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Production of Pectinase from *Bacillus sonorensis* MPTD1

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SUMMARY

Seven isolates from spoiled fruits and vegetables were screened for pectinase production using pectin agar plates and the most efficient bacterial strain, MPTD1, was identified as *Bacillus sonorensis*. Optimisation of various process parameters was done using Plackett-Burman and Box-Behnken designs and it was found that parameters like yeast extract, K_2HPO_4, incubation time, $NaNO_3$ and KCl have a negative impact on pectinase production. Parameters like pH and $MgSO_4$ and pectin mass fractions have a positive impact on pectinase production. The maximum obtained enzyme activity was 2.43 (μM/mL)/min. This is the first report on pectinase production by *Bacillus sonorensis*.

Key words: pectinase, fermentation optimization, *Bacillus sonorensis*

INTRODUCTION

Pectin is a major component of the primary cell wall of all land plants. Pectic substances are colloidal polysaccharides, with galacturonic acid backbone linked by α (1-4) linkage (*1*). Pectin acts as the inducer for the production of pectinolytic enzymes by microbial systems (*2*). Pectinases degrade complex pectin to monogalacturonic acid molecules. They are classified into seven classes: pectinesterase (EC 3.1.1.11), polygalacturonase (EC 3.2.1.15), galacturan 1,4-α-galactouronidase (EC 3.2.1.67), exopoly-α-galactouronosidase (EC 3.2.1.82), endopectate lyase (EC 4.2.2.2), exopectate lyase (EC 4.2.2.9) and endopectin lyase (EC 4.2.2.10) (*3*) based on their activity. *Bacillus* sp., *Erwinia* sp. and *Pseudomonas* sp. are major producers of pectinases (*4*). Pectinase contributes to 25 % of the global food enzyme market (*5*). Since pectinase is one of the most important industrial enzymes, several research activities are going on for the production of pectinases with improved activities, properties as well as utilisation of cost-effective substrates for better process economics.

Several reports are available on pectinase production from fungi like *Aspergillus*, *Rhizopus* and *Penicillium* (*6*). Most of the pectinase production is carried out by adopting solid-state fermentation (SSF) strategy. Acidic pectinases find wider range of applications in food industry, while alkaline pectinases find applications in various industrial processes (*7*). Pectinolytic enzymes are present on rotten fruit and vegetable wastes because they are an essential component of the natural decaying process. Pectinase has a wide variety of applications such as fruit juice clarification in fruit processing industry (*1*). Alkaline pectinase produced by *Bacillus licheniformis* can be used for the treatment of effluent generated by vegetable and food processing industries (*8*), protoplast isolation (*9*), bleaching of paper, or in textile industry for degumming of jute, sun hemp, flax, ramie and coconut fibre (*10*). The addition of pectinase together with cellulases, hemicellulases and proteinases to the tea-leaf fermenting bath increases the tea quality index by 5 % (*11*). Use of pectinase in diverse applications indicates the importance of pectinase for green process.

The target of this research is to evaluate the potential of a locally isolated pectinase--producing strain from decayed fruits and vegetables. Optimization of culture conditions using submerged fermentation (SmF) was also investigated for maximum enzyme production.

MATERIALS AND METHODS

Sample collection and isolation

Spoiled fruits and vegetables were collected from nearby fruit shops in Pappanamcode, Kerala, India, and samples of 1 g were mixed in 100 mL of normal saline, serially diluted from 10^{-1} to 10^{-6} ratio. A volume of 100 µL of each diluted sample was inoculated in pectin agar medium and incubated at 37 °C for 48 h (*12*). About 50 microorganisms were isolated on pectin agar plate. Serial dilution up to 10^{-6} was carried out to obtain isolated colonies. The pectinolytic activity was detected by visualizing a clear zone around the colony using Gram's iodine flooding method.

Selection and screening of microorganisms

Colonies from serially diluted plates with different morphologies were selected; each purified colony was maintained on pectin agar plates and stored at 4 °C. Pectin agar medium was prepared with (in g/L): $NaNO_3$ 1.0, KCl 1.0, K_2HPO_4 1.0, $MgSO_4$ 0.5, yeast extract 0.5, pectin 10 and agar 20 with pH adjusted to 7.0 (*13*). All chemicals were obtained from HiMedia, Mumbai, India. Subsequent subculturing was done for proper maintenance of the culture and further experimental studies.

Pectinase-producing microorganisms were primarily screened by plating the colonies on pectin agar plates incubated at 37 °C for 48 h. Gram's iodine assay was carried out for further screening by adding Gram's iodine solution to pectin agar plates with colonies of sample organisms; after 5 min of incubation with an intermittent gentle shaking the plates were washed with distilled water and clear zones around colonies were observed (*14*).

Enzyme extraction and polygalacturonase assay by dinitrosalicylic acid method

Enzyme was extracted by centrifuging the culture at 6000×*g* (centrifuge model C-24BL; REMI, Maharashtra, India) for 10 min at 4 °C, the supernatant was stored at -20 °C and the pellet was discarded.

The pectinase activity of the enzyme extract was assayed based on the modified method of Miller (*15*). The 3,5-dinitrosalicylic acid (DNS; Merck, Mumbai, India) in alkaline solution was reduced to 3-amino-5-nitrosalicylic acid. Pectinase activity was measured by the estimation of the amount of galacturonic acid with the DNS method using 1 % citrus pectin (Merck) as a substrate and mