentage removal of COD by *Pseudomonas aeruginosa*mpd

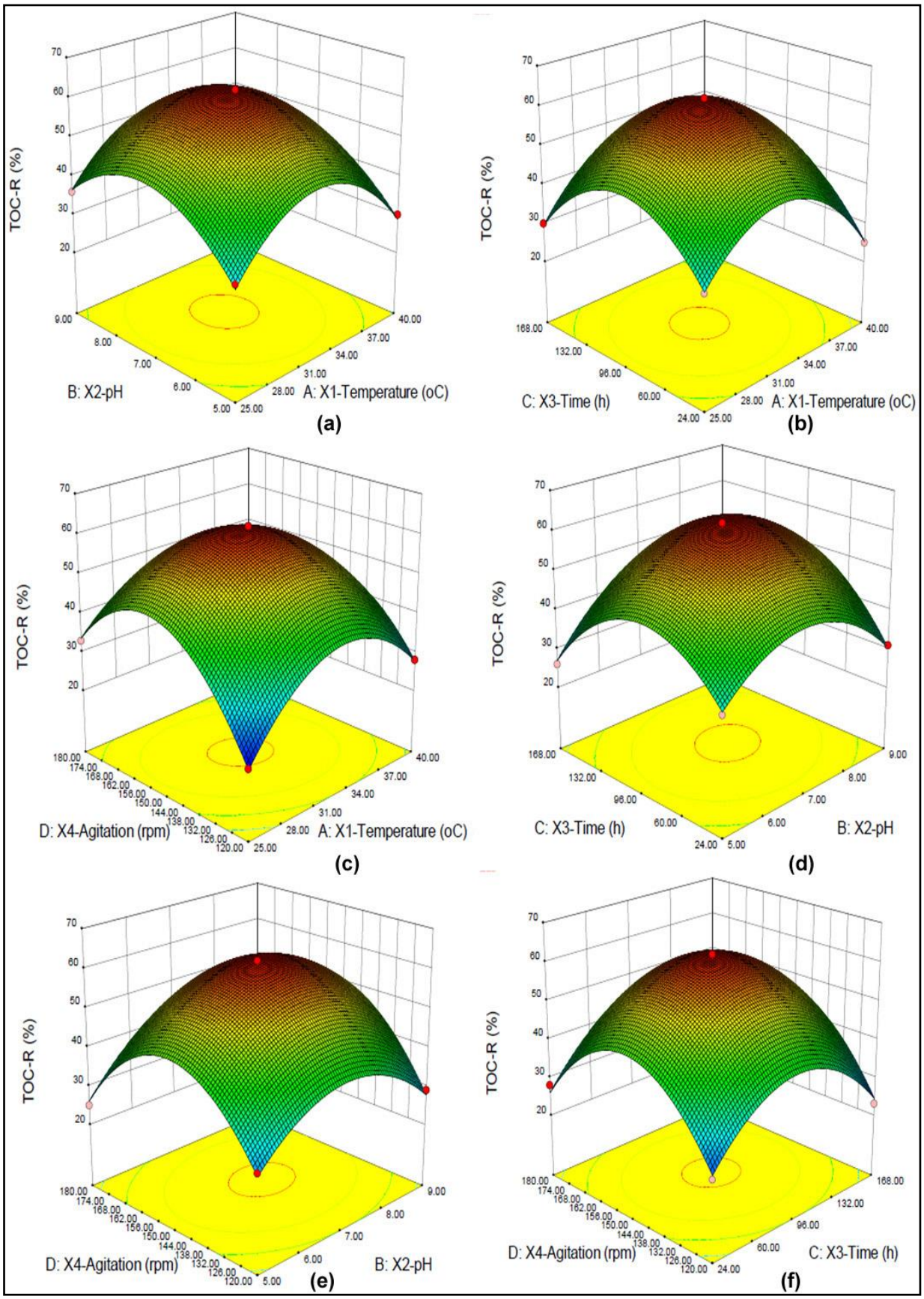

Figure 4 Response surface plot of the combined effects of pH, temperature, time and agitation on the percentage removal of TOC by *Pseudomonas aeruginosa* mpd

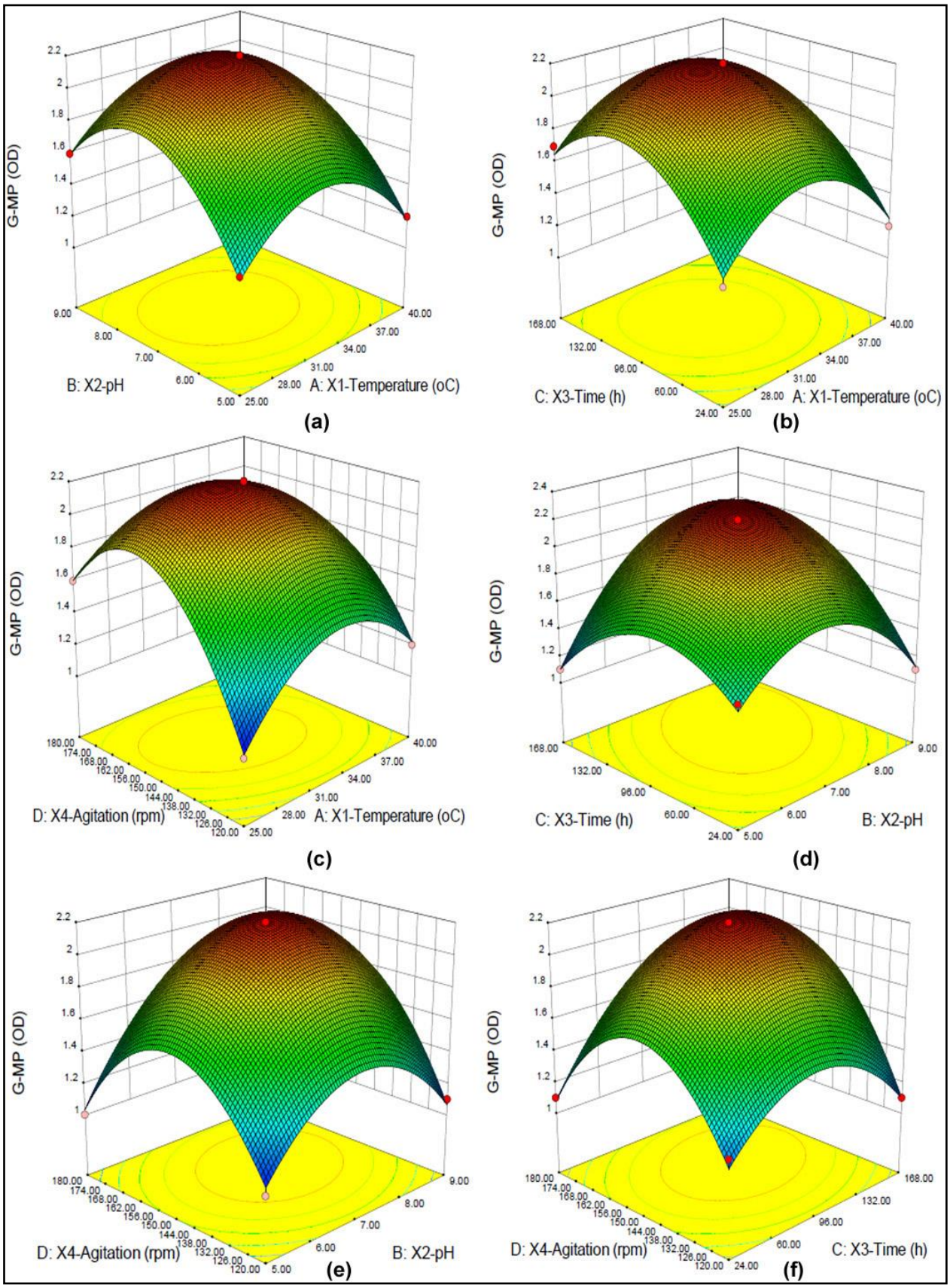

Figure 5 Response surface plot of the combined effects of pH, temperature, time and agitation on the growth of *Pseudomonas aeruginosa* mpd

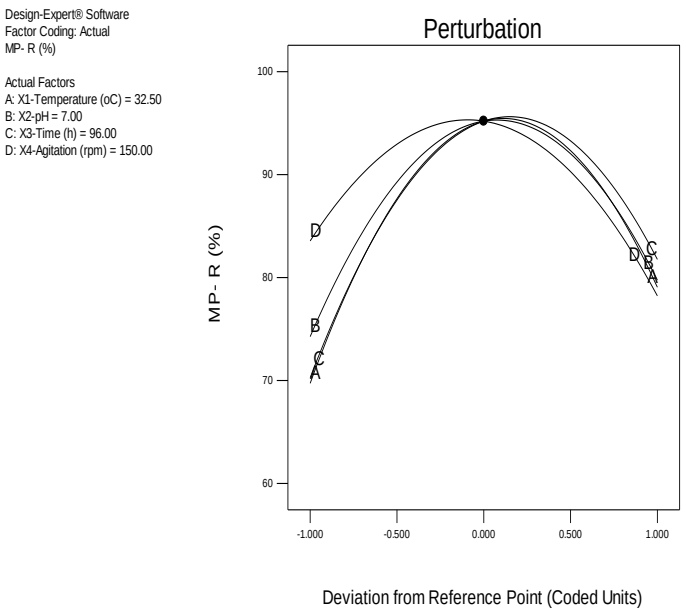

Figure 6 Perturbation graph showing the optimum values of the tested variables

However, in the presence of other carbon sources (such as glucose), initially it delayed to degrade methylparathion but with the passage of time it degraded to 95 % within 96 h, indicating that when glucose was depleted, it started to utilize methylparathion as a source of carbon. Similar results were reported by **Brajesh *et al.*, (2004)**. Glucose was chosen because it is a primarily substratum and the main carbon source for the bacteria. Glucose addition is important to improve the efficacy of bioremediation of persistent compounds like pesticides (**Sampaio, 2005; Singh, 2006; Yang *et al.*, 2009; Yugui et al., 2008**). **Qiu *et al.*, (2006)** reported that the additional nutrients such as glucose and organic nitrogen greatly enhanced the growth of *Ochrobactrum* sp B2. Singh (2006), reports that the addition of glucose produces substances of high reactivity, which react more easily with the pollutant. Previous reports concerning isolation of organophosphorus degrading microorganisms suggest that the bacteria mainly degrade the compounds cometabolically (**Horne *et al.*, 2002; Zhongli *et al.*, 2001**). Some reports showed that the isolated bacterium can utilize organophosphates as a source of carbon or phosphorus (**Subhas and Dileep, 2003**) from the hydrolysis products (**Serdar and Gibson, 1985**). In natural environments, the competition for carbon sources is immense and the utilization of pesticide as an energy source by this bacterium provides it with a substantial competitive advantage over other microorganisms (**Malghani *et al.*, 2009**).

UV-Vis Spectral Analysis

In order to investigate the formation and eventual disappearance of intermediate compounds in the reaction mixture, the biotreated synthetic wastewater containing methylparathion was monitored using UV-Vis spectroscopy as a function of time. The UV-Vis spectroscopy scanning profile shows a peak formation with lambda max (λmax) at 277 nm as shown in Figure 7. The extended biotreatment after 96 h shows the same band decrease its intensity and eventually disappeared. The absorbance value was found to be reduced at maximum time of 96 h at optimized process variables. The wavelength at 277nm shows a displacement to higher wavelengths and formation of band at 400 nm that can be attributed to the p-nitrophenol absorption bands. **Zhongli *et al* (2001)** reported that the maximum absorption peak of methylparathion was recorded at 273nm by *Plesiomonas* strain (M6). **Wu and Linden (2008)** reported that the parathion produces a maximum absorbance (λmax) at 275nm. Further, the biotreated samples were analysed by HPLC for the confirmation of the residual MP and intermediates formation.

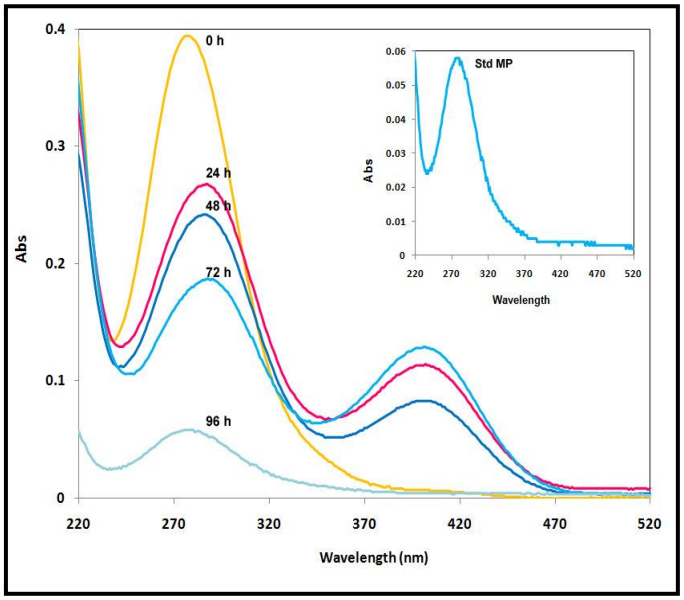

Figure 7 UV –Vis NIR Spectroscopic scanning profile of MP degradation by *Pseudomonas aeruginosa* mpd at different treatment time (h)

HPLC Analysis

The biotreated samples were analysed by HPLC for the confirmation of the residual methylparathion and their byproducts or intermediatediates formation. The retention time for methylparathion was found to be 3.3min which was confirmed by the spectra as shown in Figure 8. The percentage degradation of methylparathion by *Pseudomonas aeruginosa* mpd was found to be 95 %. Treated samples showed that the peak reduction at 3.3 retention time (RT), hence it proves the degradation of methylparathion by biotreatment (*Pseudomonas aeruginosa* mpd) process. The peak at retention time of 4.0, 4.4 and 10 min in treated sample were observed as the intermediate products of methylparathion degradation during the biotreatment process. Moreover, methylparathion was rapidly oxidized into other organic compounds.

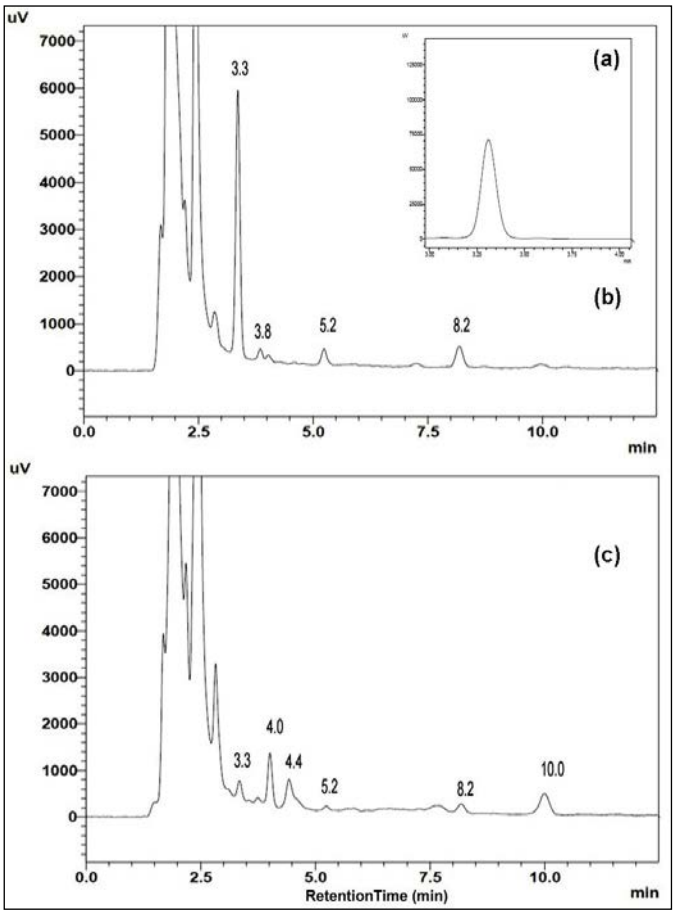

Figure 8 HPLC profiles of methylparathion biodegradation by *Pseudomonas aeruginosa* mpd after 72h (a) Standard, (b) Control and (b) Treated after 72 h

CONCLUSION

RSM was used in this study to establish the optimum variables initial pH, time, culture temperature and agitation for methylparathion biodegradation. It was concluded that the optimal conditions for methylparathion removal are pH 7 and 32.5 ^{o}C temperature and agitation at 150 rpm for 96 h of incubation period. The predicted extent of methylparathion biodegradation by this strain of *Pseudomonas aeruginosa* under these optimum conditions was 95.2 %, and the experimental results were in close agreement with this prediction. The point prediction from the analysis of variable for response surface model for methylparathion removal (95.2 %), COD removal (82 %), (c) TOC removal (61.2 %) and (d) growth (2.18 OD) by *Pseudomonas aeruginosa* (mpd) from waste water with 1000 mg/L of medium at optimum conditions of pH, temperature and agitation for 96 h of incubation period. The predicted optimal and experimental measured methylparathion removal efficiencies agreed well with high coefficients of determination (R^2 = 0.9994, R^2_{adj} = 0.9989), and the COD removal (R^2 = 0.9981 R^2_{adj} = 0.9962) and TOC removal (R^2 = 0.9950, R^2_{adj} = 0.9901) are also agreed well. Moreover the growth of the strain in terms of its OD were also agreed well (R^2 = 0.9915, R^2_{adj} = 0.9830). Hence this study was an attempt for methylparathion removal using *Pseudomonas aeruginosa* strain with RSM model, has helped to recognize the important operating variables and optimum levels with least effort and time. The isolate of the present study was found to have potential in methylparathion removal at optimized condition and suggested for biotreatment of methylparathion wastewater. This study will form the basis for the further utilization of the bacterial strain, grown on suitable substrates, in biofiltration systems for the treatment of wastewaters.

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THERMOPHILIC *BACILLUS* LICHENIFORMIS RBS 5 ISOLATED FROM HOT TUNISIAN SPRING CO-PRODUCING ALKALINE AND THERMOSTABLE α-AMYLASE AND PROTEASE ENZYMES

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ABSTRACT

Bacillus licheniformis RBS 5 was isolated from thermal spring in Tunisia. The isolate coproduce α-amylase and protease enzymes. The α-amylase activity showed an optimal activity at approximately 65°C and in wide pH interval ranging from 4 to 9. This enzyme was stable over the range of 45 to 70°C after 30 min of incubation and in the pH range of 8 to 10. Protease activity was optimal; at 80°C, pH 12. This enzyme was stable until 60°C over the pH range of 10 to 12. EDTA at concentration of 5 mM reduces slightly both activities evoking the serine alkaline protease. Cationic ions (Ca^{2+}, Cu^{2+}, Zn^{2+}, and Mg^{2+}) have an inhibition effect on α-amylase. However, protease activity was enhanced by Ca^{2+}, Cu^{2+} and Mg^{2+}); the other cations reduce slightly the proteolytic activity. SDS and H_2O_2 were found as inhibitors for both activities whereas Triton X-100 and perfume have no effect. Taken together, these traits make protease activity of *B. licheniformis* RBS 5 as efficient for use in detergent industry.

Keywords: Thermophilic *Bacillus licheniformis*, α-amylase, protease, detergent additive

INTRODUCTION

α-Amylases (EC 3.2.1.1) or α (1-4) D-glucan-4-glucanhydrolase are endohydrolases, that specifically cleave the glycosidic bonds in starch to produce glucose, maltose, or dextrins, and glucoamylase (EC 3.2.1.3). They cut α (1-4) and α (1-6)-glycosidic linkages, releasing glucose from the non-reducing ends of starch, they are widely used in the industrial conversion of starch into sugars **(Xiao *et al*., 2006)**. Amylases have a significant commercial importance and they occupy ca. 25% of the world enzyme market **(Burhan *et al*., 2008)**. These enzymes have numerous applications in various industrial processes including the nutritional, textiles **(Gupta *et al*., 2003)** and starch industry **(Prakash *et al*., 2009)**. Thermostable α-amylases have been isolated from diversified sources including plants, animals, and microbes, where they play a dominant role in carbohydrate metabolism. However, microbial sources, namely fungal and bacterial, are used for the industrial production due to advantages such as cost effectiveness, consistency, less time and space required for production, and ease of process modification and optimization. Proteases play a crucial role in physiology and pathology **(Qiao *et al*., 1997; Hedstrom, 2002)**. Microbial proteases account for ca. 59% of the total worldwide enzyme sales **(Wei, 2007),** they play an important role in biotechnological processes **(Sellami *et al*., 2008)**. Proteolytic enzymes can be classified as acidic, neutral and alkaline proteases with regard to their pH working range. Neutral and alkaline proteases hold great potential for application in the detergent- **(Rao *et al*., 1998; Banerjee *et al*., 1999; Anwar and Saleemudin, 2000; Gupta *et al*., 2003)**, pharmaceutical- and medical fields **(Kudrya and Simonenko, 1994)**, and in the food- **(Anwar and Saleemudin, 1998),** tannery **(Wellingta and Weire, 2003)** and leather industries **(Anwar and Saleemudin, 1998)**.
Thermophilic bacilli that grow optimally over the temperature range of 45 to 70 °C have been isolated from both thermophilic and mesophilic environments. The interest for these bacteria arises from their biotechnological importance as sources of thermostable enzymes (proteases, amylases, pullanases, glucose-isomerases, lipases, xylana-ases and DNA restriction endonucleases) and products for industrial use **(Burhan, 2008**; **Adiguzel *et al*., 2009).**
Research on thermo- and extremophiles, as promising sources for highly stable enzymes, has remained an active research subject **(Adiguzel *et al*., 2009)**. Thermophiles represent an obvious source of thermostable enzymes, it being reasonable to assume that such character will confer on their proteins a high thermal stability **(Szilagyi and Zavodszky, 2000)**. Enzymes isolated from these microorganisms are not only thermostable and active at high temperature, but are also often resistant to and active in the presence of organic solvents and detergents **(Saeki *et al*., 2007)**.
During the screening for thermophilic bacteria with extracellular hydrolyses from thermal Tunisian springs, a thermophilic *Bacillus lichenifomis* isolate (strain RBS 5) that displays high thermostable protease and α-amylase was isolated and identified. In addition characterization of these protease and α-amylase were performed.

MATERIALS AND METHODS

Isolation and identification of the strain

RBS5 strain was isolated from thermal springs in Korbos, Tunisia, using specific protocol as reported previously (**Adiguzel *et al.*, 2009)**. This isolate produce clear zones on starch and/or milk nutrient agar plates. The isolate is rod-shaped and is able to grow from 55 °C to ca. 80 °C. The biochemical tests were carried out using the API 50CHB system **(Edward *et al*., 2003)**.
The DNA from bacterial cultures was extracted using a Wizard Genomic DNA Purification Kit. The amplification of the 16S ribosomal ribonucleic acid (rRNA) gene was performed using universal primers Fd1 and Rd1 (Fd1, 5′-AGAGTTTGATCCTGGCTCAG-3′ and Rd1, 5′-AAGGAGGTGATCCAGCC-3′). PCR product of ~1.5 kb corresponding to base positions 8-1542 based on *Escherichia coli* numbering of the 16S rRNA gene **(Winker and Woese, 1991)**. The sample was placed in a hybrid thermal reactor thermocycler (BIOMetra, Leusden, The Netherlands), denatured for 1 minute at 95 °C and subjected to 30 cycles for 20 seconds at 95 °C, 30 seconds at 55 °C, and 1 minute and 30 seconds at 72 °C. This was followed by a final elongation step for 5 minutes at 72 °C. The PCR product was analyzed on 1.5% (w/v) agarose gel and sent to GATC (Germany) for sequencing. The nucleotide sequence of the 16S rRNA gene was determined using the dideoxy chain-termination method**.** The homology search was performed using the BLAST algorithm.

Culture conditions

For the screening of amylolytic activity, RBS 5 was grown on starch agar plate containing 1 g soluble starch, 0.4 g yeast extract, 0.1 g KH_2PO_4, 0.15 g Mg SO_4.7 H_2O and 15 g Agar in 1 L of distilled water at 70°C for 48 h **(Saxena *et al*., 2007)**. The bacterial isolate was inoculated on skim milk agar plates prepared with 250 ml skim milk, 3 g yeast extract, 5 g peptone, 20 g agar in 1 L of distilled water adjusted to pH 8.0 and incubated at 55 °C for 24 h (**Johnvesly and Naik, 2001)**. A clear zone of hydrolysis revealed the presence of proteolytic activities depending on the clearance and diameter zone of substrate hydrolysis.

Detection of enzyme activity by zymography

RBS 5 enzyme extract was subjected to electrophoresis (Mini Protein II Electrophoresis Cell, Bio-Rad) on 30% acrylamide gel under denaturing conditions. The SDS-PAGE procedure was completed with a 12.5% gel, following the method of **Laemmli (1970).** The gel was washed with Triton X-100 (2.5%) for one hour to remove the SDS. Electrophoresis was performed at 100 mA for 1 hour at 4 °C in 1.5 M Tris-HCl buffer at pH 8.8 **(Hmidet *et al*., 2009)**. After migration, the gel was soaked in a 1.0 M Tris-HCl buffer at pH 6.8. α-amylase and protease activities were revealed by the iodometric technique using Lugol solution to detect α-amylase and a 1% of casein solution to detect protease enzymes with the specific enzyme substrate method. Detection of protease activity was performed on polyacrylamide gel by staining for 1 H in Coomassie blue (G-250).

Enzyme assay α-amylase activity

The culture was grown at 70 °C for 72 h in a shaking incubator (150 rpm). The pH of the medium is 7.0. The broth was centrifuged at 10,000 rpm at 4 °C for 20 min and the clear supernatant was used as crude enzyme **(Saxena *et al*., 2007).** α-amylase activity was detected phenotypically: starch forms a deep blue complex with iodine **(Gupta *et al*., 2003),** and with the progressive hydrolysis of the starch, the color changes to red brown. The α-amylase activity was determinate by incubating of 0.5 mL of each enzymatic aliquot with 1% of soluble starch dissolved in 0.1M phosphate buffer (pH 6.5) at 70 °C for 15 min **(Konsoula and Liakopoulou, 2007)**. The reaction was stopped by adding 1ml of 0.1 N of HCl and the final volume was adjusted to 40 mL with distilled water **(Konsoula and Liakopoulou, 2007)**. The optical density of the precipitate was determined at 620 nm. All the experiments were performed in triplicates.

Protease enzyme assay

The Protease was produced in 1% glucose (w/v), 0.5% casein (w/v) and 0.1% $MgSO_4$.7 H_2O (w/v). The culture was grown for 72 hours at the optimum temperature for bacterial growth (**Johnvesly and Naik, 2001)**. Protease activity in the cell-free supernatant was measured; alkaline protease activity was determined by using 1% casein as a substrate in 25 mM Glycine-NaOH buffer (pH 11). The assay was carried out routinely in a mixture containing 0.5 mL of a suitably diluted enzyme with 0.95 ml of Glycine-NaOH solution and 1 ml casein solution. After 20 min incubation in the optimum temperature for bacterial growth, the reaction was terminated by the addition of 2 ml of 10% TCA (trichloroacetic acid) solution. The mixture was slightly agitated and was centrifuged at 8000 rpm for 30 min. The optical density of the precipitate was determined at 280 nm (**Johnvesly and Naik, 2001)**.

Effect of pH on the activity and stability

The optimum pH and stability of the enzymatic preparation was monitored over a pH range of 4.0 to 13.0 in different buffers and the residual enzymatic activities were determined under standard assay conditions. The following buffers were used: 100 mM glycine-HCl, pH 2.0-4.0; 100 mM Sodium acetate buffer, pH 4.0-6.0; 100 mM Tris-HCl buffer, pH 7.0-8.0; 100 mM glycine-NaOH buffer, pH 9.0-10.0; 100 mM Na_2HPO_4-NaOH buffer pH 11.0; 100 mM KCl-NaOH, pH 12-13 **(Hmidet *et al*., 2009).**

Effect of temperature on the activity and stability

To investigate the effect of temperature, enzyme activity was tested at different temperatures between 40 to 100 °C at pH 12.0 for protease activity and at pH 10.0 for α-amylase activity. Thermal stability was examined by incubating the enzyme preparation at temperature of 30 min **(Hmidet *et al*., 2009)**. The remaining enzyme activities were measured under standard assay conditions. Non-heated crude enzyme was used as a control (100%).

Effects of inhibitors and metal ions on enzymes activities

The effects of enzyme inhibitors on α-amylase and protease activities were studied using Ethylenediaminetetra acetic acid (EDTA), and β-mercaptoethanol. The RBS 5 crude enzyme was pre-incubated with inhibitors for 30 min. The remaining α-amylase and protease activities were determined under standard assay conditions. The activity of the crude enzyme assayed in the absence of inhibitors was taken as 100% **(Hmidet *et al*., 2009)**. Effects of various metal ions (at 2 and 5 mM concentrations) on α-amylase and protease activities were investigated by adding divalent metal ions (Zn^{2+}, Ba^{2+}, Ca^{2+}, Hg^{2+}, Cu^{2+} et Mg^{2+}) to the reaction mixture. The activity of the crude enzyme without metallic ions was taken as 100%.

Effect of surfactants and detergents on enzyme stability

The suitability of RBS 5 crude enzyme for use as a detergent additive was tested by studying its stability in the presence of several surfactants such as SDS (sodium dodecyl sulphate), Triton X-100, and oxidizing agents such as hydrogen peroxide (H_2O_2) and perfume. The Crude enzyme was incubated with different additives for 1 hour at 40 °C **(Hmidet *et al*., 2009)**, then the residual enzyme activities were determined under standard assay conditions.

Stability of proteolytic crude extract of RBS 5 with commercial detergents powder

The suitability of RBS 5 crude extract to use in commercial laundry detergents was also studied. The solid detergents tested were Dixan (Henkel, Spain), Nadhif (Henkel, Tunisia) and Ariel (Switzerland). Solid detergents were diluted in tap water to give a final concentration of 7 mg/ml. The endogenous enzymes contained in these detergents were inactivated by heating the diluted detergents for 30 min at 80 °C. The crude enzyme were added to solid detergents diluted in tap water and incubated in various detergent solutions for 1 hour at different temperatures; the residual enzyme activities were determined under standard assay conditions. The stability towards commercial detergents was described with two commercial detergent enzymes under the same conditions as with the RBS 5 enzymes. The enzyme activity of the control sample (without any detergent), incubated under the same conditions, was taken as 100% **(Hmidet *et al*., 2009)**.

RESULTS

Identification of RBS 5 Strain

RBS 5 strain was isolated from thermal springs in Korbos, Tunisia. The identification of the isolate was based on both catabolic and molecular methods. Morphological and physiological characteristics showed that RBS 5 belong to the *Bacillus licheniformis*. It was a spore-forming and Gram-positive bacterium, it was rod-shaped, and it was able to grow from 50 °C to 80 °C.

The amplification of the 16S ribosomal ribonucleic acid (rRNA) gene using universal primers Fd1 and Rd1 (Fd1, 5′-AGAGTTTGATCCTGGCTCAG-3′ and Rd1, 5′-AAGGAGGTGATCCAGCC-3′) designed from conserved regions within the rRNA operon of E. coli was performed. The sequence showed a similarity of 99% with the 16S rRNA gene of *Bacillus licheniformis* strain NBSL59 (accession number JN624930). Based on the obtained data, RBS5 seemed to be closely related to the *B. licheniformis* species. Therefore, we proposed the alignment of this organism as the *B. licheniformis* RBS5 strain.

Amylolytic and proteolytic activities produced by RBS 5

Enzymatic activities produced by RBS5 were detected on polyacrylamide gels. α-amylase activity appeared on SDS-PAGE gels as unique clear band, indicating that the isolate harbored only one α-amylase enzyme (Figure 1). Similarly, protease activity was detected also as a single band (Figure 1).

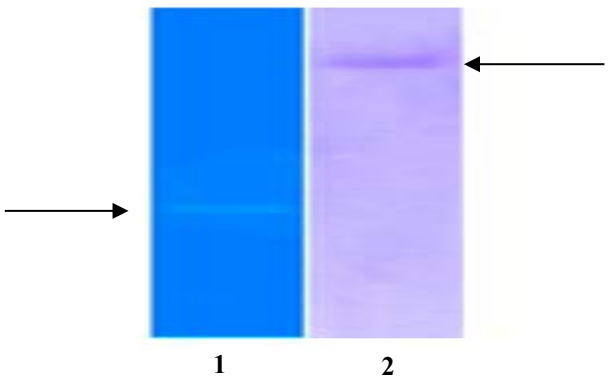

Figure 1 Zymogram showing proteolytic (1) and amylolytic (2) activities of *B. licheniformis* RBS5

Effect of pH on α-amylase and protease activities and stability

The effect of pH on proteolytic and amylolytic activities of RBS 5 crude enzyme was determined over a pH range from 4.0 to 13.0. The general activity of α-amylase was at the pH range from 4 to 9 (Figure 2). Concerning α-amylase stability, the activity was highly stable in the pH range 8.0 to 10.0 retaining more than 95% of its initial activity (data not shown) and decreased till 30% at pH 11. The RBS 5 crude extract protease was active over a broad pH range of 3.0 to 13.0 with optimal activity at pH 11.0 (Figure 2). The crude enzyme retained 100% of its proteolytic activity between pH 10.0 and 12.0, and ca. 50% of its activity at pH 13.0 (data not shown).

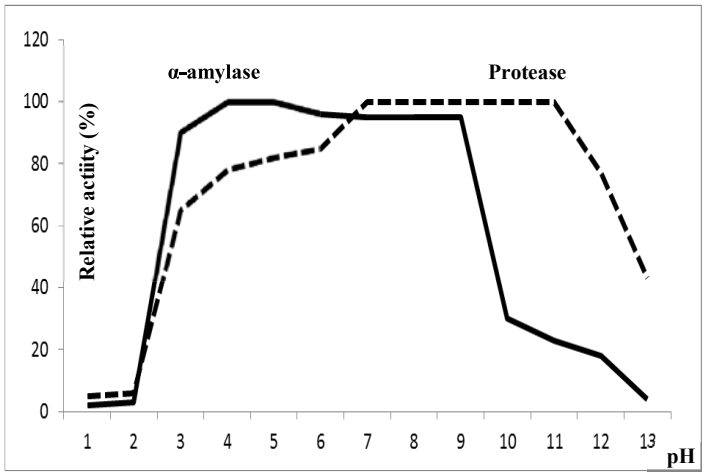

Figure 2 Effect of pH on enzyme activities of *B. licheniformis* RBS 5.

Effect of Temperature on the activity and stability of α-amylase and protease

α-amylase activity was essayed at different temperature and was active between 45 °C to 80 °C with an optimum at ca. 65 °C. For the proteases enzyme, activity increased with temperature within the range of 60-95 °C, and had optimum activity at 80 °C (Figure 4A and 4B).
The Effect of temperature on enzyme stability was studied. α- amylase retained its full activity at temperatures between 45 and 70 °C after incubation for 30 min. However, for higher temperatures, the α-amylase activity showed clear decrement and retained only 40 % of its initial activity at 80 °C. For the protease activity, the initial full activity was conserved until 60 °C during 30 min of incubation, moreover, this activity shutdown to 10% at 95 °C.

Effects of various enzyme inhibitors and metal ions on α-amylase and protease activities

The effects of various enzyme, inhibitors, were studied and are reported in Table 1**.** The amylolytic activity was slightly inhibited with 2 mM of EDTA, however, with 5 mM EDTA the retained activity was 10%. The effect of EDTA on protease activity was concentration dependent, being without effect by using 2 mM and caused 40 % - activity loss by 5 mM of EDTA. The β-mercaptoethanol showed a slightly inhibition of activity for both protease and amylase activities being 87% and 92, respectively. The effects of various cations at a concentration of 5mM on protease and α-amylase activities were studied at optimal activity conditions (Tab 2). The α-amylase activity was clearly inhibited by Ca^{2+}, Zn^{2+}, Mg^{2+}, Hg^{2+}, and Ba^{2+}, and the retained activity varried between 11% and 27%. The Cu^{2+} also exhibited inhibition effect, less than the before mentioned cations. Protease activity increased in the presence of Ca^{2+}, Cu^{2+}, Mg^{2+} by 120%, 103% and 106%, respectively. A slight inhibition was observed by using Hg^{2+}, Zn^{2+} and Ba^{2+} cations (Tab 2).

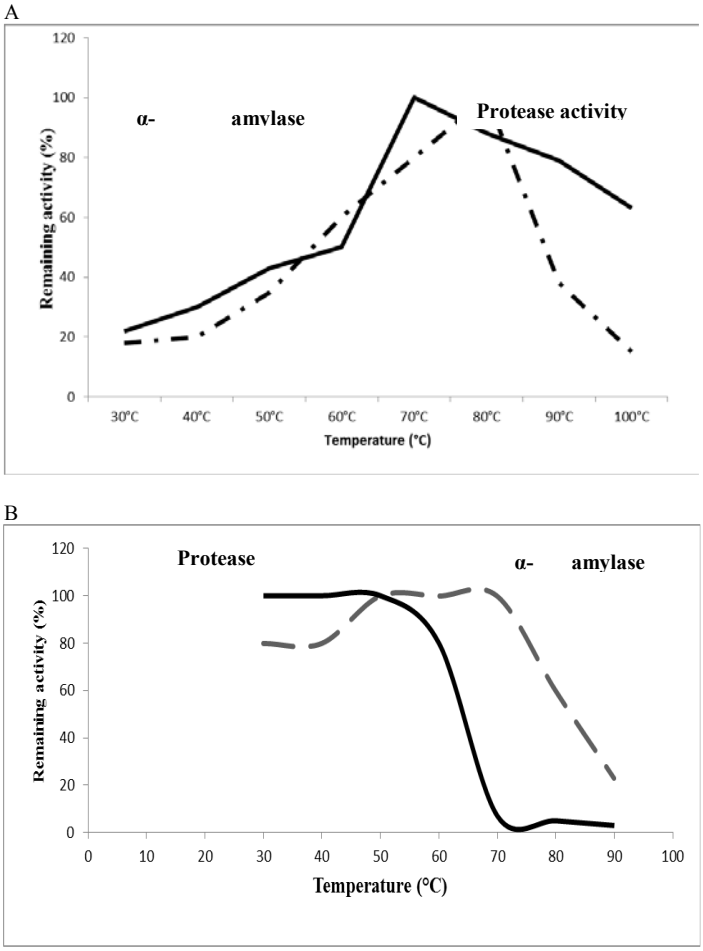

Figure 3 Effect of temperature on enzyme activities (A) and stability (B) of RBS 5.

Table 1 Effect of enzyme inhibitors on α-amylase and protease activities of RBS 5

Inhibitors	Concentrations (mM)	Relative activity	
		α-amylase	Protease
None	0	100	100
EDTA	2	80	100
	5	10	60
β-mercaptoethanol	2	92	87

Table 2 Effect of some metal ions (5mM) on α-amylase and protease activities of RBS 5

Metal ions (5 mM)	Residual activity (%)	
	α-amylase	Protease
None	100	100
Ca^{2+}	21	120
Cu^{2+}	61	103
Zn^{2+}	13	67
Mg^{2+}	11	106
Hg^{2+}	19	92
Ba^{2+}	27	90

Effect of surfactants and oxidizing agents on α-amylase and protease stability

The effect of surfactants and oxidizing agents were assayed under standard conditions (Tab 3). Both activities were highly stable in the presence of non-ionic surfactants, retaining 100% of the initial activities in the presence of 1mM Triton X-100 after 30 min of incubation at 60 °C. The assay of SDS at 5 mM caused a slight inhibition for the α-amylase and strong inhibition of protease. However, 1 mM of SDS has low inhibition effect in α-amylase and protease activities retaining ca. 94% and 93% of the initial amylolytic and proteolytic activities, respectively. In addition, we investigated the effect of the oxidizing agent hydrogen peroxide (H_2O_2) on the stability of RBS 5 enzymes after pre-incubation for 30 min at 60 °C. As shown in Table 3**,** both amylolytic and proteolytic

activities were significatively inhibited by H_2O_2. The α-amylase and protease enzymes retained 63% and 61% of its initial activities, respectively. Furthermore, a local commercialized perfume used at 1 (v/v) concentration does not affect proteolytic and amylolytic activities of RBS 5.

Table 3 Stability of α-amylase and protease of *B. licheniformis* RBS 5 in the presence of various components.

components		Concentration	Residual activities of RBS 5 (%)	
			α-amylase	Protease
	None	0	100	100
	SDS	1 (w/v)	94	93
Surfactants	SDS	5 (w/v)	82	14
	Triton X-100	1 (v/v)	100	100
Oxidizing agents	H_2O_2	0.5 (v/v)	63	61
Anti-redeposition agents	Local commercialized Perfume	1 (v/v)	100	100

Stability of proteolytic crude extract with commercial detergents powder

In order to check the suitability of the enzyme preparation with detergents powder, the crude enzyme was incubated for 1 h at 40 and 50 °C with various commercial laundry detergents. The data presented in Figure 4 showed that, RBS 5 crude enzyme was extremely stable towards detergents Ariel powder tested at 40 °C and 50 °C that retained 100% of its initial activity, After 1 hour of incubation at 40 °C more than 83% of activity with Dixan was conserved and 70% with Nadhif. Interestingly, at 50 °C, crude enzyme mixed with Ariel retained 99% and only 20% with Dixan and 14% of its activity in the presence of Nadhif. Enzyme activities of control samples without any detergent, incubated under the similar conditions, were not affected.

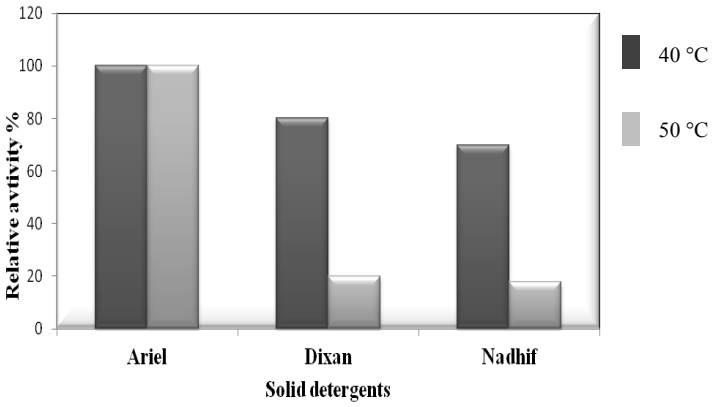

Figure 4 Stability of the protease activity in the presence of various commercial powder detergents (Ariel, Dixan and Nadhif).

DISCUSSION

In this work, we report the characterization of thermostable α-amylase and alkaline protease produced by *Bacillus licheniformis* RBS 5 strain isolated from hot Tunisian springs (korbos). The *B. licheniformis* RBS 5 was shown to co-produce thermostable α-amylase and protease. On SDS-PAGE, both activities were subsequently detected as a single band for each one suggesting the presence of two separate extracellular enzymes α-amylase and protease. Many authors have reported the occurrence of one α-amylase and more than one protease that were found in *B. licheniformis* isolate **(Akel *et al*., 2009; Hmidet *et al*., 2009).** Similarly to our finding, other reported production of unique α-amylase in thermophilic *Bacillus* sp. strain TS-23 **(Chi *et al*., 2010**) and unique protease that was produced by *Bacillus*. sp PS719 **(Nongporn *et al*., 1999)**. Both pH and temperature had the great influence on the enzymatic activities and their stability. RBS 5 coproduces two enzymatic activities: i) an α-amylase activity with an optimum production at 65 °C, and the activity remaining until 80 °C to produce 40% and ii) a protease activity with an optimum at 80° C that is still active at 95 °C conserving 10% of proteolytic activity. Similar results were reported for *Bacillus* species: *B. ferdowsicous* **(Konsoula and Liakopoulou, 2007)**, *Bacillus* sp. JB-99 (**Johnvesly and Naik, 2001)** and *B. licheniformis* NH 1 **(Hmidet *et al*., 2009**). High activity of RBS 5 α-amylase was reached at pH 4 and maintained in large pH interval, being 4 to 9. Similarly, *B. subtilis* JS-2004 has been reported to have an optimum activity at pH 8 **(Asgher *et al*., 2000)** and for *B. licheniformis* MIR 29 it was up to 9 **(Ferrero *et al*., 1996)**. Amylase production reported for *B. licheniformis* Shahed-07 **(Rasooli *et al*., 2008)** and *B. thermooleovorans* NP54 **(Malhotra *et al*., 2000)** was strictly at neutral pH. Protease produced by *B. licheniformis* RBS 5 produce higher activity at pH 11. The crude enzyme retained 100% of its proteolytic activity between pH 10.0 and 12.0, and ca. 50% of its activity at pH 13.0. Alkaline protease was reported from B. *subtilis* BP-36 with a pH range of stability 9.0 to 11.0 and an optimum pH at 9.0 **(Mashayekhi *et al*., 2012)** and at pH 10 for *B. sphaericus* **(Mourad *et al*., 2011)**. Several investigators have reported the production of alkaline proteases over this pH range **(Singh *et al*., 1999**; **Johnvesly and Naik, 2001)**. It is important to obtain enzymes with high stabilities and activities at alkaline pHs and at high temperatures **(Wellingta and Meire, 2003)**, which are established in industrial scale **(Pernilla *et al*., 2007)**. Today a large number of microbial α-amylases and protease are marketed with applications in different industrial sectors and have a biotechnological perspective **(Gupta *et al*., 2003)**.

Effects of various enzyme inhibitors and metal ions on protease and α-amylase activities were investigated in this work. The addition of EDTA and β-mercaptoethanol at a concentration of 2 mM kept the protease activity and only 80% of activity was retained for α-amylase. However at 5 mM, only 10% and 60% of α-amylase and protease activities were retained, respectively, indicating the no requirement for metal cofactor. The stability of the enzyme in presence of EDTA is advantageous for use of enzyme as detergent additive **(Akel *et al*., 2009)**. It might be due to detergents that contain high amount of chelating agents, which function as water softeners and also assist in stain removal. These agents specifically bind to, and chelate metal ions making them unavailable in the detergent solution **(Beg and Gupta, 2003)**. β-mercaptoethanol is a reducing agent that affected slightly α-amylase activity; similar results was obtained by **Azad *et al*., 2009**; Stability with β-mercaptoethanol brings change in the conformation of the enzyme in the active site by breaking disulfide bonds, results in loss of enzyme activity. This indicates that the disulfide bonds play an important role in maintaining the structure of α-amylase **(Pernilla *et al*., 2007)**. β- mercaptoethanol decrease protease activity, that suggests the serine alkaline nature of our protease enzyme **(Anupama and Jayaraman, 2011).**

The effect of metal ions on α-amylase activity showed that *B. licheniformis* RBS 5 enzyme extract was strongly inhibited by Zn^{2+}, Mg^{2+}, Hg^{2+} and Ca^{2+} with residual activities values of 13%, 11%, 19% and 27% respectively. In the presence of Cu^{2+}, the activity was not very influenced, it was 61%. These findings are quite similar in part to what had been observed with *Bacillus ferdowsicous* **(Asooodeh *et al*., 2010)**. Protease activity was weakly affected by the presence of metal ions, with the exception of Zn^{2+} which inhibited the activity to 67% relative to total activity. Similar results have been reported for *Bacillus stearothermophilus* **(Jae *et al*., 2002)**. Most alkaline proteases are significantly stabilized by the addition of Ca^{2+} **(Gupta *et al*., 2003)**. The improvement of activity in the presence of $CaCl_2$ solution can be explained by Ca^{2+} binding at the catalytic site **(Lee and Jang, 2001)**. It has been reported that Ca^{2+} binding is a metal ion protects the enzyme against thermal denaturation and that it plays a vital role in stabilizing the conformation of the enzyme at high temperatures **(Wellingta and Meire, 2003)**.

We have studied the influence of surfactants, oxidizing agents and local commercialized perfume on the α-amylase and protease activities. Triton X-100, SDS and 1% of perfume kept nearly the total original activity. The protease was stable and remained active in presence of 1% of SDS.The same result has been reported for *B. mojavensis*, *Bacillus*. sp. RGR-14 and *Bacillus*. sp. KSM-KP 43 **(Gupta *et al*., 2003; Saeki *et al*., 2007)**. A 0.5% of H_2O_2, reduced the α-amylase and protease activities to 63 and 61% of the initial activities, respectively.

To assess the stability of alkaline protease and the suitability for use in commercial detergents, RBS 5 proteolytic crude extract was incubated for 1 hour at 40 °C and 50 °C, in the presence of various detergents marketed in Tunisia (Ariel, Dixan and Nadhif). The results indicated that the enzyme extract showed high stability when it was mixed with various commercial detergents powder at a concentration of 7 mg/mL at 40 °C. RBS 5 alkaline protease showed a higher stability with Ariel (100% residual activity) comparing to Dixan and Nadhif (80% and 60% of residual activity) respectively, when similar results have been reported for *B. licheniformis* RP1 and *B. mojavensis* A21 with excellent stabilities obtained with a range of commercial detergents powder at a concentration of 0.7 g/L **(Sellami Kammoun *et al*., 2008; Haddar *et al*., 2009; Jellouli *et al*., 2011)**. At 50 °C, proteolytic crude extract was not stable and activity was lost to reach 20% and 18% respectively, for Dixan and Nadhif but was stable for Ariel.

CONCLUSION

The α-amylase and protease activities produced by *B .licheniformis* RBS 5 isolate, were stable over a wide range of pH and temperature. In addition, the crude extract showed compatibility with various commercial detergents tested, it enhanced washing performance of the detergent.

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SCREENING OF ANTIMICROBIAL, CYTOTOXIC AND PESTICIDAL ACTIVITIES OF *COCCINIA GRANDIS* (L.) VOIGT

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ABSTRACT

This study was undertaken to assess the antibacterial, antifungal, cytotoxic and pesticidal activities of *Coccinia grandis* roots extract. The antimicrobial activity was evaluated using disc diffusion method against some pathogenic microorganisms. Cytotoxicity was determined using brine shrimp lethality bioassay method. The plant extract was screened for pesticidal activity towards *Sitophilus oryzae* adults. Minimum inhibitory concentration (MIC) was also studied against test organisms, using serial dilution technique to determine the antibacterial potency. In antibacterial screening, large inhibition zones were observed against the tested gram-positive (*Bacillus subtilis*, *Sarcina lutea* and *Stapphylococcus aureus*) and gram-negative (*Salmonella typhi* and *Shigella dysenteriae*) bacteria. In antifungal screening, the extract showed moderate antifungal activities against the tested fungi (*Candida albicans* and *Colletotrichum falcatum*). In cytotoxicity activity test, LC_{50} (lethal concentration, 50%) of the extract against brine shrimp nauplii was 15.00µg/ml. The plant extract also showed moderate pesticidal activities towards *S. oryzae* adults. These results suggested that the plant extract has significant antibacterial activity against the tested bacteria, moderate antifungal, cytotoxic and pesticidal activity towards the tested fungi, brine shrimp nauplii and *S. oryzae* adults, respectively.

Keywords: *Coccinia grandis*, methanol extract, antimicrobial activity, cytotoxicity, pesticidal activity

INTRODUCTION

Coccinia grandis (L.) Voigt (family of Cucurbitaceae), locally known as Telakucha, is an important medicinal plant, distributed in Indian sub-continent, Eastern Africa, and Central America. Different parts of the plant are used by humans mostly as a food crop in several countries in Australia, Asia, Caribbean, the southern United States, and Pacific Islands (**Pekamwar *et al.*, 2013**). Fruits may be eaten immature and green, or mature and deep red **(Hasanuzzaman *et al.*, 2013)**. The young shoots and leaves may also be eaten as greens. The fruits, stems, roots and leaves of the plant are popularly used in the treatment of edema, eye diseases, carminative, hypertension, fever, anti-inflammatory, headache, typhoid, sunstroke, hypnotic, jaundice, stomach pain, anti-pyretic, mental disease, leucorrhea, alopecia, dermatitis, eczema, emetic, dysentery, scabies and blood purifier (**Sivaraj *et al.*, 2011; Abbasi *et al.*, 2009**). Phytochemical screening of *Coccinia grandis* revealed the presence of saponins, cardenolides, flavonoids and polyphenols that may be attributed to antibacterial activity (**Sivaraj *et al.*, 2011**). The root of this plant contains resin, alkaloids, starch, fatty acids, carbonic acid, triterpenoid, saponin coccinoside, flavonoid glycoside, lupeol, β-amyrin, β-sitosterol, taraxerol (**Deokate and Khadabadi, 2011**).

The frequency of life-threatening infections caused by pathogenic microorganisms has increased worldwide and is becoming an important cause of morbidity and mortality in immunocompromised patients in developing countries. To overcome this problem, there is a constant need for new and effective infection fighting strategies. Therefore, there is a need to develop alternative therapeutic agents for the treatment of infectious diseases from medicinal plants (**Tumpa *et al.*, 2015**). Medicinal plants continue to be used world-wide for the treatment of various diseases and have a great potential for providing novel drug leads with novel mechanism of action (**Singh *et al.*, 2012**). There are many reports on *Coccinia grandis* plant including, antibacterial and antifungal activity of leaves extract (**Bhattacharya *et al.*, 2010**), antiplasmodial activity against the *Plasmodium falciparum* (**Ravikumar *et al.*, 2012**), anti-inflammatory activity of leaves and stem extracts (**Deshpande *et al.*, 2011**), anthelmintic activity (**Tamilselvana *et al.*, 2011**), antioxidant activity (**Ashwini *et al.*, 2012**), antipyretic activity (**Aggarwal *et al.*, 2011**), anticancer activity of leaves extract (**Bhattacharya *et al.*, 2011**), antitussive activity of fruit extract (**Pattanayak and Sunita, 2009**), antiulcer activity of leaves extract (**Santharam *et al.*, 2013**) and others (**Kumar, 2012; Ravikumar *et al.*, 2012**). But there is no sufficient report on antimicrobial, cytotoxic and pesticidal activity on this valuable plant root extract.

The present study was designed to determine the role of methanolic extract of *Coccinia grandis* roots for potential antibacterial and antifungal activities against some pathogenic bacteria and fungi. The cytotoxicity and pesticidal activities of the plant extract were also determined using brine shrimp nauplii and *S. oryzae* adults, respectively.

MATERIAL AND METHODS

Plant material

Coccinia grandis plants were collected from Rajshahi University Campus, Rajshahi, Bangladesh and were identified by Md. Shahed Alam, Senior Technical Officer, Herbarium Museum, Department of Botany, University of Rajshahi, Bangladesh, where its voucher specimen was deposited for reference. Roots of these plants were used as plant material for this present investigation.

Chemicals and reagents

Methanol and DMSO (dimethyl sulfooxide) were purchased from Merck, Germany. Kanamycin was purchased from Square Pharmaceuticals Ltd., Bangladesh. Gallic acid and vincristine sulfate were purchased from Cipla Ltd., Goa, India. All the chemicals and reagents used throughout the investigation were of reagent grade.

Organisms

Antibacterial activity and MIC values were determined against six gram-positive bacteria (*Bacillus cereus, Bacillus subtilis, Bacillus megaterium, Sarcina lutea, Staphylococcus aureus,* and *Stapphylococcus-β-haemolyticus*) and seven gram-negative bacteria (*Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi, Shigella boydii, Shigella dysenteriae, Shigella flexneri* and *Shigella sonnei*). Antifungal screening was carried out against seven fungi (*Aspergillus fumigatus, Aspergillus flavus, Aspergillus niger, Candida albicans, Colletotrichum falcatum,*

Rhizopus oryzae and *Tricophyton rubrum*). Cytotoxicity was determined against brine shrimp nauplii (*Artima salina*). Brine shrimp nauplii were obtained by hatching brine shrimp eggs (Carolina Biological Supply Company, Burlington, NC, USA) in artificial sea water (3.8% sodium chloride solution) for 48h. These organisms were collected from the Microbiology Laboratory, Department of Microbiology and Institute of Nutrition and Food Sciences (INFS), University of Dhaka, International Centre for Diarrhoea Diseases Research Bangladesh (ICDDRB), Dhaka, Bangladesh. Pesticidal activity was tested against *Sitophilus oryzae* L. adult insect. The insects were collected from the "Integrated Pest Management Laboratory" Institute of Biological Sciences, University of Rajshahi, Bangladesh.

Media

Nutrient agar media (Difco laboratories) pH 7.2, nutrient broth media (Difco Laboratories) pH 6.8, Sabouraud dextrose agar media (Biolife Vole Monza) pH 5.6 and artificial seawater (3.8% sodium chloride solution) pH 8.4 were used for antibacterial screening, MIC determination, antifungal screening and cytotoxicity determination, respectively. A standard mixture of rice and powdered brewer's yeast in the ratio of 19:1 was used as food medium to culture *S. oryzae*.

Plant material extraction and fractionation

Collected roots of the plants were cut, air-dried powdered in a grinding machine and stored in an airtight polybag. Powdered dried roots (400g) of the plant were extracted (cold) with methanol (1.25 Liter) in flat bottom conical flask, through occasional shaking and stirring for 10 days (**Jeffery *et al*., 2000**). The content was pressed through the markin cloth to get maximum amount of extract. The whole mixture was then filtered by Whatman filter paper No. 41 and the remaining filtrate was dried (**Hussain *et al*., 2010**) in vacuo to afford a blackish mass. The output extract and fraction were collected to glass vials and preserved in a refrigerator at 4°C.

Antibacterial screening

Antibacterial screening was performed by disc diffusion method (**Hussain *et al*., 2010**) against six gram-positive and seven gram-negative bacteria at the concentration of 300µg/disc, which is a qualitative to semi quantitative test. Briefly, 20 ml quantities of nutrient agar were plated in petri dish with 0.1 ml of a 10^{-2} dilution of each bacterial culture. Filter paper discs (6 mm in diameter) impregnated with the concentration of plant extract was placed on test organism-seeded plates. Methanol was used to dissolve the extract and was completely evaporated before application on test organisms seeded plates. Blank disc impregnated with solvent methanol followed by during off was used as negative control. The activity was determined after 16 h of incubation at 37°C. The diameter of zone of inhibition produced by the plant extract were then compared with the zones produced by standard antibiotic (kanamycin 30 µg/disc).

Determination of minimum inhibitory concentration (MIC)

Serial tube dilution technique (**Hussain *et al*., 2010**) was used to determine MIC of the extract against six gram-positive and seven gram-negative bacteria. The plant extract (1.0 mg) was dissolved in 2 ml distilled water (2 drops tween-80 was added to facilitate dissolution) to obtain stock solution. After preparing the suspensions of test organisms (10^7 organisms per ml), 1 drop of suspension (20µl) was added to each broth dilution. After 16 h incubation at 37°C, the tubes were then examined for the growth. The MIC values of the extract were taken as the lowest concentration that showed no growth. Growth was observed in those tubes where the concentration of the extract was below the inhibitory level and the broth medium was observed to be turbid (cloudy). Distilled water with 2 drops of tween-80 and kanamycin were used as negative and positive control, respectively.

Antifungal screening

The antifungal activity of the extract was tested by disc diffusion method (**Hussain *et al*., 2010**) against the five pathogenic fungi at the concentrations of 300µg/disc for each. Here, 20 ml quantities of nutrient agar were plated in petri dish with 0.1 ml of a 10^{-2} dilution of each bacterial culture. Filter paper discs (6 mm in diameter) impregnated with the concentration of plant extract was placed on test organism-seeded plates. Methanol was used to dissolve the extract and was completely evaporated before application on test organisms seeded plates. Blank disc impregnated with solvent methanol followed by during off was used as negative control. The activity was determined after 72 h of incubation at room temperature (32°C). The diameter of zone of inhibition produced by the extract were then compared with the zones produced by standard antibiotic (kanamycin 30 µg/disc).

Cytotoxicity bioassay

Cytotoxicity of *C. grandis* roots was screened against *Artemia salina* in a one day *in vivo* according to published protocols (**Rahman *et al*., 2010**). Brine shrimp nauplii were obtained by hatching brine shrimp eggs in artificial sea water (3.8% sodium chloride solution) for 48 h in 25°C. Dissolution for extract was performed in artificial sea water using DMSO. Serially diluted test solutions (0.5, 1, 2, 5, 10, 20 and 40µg/ml) were added to the sea water (5 ml) containing 10 nauplii. After incubation for 24 h at 25°C, the numbers of survivors was counted. From this data, lethal concentration (LC_{50}) and 95% confidence intervals of the test samples were calculated using probit analysis method described by Finney (**Finney, 1971**). Each sample was used in triplicate for the determination of the LC_{50} (50% lethal concentrations, µg/ml). Gallic acid and vincristine sulfate were used as standards in this bioassay.

Assessment of pesticidal activity

Pesticidal activity of *C. grandis* root extract was assessed against *S. oryzae* adults as previously described (**Roy *et al*., 2005**). The mortality test was performed at the concentrations of 100, 50, 25 and 12 mg/ml. 1ml solution of each doses were dropped onto the petri dishes (6 cm diameter), spread and then air dried for five minutes. Ten adult insects (10-12 days old) were release into each petri dish, kept in room temperature and mortality (%) were recorded at 24, 48 and 72 h after treatment. For determining repellency test, required amount of extract was dissolved in methanol to obtain the concentrations as 100, 50, 25 and 12mg/ml. Filter papers (Whatman No. 40, diameter 9 cm) were cut into two half, and 1 ml solution of each dose was applied to each half uniformly with a dropper. The treated half of the papers were then air-dried at attached with the untreated half with a cello-tape at middle and paper was then placed on the petri dish. Ten adult insects were release in treated papers and petri dishes were placed in the laboratory at room temperature. The insects present on each half of the paper strip were counted at 24, 48 and 72 h after treatment.

Statistical analysis

The experimental results are presented as mean for three triplicates for studied parameters. The median lethal concentration (LC_{50}) and 95% confidence intervals of the test samples were calculated using probit analysis method described by Finney (**Finney, 1971**).

RESULTS AND DISCUSSION

Antibacterial activity

The results representing antibacterial activity of methanol extract of roots are presented in **Table 1**. The highest inhibition zones of root extract were 26.0 mm diameter found against *B. subtilis*, *S. aureus* and *S. lutea* (gram-positive) followed by 25.0 mm diameter against *S. typhi* and *S. dysenteriae* (gram-negative) at the concentration of 300 µg/disc. The lowest activity of plant extract was 16.0 mm diameter of zone inhibition observed against *Bacillus cereus* at the concentration of 300µg/disc. But, the extract found no inhibition zone against *S. sonnei* and *S. flexneri* at the concentration of 300 µg/disc. Negative control exhibited no zone of inhibition against all the organisms. In comparison to reference standard antibiotic (kanamycin, 30 µg/disc), the methanol extract of root exhibited significant antibacterial activities at the concentration of 300 µg/disc against almost all of the organisms tested (**Table 1**). **Bhattacharya *et al.* (2010)** evaluated the aqueous extract of leaves of *Coccinia grandis* for antibacterial activity against *Shigella flexneri* NICED, *Bacillus subtilis*, *Escherichia coli*, *Salmonella choleraesuis*, *Shigella dysenteries*, and *Shigella flexneri*. Ethanolic extract of *Coccinia grandis* leaf showed high antibacterial activity against *S. pigeons*, *E. coli*, *B. ceres*, *K. pneumonia* and *S. aureus* (**Sivaraj *et al*., 2011**). **Hasanuzzaman *et al*., (2013)** reported 12 mm of zone of inhibition of *Coccinia grandis* root extract against *Staphylococcus aureus*. Previous studies on antibacterial activity of fruits, leaves and stem of *Coccinia grandis* (**Aggarwal *et al*., 2011; Hussain *et al*., 2010**) have also detected the significant activity of methanol extract against different pathogenic bacteria providing support to the fact that methanol is a better solvent for extraction and screening of phytochemicals having antimicrobial activity.

Table 1 Antibacterial activities of methanol extract of *Coccinia grandis* roots.

Test organisms	Diameter of zone of inhibition (in mm)	
	Methanol extract (300µg/ disc)	Kanamycin (30µg/disc)
Gram-positive		
Bacillus cereus	16	24
Bacillus subtilis	26	31
Bacillus megaterium	23	32
Sarcina lutea	26	26
Stapphylococcus aureus	26	33
Stapphylococcus-β-haemolyticus	18	28
Gram-negative		
Escherichia coli	22	29
Pseudomonas aeruginosa	19	27
Salmonella typhi	25	31
Shigella boydii	23	31
Shigella dysenteriae	25	32
Shigella flexneri	0.0	21
Shigella sonnei	0.0	19

Note: Data are represented in the form of mean of three tested of the standard groups.

Minimum inhibitory concentration (MIC) measurement

The Minimum inhibitory concentration (MIC) values of the extract against tested bacteria were shown in **Table 2**. The MIC values were 64, 16, 32, 16, 16, 64, 32, 64, 32, 64, 32, 64 and 128 µg/ml respectively, against the tested organisms (six gram positive and seven gram negative). The MIC values against the tested gram positive bacteria ranged from 16 to 64 µg/ml and against gram negative bacteria from 32 to128 µg/ml. Negative controls exhibited no inhibition against all the organisms. The standard antibiotic (kanamycin) had MIC values varying 4 to16 µg/ml against the tested organisms. Antibacterial potency of the plant extract against these bacteria expressed in MIC values indicated that the plant extract is more effective against gram-positive bacteria than gram negative bacteria. **Sivaraj *et al*., (2011)** reported with 31.25 µg/ml MIC values of *Coccinia grandis* leave extract against *Stapphylococcus aureus*. **Bhattacharya *et al.* (2010)** showed 1000-1750 µg/ml of MIC values of *Coccinia grandis* leaves extract against some gram positive and gram negative bacteria. **Hasan and Rahman, 2011; Saikot *et al*., (2012); Khan *et al*., (2013)** reported similar MIC values for different plant extracts which supported our present findings.

Table 2 Minimum inhibitory concentration (MIC) of methanol extract of *Coccinia grandis* roots.

Test organisms	Methanol extract (µg/ml)	Kanamycin (µg/ml)
Gram-positive		
Bacillus cereus	64	16
Bacillus subtilis	16	4
Bacillus megaterium	32	4
Sarcina lutea	16	4
Stapphylococcus aureus	16	8
Stapphylococcus-β-haemolyticus	64	8
Gram-negative		
Escherichia coli	32	8
Pseudomonas aeruginosa	64	4
Salmonella typhi	32	4
Shigella boydii	64	8
Shigella dysenteriae	32	8
Shigella flexneri	64	8
Shigella sonnei	128	16

Note: Data are represented in the form of mean of three tested of the standard groups.

Antifungal activity

The antifungal activities of methanol extract of the plant root (300µg/disc) and standard kanamycin (30µg/disc) were determined against seven pathogenic fungi (**Table 3**). The highest activity was 19.0 mm diameter of zone inhibition observed against *C. albicans* and *C. falcatum* followed by 17.0 mm diameter of zone inhibition against *A. niger* at the concentration of 300 µg/disc. The lowest activity was 7.0 mm diameter of zone inhibition found against *Aspergillus fumigatus* at the concentration of 300µg/disc. The lowest activity was 6 mm diameter of zone inhibition found against *A. fumigatus* at the concentration of 300µg/disc. The plant extract showed no inhibition zone against *A. flavus* and *T. rubrum.* Negative control exhibited no zone of inhibition against all the organisms. In comparison to reference standard antibiotic (kanamycin, 30 µg/disc), the methanol extract of root exhibited significant antifungal activities at the concentration of 300 µg/disc against almost all of the organisms tested (**Table 3**). **Bhattacharya *et al*., (2010)** evaluated the antifungal activity of the *Coccinia grandis* leaves extract against the *Candida albicans*-II, *Candida tropicalis*, *Aspergillus niger*, *Saccharomyces cerevisiae*, *Candida tropicalis* II, *Cryptococcus neoformans* and *Candida albicans* ATCC. **Satheesh and Murugan (2011)** reported that *C. grandis* leaves have a strong inhibition activity against pathogenic fungus *C. albicans*, *M. indicus*, *P. notatum*, *A. flanus* and *C. neoformans.* Previous studies on antifungal activity of different plants crude extracts **(Hasanuzzaman *et al.*, 2013; Khan *et al.*, 2013; Hasan *et al*., 2009)** have also detected the significant activity against some pathogenic fungi which support our present findings.

Table 3 Antifungal activities of methanol extract of *C. grandis* roots.

Test organisms	Diameter of zone of inhibition (in mm)	
	Methanol extract (300µg/ disc)	Kanamycin (30µg/disc)
Aspergillus fumigatus	7	19
Aspergillus flavus	0.0	17
Aspergillus niger	17	23
Candida albicans	19	24
Colletotrichum falcatum	19	22
Rizopus oryzae	15	21
Tricophyton rubrum	0.0	19

Note: Data are represented in the form of mean of three tested of the standard groups.

Cytotoxicity bioassay

The LC_{50} values of the brine shrimp lethality bioassay obtained for root extract of the plant and that of the positive controls gallic acid and vincristine sulfate, has been presented in **Table 4**. The extract showed significant cytotoxicity against brine shrimp nauplii. The LC_{50} value of the plant extract was 15.00 µg/ml, whereas the cytotoxicity of standard gallic acid and vincristine sulfate LC_{50} values were 7.50 and 2.90 µg/ml respectively. No mortality was found in the control group. **Hasanuzzaman *et al*., (2013)** showed significant cytotoxicity with LC_{50} of 2.49 µg/ml in ethanolic extract of *C. grandis* roots. **Saikot *et al*., (2012)** showed cytotoxicity against brine shrimp nauplii with LC_{50} of 7.06 µg/ml in acetone extract of *Abroma augusta* leaves. **Hasan and Rahman, (2011)** reported cytotoxicity with LC_{50} of 35.45 µg/ml in ethanol extract of *Polygonum hydropipper* stem. These cytotoxicity results support our present findings.

Table 4 Cytotoxic activity of methanol extract of *Coccinia grandis* roots on brine shrimp nauplii.

Samples	LC_{50} (µg/ml)	95% confidence limits (µg/ml)	Regeneration equation	X^2 value
Plant extract	15.00	10.5-25.00	Y=2.65+2.01X	1.71
Gallic acid	7.50	5.10-13.50	Y=3.83+1.52X	1.25
Vincristine sulfate	2.90	1.34-5.55	Y=2.16+1.98X	0.61

Note: LC_{50} values, confidence limits, regeneration equations and X^2 values were calculated by probit analysis.

Assessment of pesticidal activity

In the present investigation, pesticidal activity of methanol extract of the plant root against *Sitophilus oryzae* has been determined. The mortality (%) and repellency (%) of *S. oryzae* adults in different concentrations, at different exposure periods has been given in **Fig. 1 and 2**. The highest percentage of mortality was 73.3% found at the concentration of 100mg/ml after 72 hours of treatment, followed by 66.6% at the same concentrations after 48 hours. On the other hand, no mortality (0.0%) was observed in the concentration of 12mg/ml after 24 hours (**Fig. 1**). Similarly, the highest percentage of repellency was 80.0% observed at the concentration of 100mg/ml after 72 hours followed by 73.3% at the same concentration after 48 hours**.** On the left hand, the lowest percentage of repellency was 6.6% found at the concentration of 12mg/ml after 24 hours **(Fig. 2)**. The control group had no mortality and repellency; therefore, it has not been presented. **Mobki *et al*., (2014)** reported with 83.3% mortality and 95% repellency of *Tribolium castaneum* larvae using garlic extract. **Roy *et al*., (2005)** reported with 56.71% mortality and 55.34% repellency of *Sitiphilus oryzae* using *Blumea lacera* plant extract. **Rahman *et al*., (2007)** reported with highest 34.0% mortality and 22.43% repellency in *Sapindus mukorossi* fruits extract against *Sitiphilus oryzae* adult insect. These results was similar to our present findings.

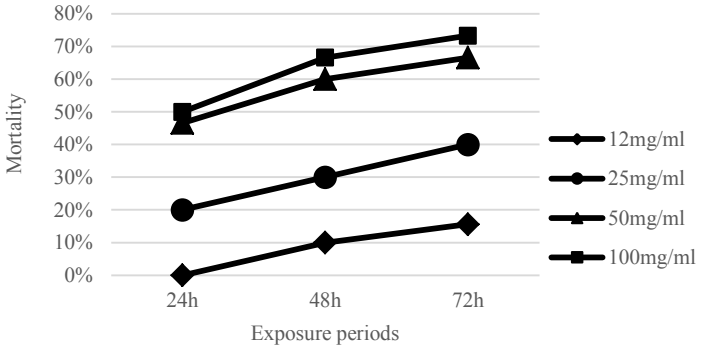

Figure 1 Regression line of mortality (%) on different doses (mg/ml) of root extract of methanol on *S. oryzae* at different exposure periods.

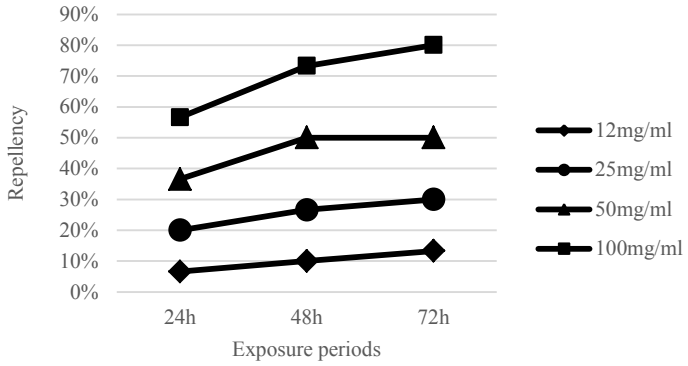

Figure 2 Regression line of repellency (%) on different doses (mg/ml) of root extract of methanol on S. oryzae at different exposure periods

CONCLUSION

The results observed in the present study demonstrated that *Coccinia grandis* roots have promising antimicrobial (300µg/disc), cytotoxic (15.00 µg/ml) and pesticidal (100mg/ml) activities. These antimicrobial, cytotoxic and pesticidal activities are probably first reported for the methanol extract of roots of *Coccinia grandis*. Further, remarkable antimicrobial, cytotoxic and pesticidal activities found by the experiments support the claims of traditional medicine. The present findings can be source of antibiotic substances for possible treatment of microbial infections. The cytotoxicity and pesticidal results revealed that the *Coccinia grandis* roots extract might be considered as a moderate toxic. However, to isolate these active phytochemicals and determine their activities are in progress.

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INCIDENCE OF FILAMENTOUS FUNGI WITH TOXIGENIC POTENTIAL ON SAMPLES OF FEED AND RAW MATERIALS FOR THEIR MANUFACTURE

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ABSTRACT

The presence and/or accumulation of mycotoxins in foods intended for human and animal nutrition is a constant concern for the harmful health effects resulting from ingestion. The aims of this communication were to analyze samples of feed and raw materials for manufacturing and to determine the presence of strains of filamentous fungi with toxigenic capacity. The values of frequency in the total samples (N = 422), indicated 63% of contamination, where *A. flavus* represented the most common (29.8%), while in feed and raw materials separately, indicated *A. flavus* has the highest value in both categories. In the analysis of different type of raw materials, *A. flavus* contaminated all types of samples, with the bran and soybean meal substrates having higher values for this *Aspergillus*, and corn substrate more fungal contamination. These results would demonstrate that the presence of mycobiota with toxigenic potential in food for animal feed is a disturbing reality.

Keywords: *Aspergillus flavus*, feed, filamentous fungi

INTRODUCTION

For the reason that many food intended for animal nutrition are an important channel introducing hazards on the human food chain, it is safety should be evaluated and valued before being fed to the animals. These following assessment processes reviewed various aspects of the presence of some species of microorganism, implying contemplate the safety of the animals as well as humans who can consume remains of them or their metabolites that can stay in food of animal origin (**FAO/WHO, 2007**).
Many species of filamentous fungi are able to produce mycotoxins, but those belonging to the genera *Aspergillus, Fusarium* and *Penicillium* are especially important in notorious analysis of the contamination of food for human and animal consumption, as there can be consequent adverse effects to health if they are ingested (**Bryden, 2011**), estimating that at least 300 of these metabolites are potentially toxic to animals and humans (**Huwig *et al,* 2001**).
Mycotoxins are defined as "secondary metabolites produced and accumulated by certain species of fungi, whose ingestion, inhalation or skin absorption reduces performance, sickness or kills animals or people" (**Pitt, 1996; Aissaoui *et al.*,1999**) thereby denominating mycotoxicosis to toxic response caused by the mycotoxin in humans and animals (**Osborne, 1982; Jand *et al.*, 2005**).
While mycotoxin production from fungi depends on environmental conditions and food storage, determining the degree of contamination of feed and raw materials, potentially indicates the presence of these metabolites in these substrates thus allowing us to monitor and control measures throughout the production chain. For this reason the objective of our study was to assess the degree of contamination in feed and raw materials used for manufacturing by identifying mycelial fungi with mycotoxigenic potential.

MATERIAL AND METHODS

Samples

We analyzed 422 samples, 236 of which were feed samples and 186 were raw materials (10 different substrates) from different companies located in the Iberian Peninsula dedicated to the animal sector.

Isolation and identification of filamentous fungi

The presence of certain species and genera of filamentous fungi with potential toxigenicity were isolated and determined. This included isolating: *Aspergillus flavus*, *Aspergillus niger* and *Fusarium* spp.
To isolate this mycobiota in feed and raw materials, the samples were suspended in 1/10 concentration of sterile Ringer's solution eukaryote. The bottle was shaken vigorously for 20 seconds in order to release fungal propagules adhered to the surface, and 0.1 mL of the sample was inoculated on Sabouraud Dextrose Agar plates with chloramphenicol (LIOFILCHEM®), and then incubated in a conventional oven at 28° C for 4-7 days.

Identification of fungi

After the incubation, the plates with positive growth of fungi were analysed and identified macroscopically and microscopically. Phenotypes such as colour were analysed macroscopically, such as colour of mycelium, hyphae and conidia. Whereas reproductive structures, form of conidiophores and conidia were analysed microscopically. The species *Aspergillus* were identified according to the information provided by **Raper & Fennell (1965)** and **Koneman *et al.*, (1999)**, and **Nelson *et al.*, (1983)** for the genus *Fusarium*.

Data analysis

The isolation frequency (Fr) and relative density (RD) of species were calculated according to **Gonzalez *et al.*, (1995)** as follows:

Fr (%): (number of samples whit a species or genus / Total number of samples) X 100
RD (%): (number of isolates of a species or genus / Total number of fungi isolated) X 100

RESULTS AND DISCUSSION

The results obtained in our study indicate the present mycobiota and were consistent with the findings by some authors as **Richard *et al.*, (2007)**, **Jeswal (1990)** and **Phillips *et al.*, (1996)** where in samples collected from in food industries that the presence of *A. flavus*, *A. niger* and *Fusarium* spp. was detected.

Contamination of potential toxigenic fungi in samples

The presence of filamentous fungi in products for human and animal nutrition and has been investigated by various authors for decades, with the objective being to take controlled measures and implement recommendations of good agricultural and manufacturing practices (**González-Pereyra *et al.* , 2008**). Our results of the presence of filamentous fungi in the total sample (feed and raw materials) indicate that 63.03% are contaminated with at least one possible strain having toxigenic capacity. Of the total feed analysed, 61.4% of contamination existed, whereas in the raw samples, there was 65.05% associated to the development of mycobiota (Table 1).

Table 1 Isolation Frecueny of samples contamined (%) whit toxigenic potential filamentous fungi analyzed in this study.

	n	n +	Fr (%)
Total samples	422	266	63.03
Feed	236	145	61.44
Raw material	186	121	65.05

Legends: n - number of samples, n+ - number of contaminated samples with a filamentous fungus analyzed in this study

Identification of filamentous fungi and total contamination of feed

The frequency values in the filamentous fungi identification found in the total samples (Fig. 1), indicated that *Aspergillus flavus* is the specie whit a higher contamination value (29.8%), followed by the *Fusarium* genera (20.8%) and *Aspergillus niger* specie (9.7%). The remaining 39.8% showed no presence by any of these toxigenic strains.
This results are in accordance whit the findings of others authors who have collected samples in food industries where exists the presence of these filamentous fungi exist (**Richard *et al.*, 2007; Jeswal, 1990; Philips *et al.*, 1996**). It is interesting that the percentage didn't present contamination for species/genera of toxigenic fungi (39.8%), in contrast to other similar studies where total feed samples showed an association to mycobiota (**Glenn, 2007**), this would indicate the presence of necessary contamination control measures in food companies, such as application of post-harvest antifungal treatments, control temperature and relative humidity in storage.

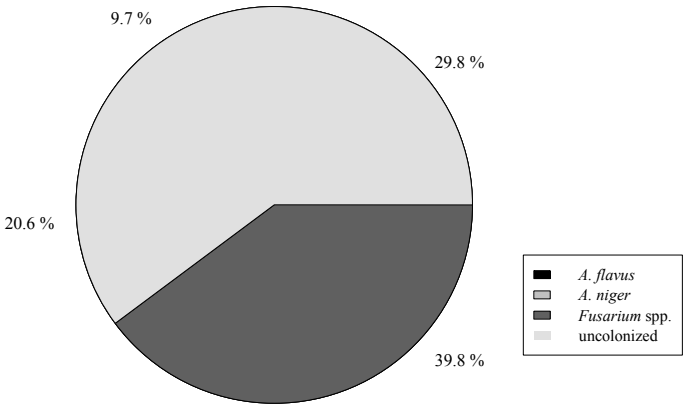

Figure 1 Distribution of potentially toxigenic filamentous fungi in all samples analyzed

Relative density of filamentous fungi whit toxigenic potential in total samples

The results indicated that *A. flavus* has the highest value of relative density in both feed and raw material, followed by *Fusarium* spp. and *A. niger* (Table 2). These results are similar to other authors, in relation to the species or genera of filamentous fungi found, however there are differences in the values of relative density, for example **González Pereyra *et al.*, (2008)** found 13.9% for *A. flavus,* 8.33% in *A. niger*, and 100% for *Fusarium* spp. Meanwhile **Rosa *et al.*, (2009)** found that *Fusarium* spp. contaminates all samples, *A. flavus* 50.0%, and *A. niger* 20%. The difference in values could be related to several factors such as: the origin of the samples, time of collection, storage and environmental characteristics and variables that favor the development of filamentous fungi.

Table 2 Relative density of species / genera of filamentous fungi on total samples analyzed in this study.

HF	Feed (n= 236)	Raw material (n=186)
Aspergillus flavus	28.38	24.15
Aspergillus niger	8.89	11.82
Fusarium spp.	24.15	18.27

Legends: HF - specie / genera of filamentous fungi analyzed in this study

In the raw materials (Table 3), the results indicated that each type of substrate is contaminated by one or more toxigenic fungi, where corn, soybean meal, and bran have the highest frequency of contamination (> 80.0%). These results agree with those obtained by authors in studies on corn when used as a raw material feed (**Mngadi *et al.*, 2008; Pereyra *et al.*, 2010**) which indicates that this is the substrate with the highest levels of contamination, a high degree of deterioration, and in some cases accumulation and mycotoxins. Also the results obtained by Lanier *et al.,* (2009) in rapeseed, are similar to species / genera of filamentous fungi determined in our study.

Table 3 Frequency and relative density of species / genera of filamentous fungi evaluated in this study on substrates used as raw materials in feed production`s.

Raw materials	n	Fr (%)	RD (%)		
			A. flavus	*A. niger*	*Fusarium* spp.
Barley	19	42.1	26.32	-	15.79
Rapeseed	7	42.9	28.57	14.29	-
Carob	8	50.0	25.0	25.0	-
Sunflower	8	62.5	37.50	12.50	12.50
Soybean meal	5	80.0	60.00	-	20.00
Corn	30	90.0	33.33	20.00	36.37
Bran	6	83.3	66.67	16.67	-
Soy	19	68.4	42.11	15.79	10.53
Wheat	29	72.4	31.03	10.34	31.03
Other ingredients	55	56.4	34.55	9.09	12.73

Legend: n – numer of samples, Fr(%) – frequency, RD(%) – relative density

CONCLUSION

The results demonstrate that the presence of mycobiota with toxigenic potential in food for animal feed is a disturbing reality and by eating the positive samples the probability and occurrence and / or accumulation of mycotoxins aflatoxins, ochratoxin, fumonisin and zearalelona type can cause a significant risk to animal and human health.
It should be emphasized that although the presence of these filamentous fungi does not necessarily indicate the presence of mycotoxin, there still is a possibility that some of the samples who did not present contamination may have accumulated mycotoxins along the productive chain **(Pitt, 1996)**, therefore the consumption of the samples whit no sign of presence of mycobiota could still present a risk to the health of the animal.
Finally, while it is important to detect potential toxigenic species in foods such as assessing of the potential risks to animal or human health by the presence of these, it is also necessary to supplement this study with quantitative detection of mycotoxins, as currently legislation regulates the presence of these metabolites and not the presence and / or concentration of filamentous fungi in products intended for human and animal consumption.

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ECOLOGY OF *PANTOEA AGGLOMERANS* 2066-7 STRAIN: A BIOLOGICAL CONTROL OF BACTERIA ONION DISEASES

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ABSTRACT

The growth response of the biocontrol agent *Pantoea agglomerans* 2066-7 to change in water activity (a_w), temperature, and pH was determined *in vitro* in basic medium. The minimum temperature at which 2066-7 was able to grow was 7°C, and the growth of 2066-7 did not change at varying pH levels (4–10.34). The best growth was obtained at a water activity of 0.98 in all media modified with the four solutes (glucose, glycerol, NaCl and polyethylene glycol). The solute used to reduce water activity had a great influence on bacterial growth, especially at unfavorable conditions (low temperature). This study has defined the range of environmental conditions (a_w, pH, and temperature) over which the bacteria may be developed for biological control of plant diseases.

Keywords: *Pantoea agglomerans*, ecology, water activity and plant diseases

INTRODUCTION

The control of diseases in fruits and vegetables is still mainly based on the use of synthetic pesticides, although the demand for produce free from chemical residues and the emergence of pesticides -resistant pathogen strains is constantly increasing.
Biological control using microbial antagonists has attracted much interest as an alternative to chemical products (**Droby and Chalutz, 1992**; **Vinas *et al.*, 1998**). However, very few of the biocontrol agents studied have been successfully commercialized.
Following application on fruits and vegetables, biocontrol agents are exposed to a range of variable biotic and abiotic stress factors, such as competition, predation, changes in temperature, osmolarity, low pH, availability of nutrients and water. Therefore, biocontrol in the field has often been limited by fluctuating environment and the narrow range of conditions over which successful establishment and effective pest and/or disease control is possible (**Hallsworth and Magan, 1994a,b**).
Dehydration of the product and maintenance in a dry environment is one of the best way to formulate microbial agents, so that they can be handled using the normal distribution and storage channels (**Rhodes, 1993**).
Unfortunately, not all microorganisms are amenable to drying and many tend to lose viability during both the drying process and storage. Biological systems are currently preserved by reducing their water content and thereby the water activity (a_w) of the medium. Thus, water activity is a measure of the availability of water for biological functions and relates to water present in a food in "free" form. In food system, total water of moisture is present in "free" and "bound" forms. Bound water is necessary to hydrate the hydrophilic molecules and to dissolve the solutes and not available for biological functions; as well, it does not contribute to water activity. The free water in a food is necessary for microbial growth. It is necessary for the transport of nutrients and the removal of waste materials, to carry out enzymatic reactions, to synthesize cellular materials, and to take part in other biochemical reactions. Each microbial species (or group) has an optimum, maximum, minimum water activity level for growth. When the water activity is reduced below the minimal level for growth of a microorganism, the cells remain viable for a while. Nevertheless, if the water activity is reduced drastically, microbial cells will lose viability, generally rapidly at first, and then more slowly (**Magan and Lacey, 1988; Plaza *et al.*, 2003; mathlouthi, 2001**).
Temperatures and pH of environment are also limited factors to the development of microorganisms. However, to assure a good application of biological control agent in the field and during storage a study of the optimal water activity, temperatures and pH of development of this agent is very important (**Cañamás *et al.*, 2009**).
In this work and at the first time in Morocco we studied the ecology of a biological control agent of onion bacteria disease. The experiments were conducted in the laboratory of Plant Protection URPP- INRA-Meknes in collaboration with the Laboratory of Plant Biotechnology and Molecular Biology, Faculty of Sciences-Meknes. The main objective of this work is to study the improvement of a_w, temperature and pH tolerance observed in *Pantoea agglomerans* 2066-7.

MATERIALS AND METHODS

Effect of temperature on growth of *P. agglomerans*

To test the ability of *P. agglomerans* 2066-7 to grow at different temperatures onto YPGA medium (5 g.L^{-1} yeast, 5 g.L^{-1} peptone and 10 g.L^{-1} glucose, 18 g.L^{-1} agar), the bacterium was incubated during 24 to 48h under 7°C, 25°C, 30°C, 36°C, 38°C and 40°C.

Effect of pH on growth of *P. agglomerans*

In order to perform the survival study, 9mL of non-acidified (control pH 6.9 ± 0.1) and liquid media at pHs 2, 3, 3.5, 4, 4.5, 5, 6, 7, 8, 9, 10 or 13 ,were inoculated with *Pantoea agglomerans* 2066-7. From the pre-culture, the liquid medium was inoculated by 20µL of 10^7 UFC.mL^{-1} of *P. agglomerans* suspension. The medium employed was "YPG", consisted of 5 g.L^{-1} yeast, 5 g.L^{-1} peptone and 10 g.L^{-1} glucose, for the culture of *P. agglomerans*. All pH determinations were made with the Beckman pH meter, and the pH was adjusted with normal HCl or normal NaOH. The growth was tested by measure of optical density by spectrophomometre (600nm).
Non- acidified basal liquid medium was used as a control.

Population dynamics of *P. agglomerans*

Population dynamics of 2066-7 *P. agglomerans* bacterium were determined on the surfaces of onion bulbs, with or without wounds. Two sets of bulbs were rinsed in fresh water after harvest. One of these sets was wounded (1 mm long and 2 mm deep). All bulbs were dipped in a 2066-7 suspension 10^8 CFU mL^{-1} for 30 s. Once dried, bulbs were placed on tray packs in plastic boxes and incubated at moist chamber at 7°C and 25°C. Populations of 2066-7 were monitored at 0 (just prior to storage), 24, 48, 288 and 312 h on bulbs stored at 25°C, and 0, 1, 2,

18, 20, and 21 days on cold stored bulbs. Four bulbs constituted a single replicate and each treatment was replicated four times. The experiment was carried out twice.
The pieces of peel surface of 1g were removed (including the wounded areas in the case of wounded bulbs) with a knife. Pieces of peel were shaken in 9 ml sterile water on agitator for 30 mins with 125 t/min. Serial dilutions of the washings were made and plated on YPGA Medium (yeast extract, 5 g $.L^{-1}$; peptone, 5 g. L^{-1}; glucose, 10g $.L^{-1}$ and agar, 15 g $.L^{-1}$). Colonies were counted after incubation at 25°C in the dark for 48 h. Population sizes were expressed as CFU ml^{-1} of bulb surface.

Effect of water activity on growth of *P. agglomerans*

Basic medium

The basic medium used in this research consisted of sucrose (10 g $.L^{-1}$) plus yeast extract (5 g $.L^{-1}$) with a pH of 6-7 and a_w of 0.995 (**Costa *et al.*, 2001**). This medium demonstrated good and cost-effective production while maintaining biological control efficacy.

Osmotic stress characterization with different solutes

To obtain information about the a_w solute profile of *P. agglomerans* bacterium, was grown in basic liquid medium and a known amount of CFU were inoculated in Petri dishes that contained the basal agar medium modified with the ionic solute; NaCl and with the nonionic solutes; glycerol, glucose and polyethylene glycol (PEG 600), to 0.98, 0.97, 0.96 and 0.95 a_w (**Teixido *et al.*, 2006**). The solid agar media were inoculated by spread plating a 20µL aliquot of a 10^5 CFU mL^{-1} bacterial suspension of *P. agglomerans* incubated at 7°C, 25°C and 30°C and then visually examined them every 24 h to determine the presence or absence of growth of colonies. This was performed for each a_w and solute condition. All treatments were carried out with four replicates, and the experiment was repeated twice.
Media from the same a_w were always sealed in plastic polyethylene bags to maintain the equilibrium relative humidity conditions and prevent water loss. Percentage of cultivability was calculated comparing CFU on treatments in relation with the control (basal agar medium).

Statistical analysis

Growth rates were subjected to the variance analysis (SAS Institute, INRA). Statistical significance was judged at the $P < 0.05$ level. When analysis revealed statistically significant differences, Duncan's multiple range test for separation of means was performed.
Response surface methodology (RSM) with a generalized linear model design was applied with the SPSS 20. Temperature (7, 25 and 30°C) and a_w (0.98, 0.97, 0.96 and 0.95) were investigated.

RESULTS

Effect of temperature on growth of *P. agglomerans*

Table 1, show the ability of *P. agglomerans* strain to growth at a temperature from 7°C to 38°C.

Table 1 Temperature influence on growth of *P. agglomerans* 2066-7 strain

Temperature	7°C	25°C	30°C	36°C	38°C	40°C
Growth rate	(+)	(+)	(+)	(+)	(+)	(-)

Legend: (+) Growth, (-) Absence of growth

Effect of pH on growth of *P. agglomerans*

Figure 1, show the adaptation of *P.agglomerans* at large range of pH conditions. The initial of colony growth was observed after 24h of incubation on the basic and acidified medium (from 4 to 10.34). The growth was absent in a pH values less than 4 and high than 10.34.

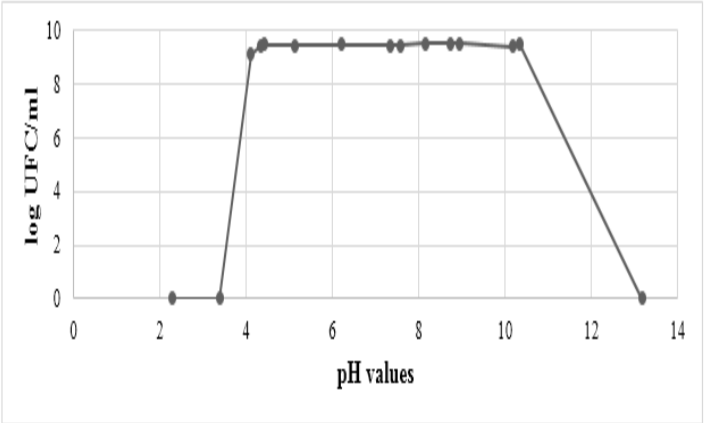
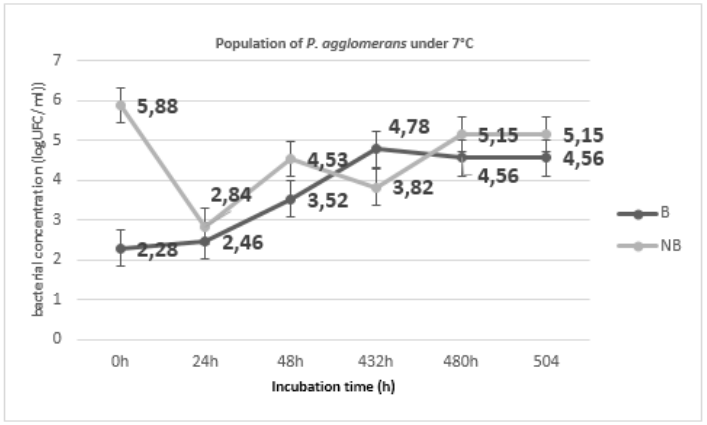


Figure 1 Effect of pH on *P. agglomerans* 2066-7 growth

Population dynamics of *P. agglomerans* bacterium

Population of *P. agglomerans* on bulb surfaces showed the same pattern under the two tested temperature conditions (Fig.2). During the first 24h, the population degreased to return to the initial concentration after 12 and 20 days of incubation under 25°C and cold temperature respectively.



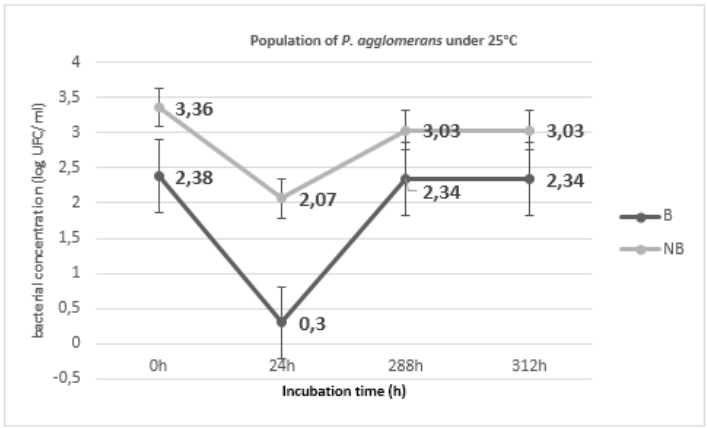


Figure 2 Population dynamics of *P. agglomerans* 2066-7 on surfaces of unwounded (NB). and wounded (B). Onion bulbs incubated at 7°C for 21 days and at 25°C for 13 days. Points represent the means of four replicates and the vertical bars are standard errors.

Osmotic stress characterization with different solutes

The cultivability of *P. agglomerans* at suboptimum a_w media modified with different solutes is shown in figure 3. The growth of the bacterium was very limited in the presence of glucose under 7°C and 30°C and in the presence of glycerol under 7°C. However, it was completely inhibited at values of $a_w \leq 0.97$ and at 0.95 under 25°C on the presence of glycerol and glucose respectively. Generally, under of greater water stress and low temperature there was increased in lag times prior to growth initiation. All solutes exhibited 100% cultivability or near than at ≥ 0.96 under 25°C. These results reflect a good adaptation of *P.agglomerans* on modified medium under 25°C.

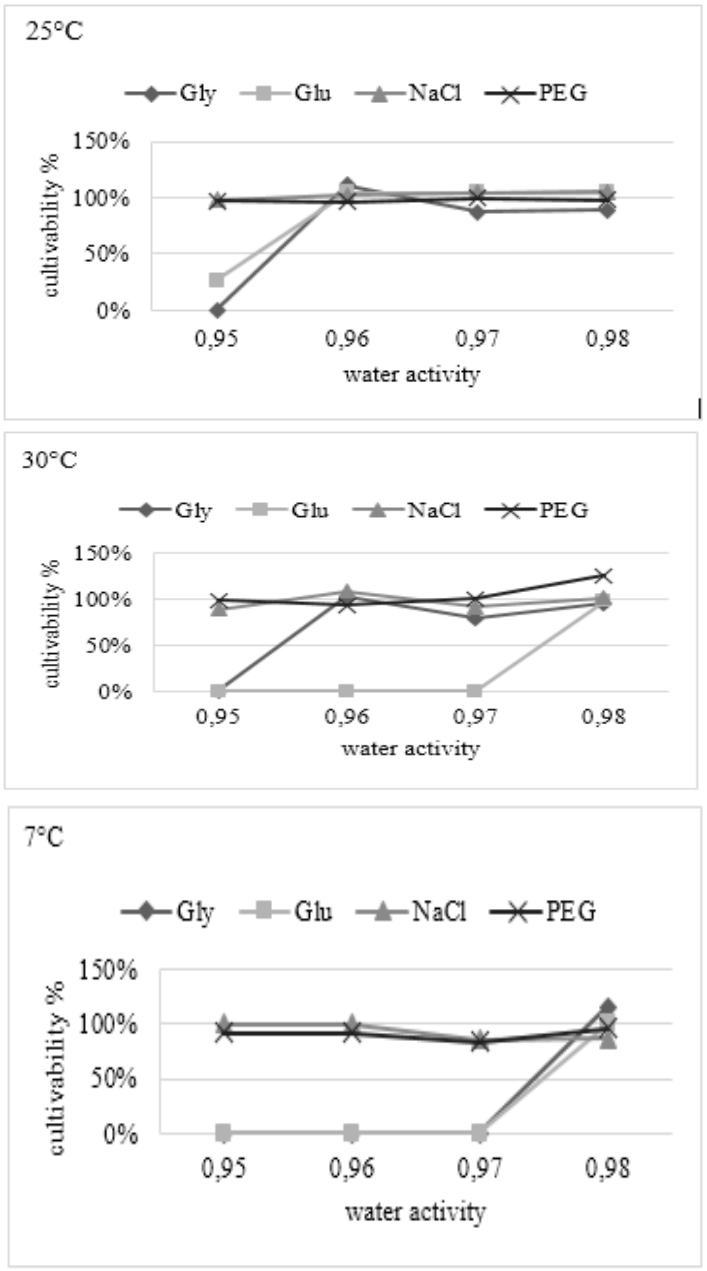


Figure 3 Percentage of cultivability (CFU on treatments in relation with CFU on control basic agar medium) of *P. agglomerans* on low water activity (a_w) modified media using different solutes: glycerol (Gly), polyethylene glycol (PEG), glucose (Glu) and NaCl. Tested a_w were 0.98, 0.97, 0.96 and 0.95. Values are mean of two experiments of four replicates each.

Figure 4, show the growth rate (μ ((LogUFC*10exp2/mL)/j)) of *P. agglomerans* at 7°C on the basic medium supplemented with various solutes us to adjust the water activity. In general, the growth rate degreased as the water activity of the medium degreased. The growth rate was highest at water activity of 0.98 whatever the solute. In the presence of polyethylene glycol or NaCl, *P. agglomerans* was able to grow at all values of a_w. However, the growth stopped at a_w of 0.95 in the presence of glucose or glycerol.

At 25°C, initial of colony growth advanced one day as compared to initiation at 7°C, moreover, the growth rate was higher at 25°C than at 7°C. At a_w of 0.96 and 0.95 the growth rate was highest on basic medium supplemented with glycerol or glucose and NaCl or polyethylene glycol respectively. Growth was stopped at 0.95 a_w in the presence of glucose or glycerol. On basic medium supplemented with polyethylene glycol or NaCl, *P. agglomerans* was able to grow at all a_w values tested at 30°C, the growth stopped at 0.97 and 0.95 on modified medium by glucose and NaCl respectively. Growth rates were much lower as at 30°C than 25°C and at 7°C than 30°C.

Statistical analysis of data, based on variance analysis with three criteria, provided evidence of significant effect ($P<0.05$) of solutes and incubation temperature and interactions thereof on the growth rate of *P. agglomerans*. Duncan's multiple range analysis confirmed a significantly higher growth rate at 0.98 that any other conditions tested, whatever the solute used to adjust the a_w of the medium (Fig.5-A). Apropos of incubation temperature, the higher growth rate was noted under 25°C with a value of 1.7718 (LogUFC*10exp2/mL)/j), under 7°C the value was 0.94 (LogUFC*10exp2/mL)/j) (Fig.5-C).

Finally, the test revealed two distinct groups for the influence of solutes; the growth rate was slightly high on medium modified with polyethylene glycol (1.69) or glycerol (1.52) that unmodified medium (1.516), and reduced when NaCl (1.50) added and reduced more strongly in the presence of glucose (0.59) (Fig.5-B).

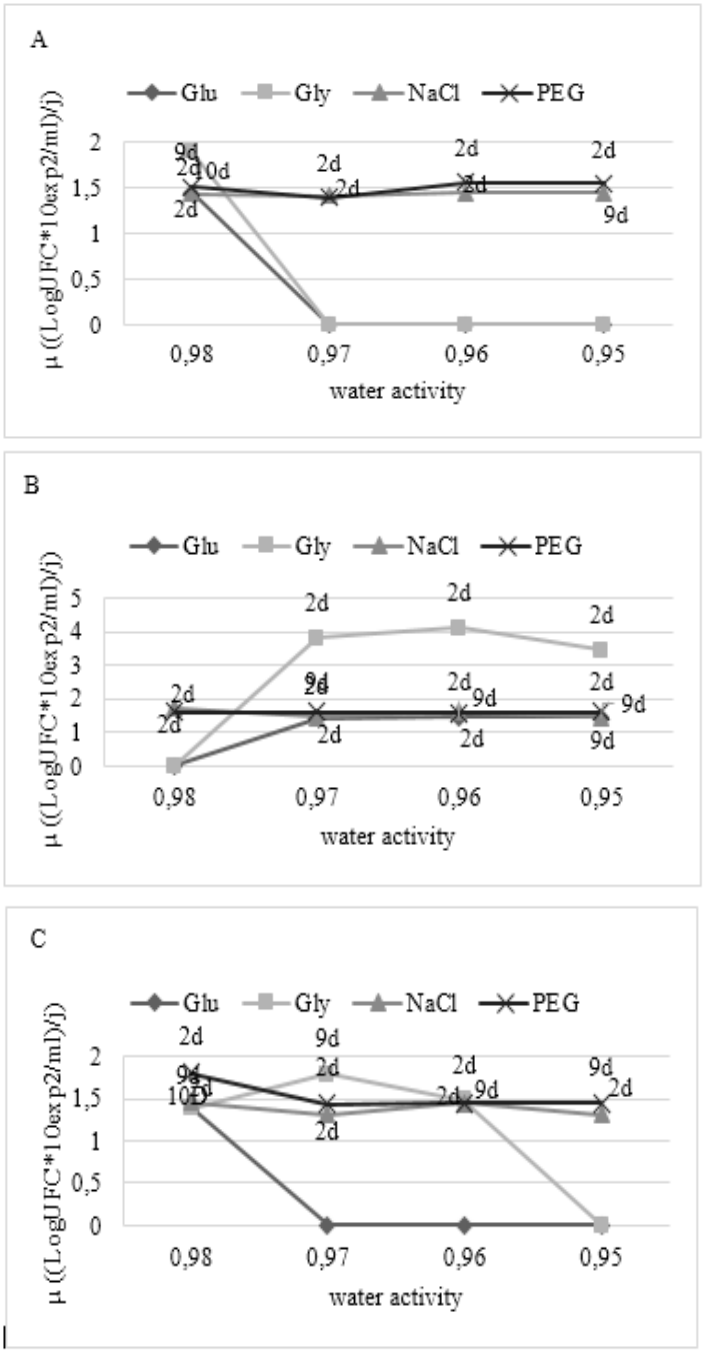
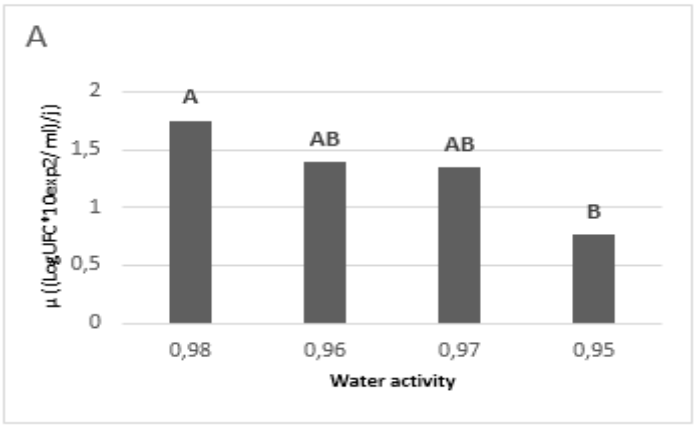
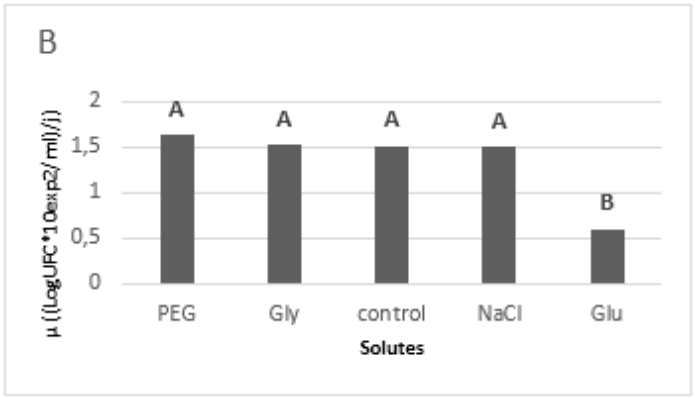


Figure 4 Effect of water activity on growth rate (μ) of *P. agglomerans* in modified medium with, glycerol (Gly), polyethylene glycol (PEG), glucose (Glu) and NaCl at 7°C (A), 25°C (B) and 30°C (C).The number of days for initiation of growth is shown.





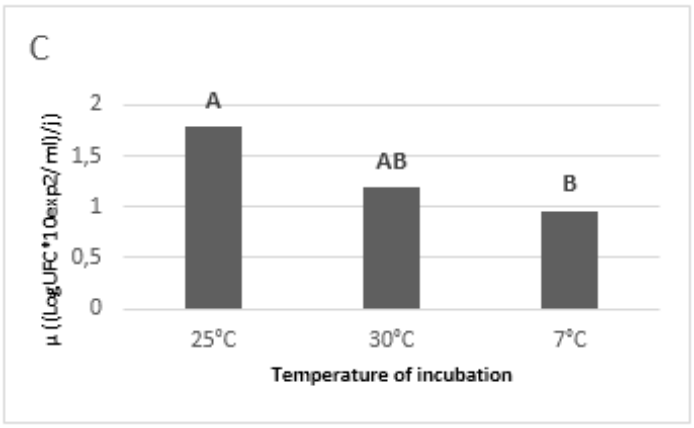


Figure 5 Comparison of *P. agglomerans* growth rate (µ) averages per treatment [water activity (A), solutes (B) and temperature of incubation (C)] performed by the Duncan's range multiple test. The treatments having same letters are not significantly different (P <0.05).

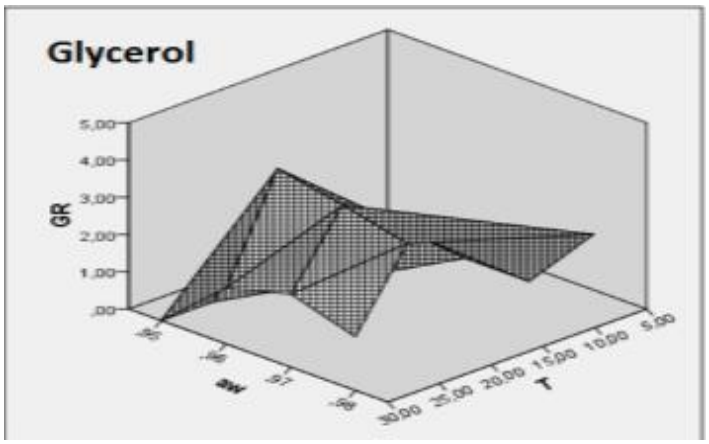
Modelling

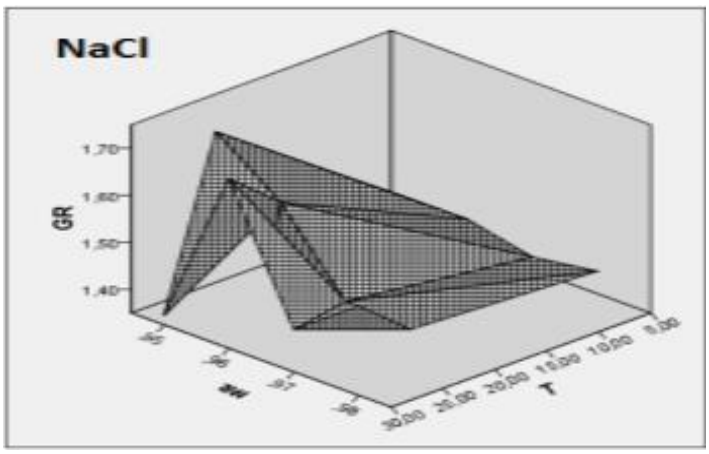
The average growth rates obtained with the model under the various conditions are reported in the table 2. No difference was observed between the observed values and those predicted by the all solutes model. To determine the conditions for growth of *P. agglomerans*, responses showing the predicted effect of a_w and temperature were down from the generalized linear model. For the four models (Fig.6), the response surface showed a growth rate sensitive to the incubations temperature and water activity.

Growth was predicted to be higher at an a_w of 0.98, whatever the temperature tested, the highest growth rate was observed, and the optimum of growth rate was noted at 0.96 under 25°C.

Table 2 Experimental and predicted values of growth rate of *P. agglomerans* obtained by applying generalized linear model for temperature and a_w with glycerol, polyethylene glycol and NaCl models

Environment factor		**Extension growth rate**					
Temperature	a_w	Glycerol		polyethylene glycol		NaCl	
		Observed	Predicted	Observed	Predicted	Observed	Predicted
7°C	0.95	0	0	1.6	1.6	1.44	1.44
25°C	0.95	0	0	1.644	1.644	1.72	1.72
30°C	0.95	0	0	1.45	1.45	1.37	1.37
7°C	0.96	0	0	1.66	1.66	1.44	1.44
25°C	0.96	4.2	4.2	1.62	1.62	1.62	1.62
30°C	0.96	1.5	1.5	1.53	1.53	1.71	1.71
7°C	0.97	0	0	1.42	1.42	1.41	1.41
25°C	0.97	3.85	3.85	1.62	1.62	1.457	1.457
30°C	0.97	1.96	1.96	1.55	1.55	1.44	1.44
7°C	0.98	1.89	1.89	1.65	1.65	1.43	1.43
25°C	0.98	3.45	3.45	1.62	1.62	1.45	1.45
30°C	0.98	1.44	1.44	2.23	2.23	1.54	1.54





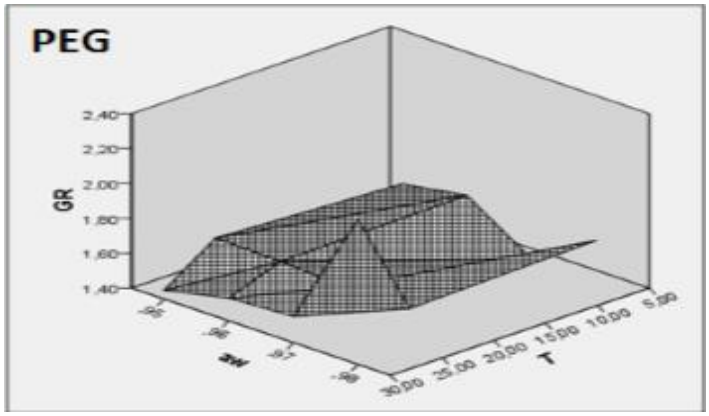


Figure 6 Response surface representing the predicted effect of water activity (aw) and temperature (T) by the three models glycerol, NaCl and Polyethylene glycol (PEG) on growth rate of *P. agglomerans* (GR).

DISCUSSION

Biological control under laboratory or controlled conditions has been demonstrated to be very effective in controlling bacteria disease in large number of studies. However, few biological control products have been commercialized and effectively used under practical conditions. The main reason for this relative lack of success is probably the fact that biological control agents are living organisms and thus limited by fluctuating environmental conditions both during application (with field conditions being the most restrictive) and the formulation process and shelf life. In this work, we tried to improve tolerance of *P. agglomerans* to low a_w and survival during the drying process with osmotic stress, survival on onion bulbs under 25°C and cold temperature (7°C), tolerance on different pH conditions and survival under different temperatures. Moreover, in this study we have modelled the growth rate of *P. agglomerans* according to temperature and a_w, two major factors affecting bacteria growth.

P. agglomerans growth was higher than on unmodified medium when polyethylene glycol was used to modify a_w. **Teixido *et al.* (2006)** found that *P. agglomerans* was less tolerant of low a_w in the presence of polyethylene glycol on medium, this difference of results may be due to the strain nature and isolation environment. In the presence of the glucose the growth was very limited under the three temperatures. Also the results show that, on basic medium supplemented or not with various solutes used to after the water activity of the medium, *P. agglomerans* grows best at temperature of 25°C and at an a_w ranging from 0.96 to 9.98. At low temperature (7°C), *P. agglomerans* can grow at a low a_w (0.95), depending on the solute used to reach this a_w. Similar studies have been carried out reported that other *P. agglomerans* strain and fugal species can grow at low a_w under 25°C (**Teixido *et al.*, 2006 ; Lahlali *et al.*, 2005**). The choice of solute used to modify the water activity of a medium has a significant impact on the growth rate of *P.agglomerans* (**Teixido *et al.*, 2006**).

Our study likewise show that polyethylene glycol, glycerol and NaCl have a lesser effect on the growth rate of *P. agglomerans* than glucose, and it was higher on the presence of polyethylene glycol than unmodified medium.

Most predictive models have been developed for pathogenic bacteria. The main problem was that the difficulty of acquiring sufficient reproducible data, suitable for modelling (**Buchanan, 1993; Gibson and Hocking, 1997**). In the presence study we have modelled the combined effects of temperature and a_w on the *P. agglomerans* growth rate. The data obtained with polyethylene glycol, glycerol and NaCl were modelled by means of linear model.

The model provide better understanding of the development of antagonist *P. agglomerans*. It give a better idea of antagonist respecting the temperature and a_w. Concerning the pH conditions, we improve the good adaptation of *P. agglomerans* under a large range of pH conditions. Moreover, the growth of *P. agglomerans* 2066-7 strain was very effective inside onion bulb wounds at 7°C and 25°C. A similar colonization of *P. agglomerans* strains was reported on apple and pear wounds under cold temperature at different atmospheric conditions (**Bennik *et al.*, 1998; Nunes *et al.*, 2001**).

This indicates an excellent adaptation of strain 2066-7 to cold storage and 25°C temperature, which is an important feature for biological control agents of onion diseases. The antagonist could survive in the microenvironment of the wound and prevent disease.

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MICROBIOTA OF *PINUS* POLLEN AS ADJUVANT FACTOR OF ALLERGY

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ABSTRACT

Bacteria, their endotoxin and mold found on pollen can be a reason of respiratory symptoms in sensitized individuals. This question concerns an anemophilous pollen more acute. In this work quantitative by dilution plating method and qualitative microbial analysis by MALDI-TOF MS Biotyper of pollen and other plants organs of *Pinus sylvestris* L., *P. nigra* Arnold, *P. mugo* Turra, *P. armandii* Franch., *P. wallichiana* A.B. Jacks from Nitra, Slovakia are performed which shows quantitative and species differences in mesophilic aerobic (0.00-6.27 log cfu/g) and anaerobic bacteria (0.00-3.70 log cfu/g), enterococci (0.00 log cfu/g), coliform bacteria (0.00-5.29 log cfu/g), lactobacilli (0.00-4.20 log cfu/g), microscopic fungi and yeasts (2.60-5.29 log cfu/g) content. Representatives of *Pseudomonas* (14), *Bacillus* (2), *Acinetobacter* (1), *Arthrobacter* (1), *Pantoea* (1), *Klebsiella* (1), *Penicillium* (6), *Aspergillus* (4), *Cladosporium* (1), *Debaryomyces* (1) genera were revealed on pine trees. The allergenic potential of the identified association of microorganisms on pollen has been evaluated based on published data. The results may be useful for aerobiologists, allergists and microbiologists, at least at the local level.

Keywords: *Pinus* species, microbiota, pollen contamination, microbiological quality, flowering, allergy

INTRODUCTION

Pinus pollen has natural properties to be allergenic: 1. it's anemophilous; 2. released in large quantities; 3. the genus is widespread in nature (**Noks, 1985; Behrendt and Becker, 2001; D'Amato *et al.*, 2007; Maňka *et al.*, 2013; Turos *et al.*, 2013**). At the same time, a *Pinus* pollen grain, for example, *Pinus sylvestris* L. is heavier (30.08 mμg) in comparison with a pollen grain of *Betula verrucosa* Ehrh. (9.48 mμg) – known potent allergen. They also differ slightly both settling velocity of grains in still air: for *P. sylvestris* L. is 3.69 cm/sec, for *B. verrucosa* Ehrh. – 2.94 cm/sec and the probable range drift of pollen by wind – 1700-1775 and 600 km, respectively (**Dyakowska and Zurzycki, 1959**). It is known more widely about the beneficial properties of pine pollen (**Lee *et al.*, 2009; Solntseva and Glazunova, 2010**). Pine pollen has long been considered a non-allergenic pollen (**Gastaminza *et al.*, 2009; Vinhas *et al.*, 2011**). The main reasons of this are the large size of a grain, its low levels of proteins and the presence of a waxy hydrophobic layer that averts protein release. Thus, the length of the polar axis of the *P. sylvestris* L. pollen grain is 67.4 (60-74) μm, *P. nigra* – 75.5 (67-87) μm. For comparison, the size of *Betula pendula* pollen grain is only 22.6 (21-25) μm (**polleninfo**). As **Přidal (2003)** determined the amount of proteins in the pollen of *Betula* is 23.02%, in *Pinus* pollen – 14.14% (**Nôžková *et al.*, 2014**). **Özler *et al.* (2009)** defined pollen of *Pinus nigra* subsp. *nigra* var. *caramanica* as important allergenic tree pollen because of content of amino acids and total protein. **Gastaminza *et al.* (2009)** detected high degree of cross reactivity among the pollen of the distinctive pine species. Cases of allergic sensitization by pine pollen are known and enough spread (**Green *et al.*, 2003; Gastaminza *et al.*, 2009**).

The genus *Pinus* includes 116 species. This genus is subdivided into subgenus *Strobus* with 43 species and subgenus *Pinus* with 73 species (**Maňka *et al.*, 2013**). *Pinus* pollen is predominant in the atmosphere of most environments: from the Arctic Circle to Guatemala, the West Indies, North Africa and Malayan Archipelago (**Green *et al.*, 2003; Maňka *et al.*, 2013**). Slovakia, as a mountainous and forested country (40.6% forest cover) in central Europe, has a large variety of vegetation zones, forest types, and a rich variety of forest tree species. *Pinus sylvestris* L. is one of the most important tree species (**Oszlányi, 1997**). Pines are frequently planted in city parks or near streets (**Ivanová and Bernadovičová, 2010; Dušička *et al.*, 2013**). Plant wealth, closeness to living environments and regional geography are additional factors that can prompt to local allergic sensitization by pine pollen (**Shah and Grammer, 2012**).

Gram-negative and Gram-positive bacteria, mould are the source of allergens. They were revealed on the surface of the pollen grains of anemophilous plants as well as endotoxin (**Śpiewak *et al.*, 1996; Heydenreich *et al.*, 2011; Mittag *et al.*, 2013**). These microorganisms are attached to the pollen exine or located within the pollen cells likely before the opening of the catkins. On the other hand they may be airborne contaminants that alight on the pollen during collection and handling (**Madmony *et al.*, 2005**). Airborne bacteria are more likely use pollen grain as a transport or source of nutrients (**González *et al.*, 2005; Madmony *et al.*, 2005; Després *et al.*, 2011**). Such pollen-associated bacteria have a privilege: ability to thrive within plant tissues gives them, in turn, with several advantages, e.g., an environment with little competition, protection from environmental stresses and a reliable food source (**Madmony *et al.*, 2005**). **Heydenreich *et al.* (2011)** showed that Gram-positive and Gram-negative bacteria on the surface of grass pollen grains may serve as adjuvants by augmenting dendritic cells maturation and inflammatory Th1, Th2 and Th17 responses helping to initiate allergic immune responses.

Based on the aforesaid the aim of this study is to analyze the surface of *Pinus* pollen grains on the presence of microorganisms, identify them and conclude about allergenic potential of *Pinus* pollen known as pollen with low allergenicity.

MATERIALS AND METHODS

Selected *Pinus* species

Five *Pinus* species were selected for microbiological analysis. They are: Scots pine (*P. sylvestris* L. (PS)), Austrian pine also called European black pine (*P. nigra* Arnold (PN)), Dwarf pine (*P. mugo* Turra (PM)), Chinese white pine (*P. armandii* Franch. (PA)), Blue pine or Himalayan pine (*P. wallichiana* A.B. Jacks (PW)). *P. sylvestris, P. nigra, P. mugo* belong to the subgenus *Pinus*, *P. armandii* and *P. wallichiana* – to the subgenus *Strobus* (**Maňka *et al.*, 2013**).

Scots pine and dwarf pine represent the two principal species of pines indigenous to Slovakia. They distribute mainly allopatric in the country (**Kormuťák *et al.*, 2013**). *P. sylvestris* L. has been chosen as the main object of this study as it is the widespread species.

All trees are growing in the same natural conditions at the Botanical garden of the Slovak Agricultural University in Nitra.

Plant and pollen materials description

Unopened male cones were collected before they were ready to shed pollen aseptically in April-May 2015. Then they were dried at room temperature overnight. Pollen was collected the next day, keeping sterile conditions. Samples were placed in a cooler and conserved for a few days until analysis at +4°C. Since some *Pinus* species flowered with a difference of one month, the microbiological analysis of pollen samples was carried out when the plant material was collected.
Samples of the other organs of *Pinus sylvestris* L. trees were collected for a more detailed analysis. They are: male cones before, during and after flowering, young and mature female cones, needles. Also male cones during and after flowering were prepared for the other *Pinus* species. Altogether thirteen pine samples were collected.
All the trees are without visible damage.

Air samples

Air samples around the trees were studied for comparison the qualitative composition of the microbiota of trees and air. Air is not a medium in which microorganisms can develop, but has a carrier particulate matter, dust and droplet which can be weighed down with microbes (**Latha and Mohan, 2013**). "Koch-type" sedimentation method was used for sampling the air (**Tóth *et al.*, 2013**). Open Petri dishes with meat peptone agar medium (Imuna, Slovak Republic) and malt extract agar base (Biomark Laboratories, India) were placed under the studied tree's crown and exposed for 10 and 15 minutes. Air sampling was performed three times on different days. Altogether fourteen air samples were investigated.

Quantitative microbial analysis

One gram of each sample of pollen were diluted in 99 mL of sterile saline solution and mixed for 30 min by vigorous shaking in Rotamax 120 Orbital shaker (Heidolph, Germany) (175 rpm) at room temperature. Ten-fold serial dilutions of suspension were made up to 10^{-3}. The serial dilutions were inoculated on appropriate nutrient media. In whole every sample was cultivated on Petri dishes quadruplicate on six kinds of nutrient media. The formed colonies on the plates were counted and expressed as log colony forming units/g (log cfu/g).
Total mesophilic aerobic and anaerobic microbiota was determined by spreading 1 mL from the appropriate dilution into sterile Petri dishes to which meat peptone agar (Imuna, Slovak Republic) was poured. Plates were incubated aerobically at 25°C and counted after 48-72 h.
Total *Enterococcus* was determined in the same way but only Slanetz-Bartley medium was used (HiMedia, India). Plates were incubated at 37°C for 48 h.
Total coliforms: A 1 mL aliquot of the appropriate dilution were transferred into Petri plates and poured with violet red bile with lactose agar (Pronadisa, Spain). Plates were incubated at 37°C and colonies were counted after 24 h.
Lactobacilli were determined by spreading of 1 mL from the appropriate dilutions on MRS Agar Modified (HiMedia, India) and incubated at 37 °C for 48-72 h.
Total microscopic fungi and yeasts were determined by spreading 0.1 mL from appropriate dilutions on Sabouraud medium (Biomark Laboratories, India). Plates were incubated at 28°C and counted after 7 days.

Qualitative microbial analysis

Qualitative microbial analysis of plant materials was performed using MALDI-TOF Mass Spectrometry (Bruker Daltonics, Germany). MALDI-TOF MS is a rapid, reliable diagnostic tool for the identification of most microorganisms (Barberis et al. 2014). For identification, the Biotyper software compares the sample spectrum to its database of spectra generated using characterized isolates (DeMarco and Burnham 2014). After counting of the colonies best of them were selected for the identification. All isolates were pick out from the Petri dishes and transferred into 300 µL of distilled water in Eppendorf tubes. Then 900 µL of ethanol was added and the tubes were centrifuged for 2 minutes at 14,000 npm. The supernatant was carefully pipetted off and discarded. The same spin was repeated on the pellet. All remaining ethanol was removed, and the pellet was allowed to dry. Ten microliters of 70% formic acid was mixed with the pellet by pipetting and vortexing. Then 10 µL of acetonitrile was added. Tubes were centrifuged for 2 minutes at 14,000 npm and 1 µL of the supernatant was applied to the MALDI target. Once dry, every spot was overlaid with 1 µL of HCCA matrix and left to dry at room temperature before analysis. Spectra were generated and MALDI-TOF analyzed on the Microflex LT (Bruker Daltonics) instrument using Flex Control 3.4 software and Biotyper Realtime Classification 3.1 with BC specific software. Criteria for successful identification as proposed by the manufacturer were a confidence score of ≥2.0 for species level and ≥1.7 for genus level (**DeMarco and Burnham, 2014**).

Statistical analysis

Results were evaluated by standard techniques using MS Excel and Statistica 10. Mann-Whitney U-test was used for pairwise comparisons of the data.

RESULTS

Microbial content of *Pinus* trees samples is presented in Tables 1 and 2.

Table 1 Microbial enumeration of *Pinus* trees samples collected in Nitra, Slovak republic in 2015, log cfu/g

Plant part	Group of microorganisms	
	Total mesophilic aerobic counts	**Total mesophilic anaerobic counts**
Male cones PS BF[a]	2.70	0.00
Male cones PS DF	3.85	0.00
Male cones PN DF	6.02	0.00
Male cones PM DF	5.16	2.30
Male cones PS AF	3.48	0.00
Needles PS AF	3.00	0.00
Female cones mature PS DF	0.00	3.00
Female cones young PS AF	0.00	0.00
Pollen PS	5.66	0.00
Pollen PN	6.27	3.70
Pollen PM	4.23	0.00
Pollen PA	5.57	2.00
Pollen PW	6.02	0.00
	***Enterococcus* counts**	**Total coliforms**
Male cones PS BF	0.00	2.00
Male cones PS DF	0.00	2.00
Male cones PN DF	0.00	2.60
Male cones PM DF	0.00	2.70
Male cones PS AF	0.00	2.00
Needles PS AF	0.00	0.00
Female cones mature PS DF	0.00	0.00
Female cones young PS AF	0.00	0.00
Pollen PS	0.00	0.00
Pollen PN	0.00	5.29
Pollen PM	0.00	5.17
Pollen PA	0.00	3.48
Pollen PW	0.00	5.01
	Total lactobacilli counts	**Microscopic fungi and yeast**
Male cones PS BF	4.08	4.54
Male cones PS DF	3.30	4.63
Male cones PN DF	0.00	4.63
Male cones PM DF	3.60	4.99
Male cones PS AF	3.48	4.18
Needles PS AF	3.30	2.60
Female cones mature PS DF	3.85	3.95
Female cones young PS AF	4.20	3.00
Pollen PS	3.90	4.32
Pollen PN	0.00	3.69
Pollen PM	3.00	4.79
Pollen PA	0.00	5.03
Pollen PW	3.48	5.29

BF – before flowering; DF – during flowering; AF – after flowering

As can be seen from Table 1 the total content of mesophilic aerobic bacteria of the investigated samples is 0.00 (young and mature female cones of *P. sylvestris*) – 6.27 (pollen of *P. nigra*) log cfu/g. A higher level of the presence of aerobic bacteria on pollen and male cones compared with female cones and needles clearly expressed. Within this group of microorganisms *Pseudomonas asplenii, P. chlororaphis, P. corrugate, P. koreensis, P. orientalis, P. tolaasii, P. rhodesiae, P. fluorescens, P. libanensis, P. veronii, P. extremorientalis, P. trivialis, P. synxantha, P. grimontii, Bacillus flexus, B. licheniformis, Acinetobacter lwoffii*, *Arthrobacter* sp. were identified using MALDI-TOF (see Table 2). All these bacteria were determined only on pollen grains.
The level of contamination by mesophilic anaerobic bacteria in comparison with the aerobic bacteria is much lower: 0.00 (all samples, except male cones of *P. mugo*, mature female cones of *P. sylvestris*, pollen of *P. armandii*) – 3.70 (pollen of *P. nigra*) log cfu/g. Samples of *P. nigra* and also *P. armandii* are the most contaminated by mesophilic bacteria among all the samples of pollen. In this group of microorganisms was not possible to identify bacteria using MALDI-TOF on a reliable level.
Enterococci were not detected on investigated *Pinus* samples. It's a good result from the sanitary point of view.
Unfortunately, total coliforms were found out on samples at the level from 0.00 (young and mature female cones, needles and pollen of *P. sylvestris*) to enough

high – 5.29 (pollen of *P. nigra*) log cfu/g. Pollen is more contaminated by coliforms than male cones. *Pantoea agglomerans* was identified using MALDI-TOF within this group of microorganisms. This is the most common species on investigated *Pinus* samples. It was revealed on all pollen samples and male cones. Also *Klebsiella pneumonia* was identified on male cones of *P. sylvestris* before and during flowering.

The amount of lactobacilli varies from 0.00 (male cones of *P. nigra*, pollen of *P. nigra* and *P. armandii*) to 4.20 (young female cones of *P. sylvestris*) log cfu/g. In general, lactobacilli are much observed on other parts of the tree rather than on pollen. During the analysis it was found that this group of microorganisms to identify the hardest. It was possible to identify only unusual to this group of microorganisms.

Colonies of microscopic fungi and yeasts are most numerical. They were detected in all the samples, without exception. The fewest of microscopic fungi and yeasts were defined on the needles of *P. sylvestris* (2.60 log cfu/g), the greatest number – on the pollen *P. wallichiana* (5.29 log cfu/g). During flowering the level of microscopic fungi and yeasts is higher on parts of the tree involved in flowering, meaning male cones and pollen. A number of yeast colonies in comparison with the colonies of microscopic fungi is more numerical. Representatives of the genera *Penicillium, Aspergillus, Alternaria, Cladosporium, Rhodotorula* and unidentified genus *Mycelium sterillium* without creation fruiting bodies were determined microscopically. Then presence of *Penicillium chrysogenum, P. digitatum, P. italicum, P. roqueforti, P. expansum, P. commune, Cladosporium herbarum, Aspergillus oryzae, A. flavus, A. versicolor, A. parasiticus,* among yeast – *Debaryomyces hansenii* was confirmed by MALDI-TOF. Also, the presence of such yeasts like *Candida sorbosa, C. guilliermondii, C. dubliniensis, Lodderomyces elongisporus* have been revealed, but at the level of less than 1.7.

Table 2 The microbial species composition isolated from the parts of *Pinus* trees

№	Identified microorganism	Part of *Pinus* trees
1	*Pseudomonas asplenii*	Pollen PS
2	*Pseudomonas chlororaphis*	Pollen PS
3	*Pseudomonas corrugata*	Pollen PS
4	*Pseudomonas koreensis*	Pollen PS
5	*Pseudomonas orientalis*	Pollen PN
6	*Pseudomonas tolaasii*	Pollen PN, PS
7	*Pseudomonas rhodesiae*	Pollen PN
8	*Pseudomonas fluorescens*	Pollen PN
9	*Pseudomonas libanensis*	Pollen PN
10	*Pseudomonas veronii*	Pollen PN
11	*Pseudomonas extremorientalis*	Pollen PN
12	*Pseudomonas trivialis*	Pollen PN
13	*Pseudomonas synxantha*	Pollen PN
14	*Pseudomonas grimontii*	Pollen PN
15	*Bacillus flexus*	Pollen PW
16	*Bacillus licheniformis*	Pollen PS, PM, PW
17	*Acinetobacter lwoffii*	Pollen PM, PS, PA
18	*Arthrobacter* sp.	Pollen PA
19	*Pantoea agglomerans*	Pollen PS, PM, PW, PA, PN, male cones PS, PM, PN DF, male cones PS AF
20	*Klebsiella pneumonia*	Male cones PS BF and DF
21	*Penicillium chrysogenum*	Pollen PS, PM
22	*Penicillium digitatum*	Pollen PS, PM, PA
23	*Penicillium italicum*	Pollen PS, PM
24	*Penicillium roqueforti*	Pollen PM, PA, PS
25	*Penicillium expansum*	Pollen PA, PS
26	*Penicillium commune*	Pollen PA
27	*Cladosporium herbarum*	Pollen PS, PN, PM, PA, PW
28	*Aspergillus oryzae*	Pollen PA, PN, PW, PS
29	*Aspergillus flavus*	Pollen PN, PW, PA, PS
30	*Aspergillus versicolor*	Pollen PS
31	*Aspergillus parasiticus*	Pollen PS, PN, PA
32	*Debaryomyces hansenii*	Pollen PS, PN, PM, PA, PW, male cones PS DF, AF, mature female cones PS

BF – before flowering; DF – during flowering; AF – after flowering

As shown the microbiological and statistical analysis, samples of pine pollen are more contaminated by microorganisms than other parts of trees (see Table 2). There are no significant differences between the number of microorganism colonies of pollen and male cones during flowering period after paired comparison (see Figure 1). However, there was found a strong tendency to the predominance of anaerobic bacteria in the pollen, in comparison with the cones at the stage of flowering (p=0.07). It is also proved that the number of fungi and yeasts colonies on the male cones after flowering less than on pollen (U=4, p=0.015).

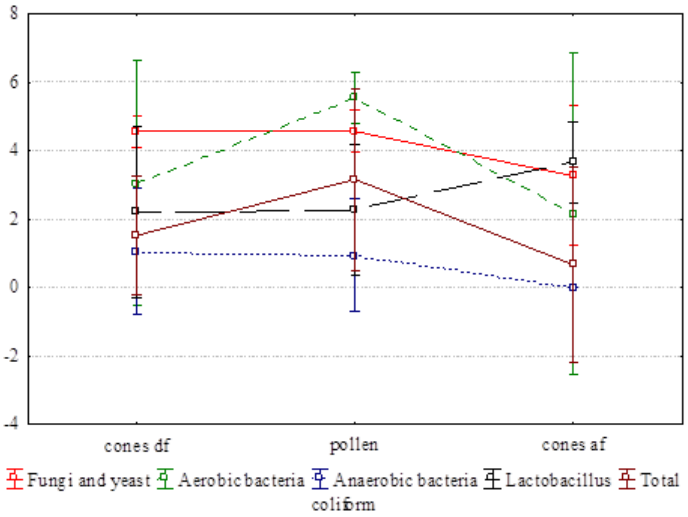

Figure 1 Comparison of the number of colonies of microorganisms groups on pollen and male cones of *Pinus* during and after flowering using Mann-Whitney U-test

Pollen of *P. sylvestris* has the most diverse composition of the microbiota. Of the 32 identified microorganisms 20 species of them belong to *P. sylvestris* samples, 15 to *P. nigra*, whereas 11 to *P. armandii*, 8 to *P. mugo*, only 6 to *P. wallichiana*. *Pantoea agglomerans, Debaryomyces hansenii* and *Cladosporium herbarum* are microorganisms the most commonly found on *Pinus* samples.

Bacillus licheniformis, Pantoea agglomerans, Penicillium commune, P. verrucosum, P. roqueforti, P. italicum, P. digitatum, P. expansum, P. lanosum, P. corylophilum, Mucor ramosissimus, M. circinelloides, Chaetomium funicola, Rhizopus stolonifer, Absidia coerulea, Alternaria alternata, Aureobasidium pullulans, Paecilomyces lilacinus, Cladosporium sp., unidentified genus *Mycelium sterillium* without creation fruiting bodies were found in the air samples around the pines at the Botanical garden. The composition of the microbiota of the air is quite diverse. According to data of the aerobiological station of Nitra region of the Slovak Republic concentrations of fungal spores were at level "very high" in the days of sampling the air (more than 150 particles per 1 m^3 of air). During the flowering season of *Pinus* only daily concentration of *Cladosporium* spores were at significant level in the Nitra region (values of average weekly concentrations of spores per 1 m^3 of air were in the range 27-141). The *Cladosporium* spores generally exceed all other airborne biologic particles in outdoor air (**Ozdemir, 2015**). Concentration levels of *Alternaria* (1-8/m^3), *Epicoccum* (0-3/m^3), *Stemphylium* (1-3/m^3), *Helminthosporium* (0/m^3) spores in the air of Nitra region were low or very low. Allergic relevant concentrations of *Pinus* pollen in the air was not observed (**www.alergia.sk**).

DISCUSSION

Thus, it has been detected and partially identified representatives of mesophilic aerobic and anaerobic bacteria, lactobacilli, coliform bacteria, microscopic fungi and yeasts on samples of pollen, male and female cones, needles of five representatives of *Pinus* species, that grow at the Botanical garden in Nitra, Slovakia. Microscopic fungi and yeasts are the largest group of microorganisms for the *Pinus* trees (mean=4.28 log cfu/g) and aerobic bacteria (4.00 log cfu/g), the least – anaerobes (0.85 log cfu/g). Young female cones are the least contaminated by microorganisms among all investigated parts of the tree (identified only representatives of lactobacilli, microscopic fungi and yeast). It is quite logical: young female cones are just beginning to take shape after pollination. There are enough nutrients on other organs of the tree for the bacteria. The presence of microscopic fungi and spores can be explained by the fact that fungi are the most common organisms in nature (**Latha and Mohan, 2013**). Moreover, pollen is better partner in the air for the airborne microorganisms (**Després *et al.*, 2012; Sapiňa *et al.*, 2013**). Mature last year's female cones and needles are also less attractive to microorganisms in comparison with pollen and male cones. Representatives of only three groups of microorganisms from the six examined were identified on it. Perhaps, this is because of the flowering period. Pollen abundantly releases, pour out on all parts of the plant, it is in the air and bacteria cannot not dealing with it.

There are differences among *Pinus* species in the quantitative composition of the microbiota of the male cones in the flowering period. These differences are most appreciable in quantitative composition of aerobes, anaerobes and lactobacilli. There are also species differences in the level of pollen microbial contamination. Pollen of *P. sylvestris* contaminated least by all groups of microorganisms (on average 2.78 log cfu/g). Pollen of *P. wallichiana* contaminated most (3.96 log cfu/g). The result is interesting because although pollen of *P. sylvestris* less contaminated quantitatively, the qualitative composition of it microbiota is most diverse. In the case of *P. wallichiana* pollen the result is opposite (see Table 2).

The level of contamination by all groups of microorganisms of *P. nigra* pollen is 3.79 log cfu/g, *P. mugo* – 3.44 log cfu/g, *P. armandii* – 3.22 log cfu/g. It was found the absence of representatives of microorganisms, at least, for two groups for all species of pollen.

With regard to the microbiota of *P. sylvestris* – species, for which the microbiological composition was done the most complete, quantitatively differences were revealed between the trees organs of this species. Thus, male cones most contaminated by coliform bacteria, fungi and yeast at different stages concerning the period of flowering, pollen – by aerobic bacteria, mature female cones – by anaerobic bacteria, young – by lactobacilli.

Quantitative microbiological analysis of *Pinus* trees and air samples near the trees showed that microscopic fungi, yeast, aerobic bacteria are the most widespread microorganisms during the flowering period of this genus. Their colonies are most often on the pollen and the male cones in the flowering period. And after this period there is a tendency to reduce them on the male cones. This indicates that the reason for the presence of microorganisms is pollen. Since pollen samples were investigated before their release into the environment, it is possible to assume that pollen contamination by these representatives occurs before the mass flowering. **Madmony *et al.* (2005)** also consider that different fungi and bacteria are located inside the pollen cells probably before the opening of the catkins. Likely the pollen contamination is caused by airborne microbiota encountered during pollen collection and handling. Content of air microbiota may be the proof. With regard to species differences in composition of the microbiota of pollen, the clear tendency for representatives of taxonomically related species subgenus *Pinus* and subgenus *Strobus* have not been identified. The representatives of these subgenera flowered with a difference of one month. Weather conditions were optimal. **Maňka *et al.* (2013)** presented results that show closeness of the pollen viability characteristics in taxonomically related species of pines growing on the same territory and shedding their pollen in the same period of time as compared with the comparing characteristics of pollen in taxonomically distant species shedding their pollen in different periods of the flowering time.

Based on the analysis of the quantitative composition of the microbiota of *Pinus* representatives can assume about an allergenic potential of pollen. Based on the analysis of qualitative composition the assumptions will be more thorough.

In general, 32 species of microorganisms belonging to the aerobic and coliform bacteria, fungi and yeasts were identified with MALDI-TOF. Their number is maybe even more diverse, but to confirm this on confidence level was not possible. Representatives of *Pseudomonas* (14 species), *Penicillium* (6 species) and *Aspergillus* (4 species) appeared the most various. In recent years, *Pseudomonas* strains have been studied with increasing interest due to their importance in medical, food and environmental microbiology and phytopathology (**Baïda *et al.*, 2002**). Genus *Pseudomonas* has environmental interest. Plant growth promoters, plant pathogens and xenobiotic degraders are among them. It is also one of the most important and best-studied bacterial taxa in soil (**Ivanova *et al.*, 2002; Bultreys *et al.*, 2003; Garbeva *et al.*, 2004; Moore *et al.*, 2006**). Really, 9 from 14 representatives of *Pseudomonas* genus can be found in the soil (**Brodey *et al.*, 1991; Bodelier and Laanbroek, 1997; Behrendt *et al.*, 2003; Kwon *et al.*, 2003; Haas and Keel, 2003**), other five were isolated from the aquatic ecosystems (**Elomari *et al.*, 1996; Dabboussi *et al.*, 1999; Baïda *et al.*, 2002; Ivanova *et al.*, 2002**). *Pseudomonas*, as different epiphytes, may influence plant productivity negatively, e.g., through induction of frost injury, or positively, e.g., by production of phytohormones that improve development. Phytopathogenic *Pseudomonas* species are distributed worldwide, causing diseases of most major groups of higher plants. They generally can be found only on diseased plants, in which they appear as relatively homogeneous populations when the pathological lesions are young (**Moore *et al.*, 2006**).

From the natural airborne allergens, pollen of grass and trees are the most important outdoor allergenic sources. Other important allergenic sources are airborne mould (**Benndorf *et al.*, 2008**). The genera of molds causing allergy and allergy-related problems most often are *Alternaria alternata, Cladosporium herbarium, Aspergillus fumigates* and *Penicillium* (**Ozdemir, 2015**). *Penicillium* species are among the most common fungi present in the environment. Observably, *Penicillium* is one of the most abundant fungal floras with the intention that there are 10^6–10^8 spores in one gram of normal soil and 10^4 spores in one milliliter of unpolluted groundwater. They are usually considered non-pathogenic to humans. *Penicillium* species can cause opportunistic infections (**Oshikata *et al.*, 2013**). *Aspergillus* infections have grown in importance in the recent years (**Hedayati *et al.*, 2007**). Several species of *Aspergillus* have been shown to be allergenic, including *A. fumigatus, A. niger, A. flavus* and *A. oryzae. A. oryzae* and *A. flavus* are the two species are so closely related. *A. flavus* is the second leading cause of invasive aspergillosis and it is the most common cause of superficial infection (**Hedayati *et al.*, 2007**). Aflatoxins, produced predominantly by fungi such as *A. flavus* and *A. parasiticus*, are among the most potent natural carcinogens known (**Yu *et al.*, 2003**). In humans there is cross-reactivity to *Aspergillus* and *Penicillium*: most sera from patients with precipitins against *Penicillium* have precipitins against *Aspergillus* (**Oshikata *et al.*, 2013**).

Also interesting representatives of the microbiota of anemophilous pine pollen is *Pantoea agglomerans*, *Pseudomonas libanensis, P. veronii, P. extremorientalis, P. grimontii* (**Špiewak *et al.*, 1996; Baïda *et al.*, 2002; Leclerc, 2003; Nam *et al.*, 2003; Egamberdieva, 2011**). *P. agglomerans* (formerly *Enterobacter agglomerans*) is phytopathogen causing human disease. **Špiewak *et al.* (1996)** showed that *P. agglomerans* is present on pollen grains as well as endotoxin – the bacterial product having strong immunomodulating properties. From the literature it is known that the abovementioned *Pseudomonas* were isolated from natural springs in Lebanon, Russia, France. *Pseudomonas extremorientalis*, *P. chlororaphis* and *P. veronii* have the ability to survive in ecologically stressed conditions, such as saline soils, contaminated soils with simple aromatic organic compounds (**Nam *et al.*, 2003; Egamberdieva, 2011**).

We classified representatives of identified microorganisms as to their pathogenic properties based on published data. Thus, eight microorganisms belong to phytopathogens (*Pseudomonas asplenii, P. corrugata, P. tolaasii, P. digitatum, P. italicum, P. expansum, C. herbarum, P. agglomerans*), 4 species to human pathogen (*P. fluorescens, K. pneumonia, P. chrysogenum, P. commune*), with possible pathogenic properties 6 microorganisms (*P. trivialis, A. lwoffii, A. oryzae, A. flavus, A. versicolor, A. parasiticus*), not pathogen – 5 (*P. chlororaphis, B. licheniformis, Arthrobacter* sp., *P. roqueforti, D. hansenii*), unknown pathogenicity – 9 representatives of identified species (*P. orientalis, P. koreensis, P. rhodesiae, P. libanensis, P. veronii, P. extremorientalis, P. synxantha, P. grimontii, B. flexus,*). So, according to literature data, 56% of the identified microorganisms in samples of *Pinus* representatives have pathogenic nature, 16% – not pathogenic and 28% – unknown. According to the «List of airborne pathogens, including allergenic, toxigenic, and suspected respiratory and non-respiratory pathogens» eight identified microorganisms (*K. pneumonia, Acinetobacter, P. chrysogenum, P. commune, P. expansum, A. flavus, A. versicolor, C. herbarum*) correspond to the list (**Breitenbach and Simon-Nobbe, 2002; Schwab *et al.*, 2004; Fomicheva *et al.*, 2006; Hedayati *et al.*, 2007; Debarry *et al.*, 2010**). Cases of allergic sensitization are known also for *A. oryzae* and *D. hansenii* (**Barbesgaard *et al.*, 1992; Yamamoto *et al.*, 2002**). Frequency of occurrence of allergenic species on pollen of *P. sylvestris* is 9 from 10, *P. armandii* – 8 from 10, *P. nigra*, *P. mugo* and *P. wallichiana* – 5 from 10. That is, theoretically, all the investigated pollen samples of *Pinus* carry allergenic stimuli on themselves.

Also among the identified microorganisms are those that have useful properties. For example, *Pseudomonas chlororaphis* (acting against various fungal plant pathogens by creating phenazine), *Pseudomonas synxantha* – a fluorescent rhizosphere bacterium with nematicidal properties, *Bacillus licheniformis,* that demonstrates antifungal activity by producing an antibiotic that acts against fungi, *Penicillium roqueforti* are used to produce compounds that can be employed as antibiotics, flavours and fragrances (**Wechter *et al.*, 2002; Rij *et al.*, 2004; Pringle, 2005; Ropars *et al.*, 2012**). Among «bad» microorganisms there are representatives also with useful properties. For example, *Aspergillus versicolor* is very effective at removing lead ions, *Aspergillus oryzae* is used in rice saccharification for sake brewing (**Kitamoto, 2002; Fomicheva *et al.*, 2006**).

On the basis of microbiological analysis, and analysis of literature data, making a conclusion about the potential allergenicity of *Pinus* pollen it can be assumed that pollen of *P. sylvestris* would most allergenic. Most of microorganisms were identified on these samples (20 of 32), the frequency of occurrence of allergenic species is maximal – 9 out of 10, but in general, the pollen of *P. sylvestris* least contaminated by microorganisms (2.78 log cfu/g), the third highest level of contamination by aerobic bacteria (5.66 log cfu/g), and the fourth – of contamination by fungi and yeasts (4.32 log cfu/g). The percentage of occurrence of allergenic species of microorganisms among identified should be consider as more important factor. It is 45% for *P. sylvestris* pollen. According to the same criteria pollen of *P. nigra* possess the minimum allergenic potential – 33.3%. This pollen sample is characterized by a variety of the presence of *Pseudomonas. P. mugo* (62.5%) and *P. armandii* (72.7%) have the average potential of allergenicity. Pollen of *P. wallichiana* has a minimum number of the identified microorganisms – only 6 out of 32, but it is more contaminated with microorganisms (3.96 log cfu/g), has the maximum values of the presence of fungi and yeast (5.29 log cfu/g), a second indicator of contamination by aerobic bacteria (6.02 log cfu/g), and 83% of the identified species of microorganisms among the presented on the pollen grains of *P. wallichiana* have the allergenic properties.

As a conclusion, available microbiota on pine pollen or associated with pines can be an addition factor of allergic sensibilization of sensitive persons. The gained results may be useful for aerobiologists, allergists and microbiologists, at least at the local level.

CONCLUSION

Microbiota of anemophilous *Pinus* pollen has been analyzed to evaluate it allergenic potential. Microbiota of other plant organs of *Pinus* trees has been examined to compare it with pollen. The content of revealed microbiota is multifarious: absence of representatives of *Enterococcus*, minimum mesophilic anaerobic bacteria and maximum microscopic fungi, yeast and mesophilic aerobic bacteria. Among them *Pseudomonas, Penicillium* and *Aspergillus* are most widespread on pine pollen grains. Among 32 identified microorganisms 56% of them have pathogenic nature and 16% – not pathogenic. All the investigated *Pinus* pollen contact with potentially allergenic species of

microorganisms, *P. wallichiana* mostly, *P. nigra* least of all. Based on literature data airborne *Pinus* pollen can be a contributing factor in development of respiratory allergy. For the development of the allergenic properties of pine pollen for sensitive person match a range of other environmental factors is necessary. For example, the mass distribution of the species, weather conditions, the negative impact of anthropogenic factors, etc.

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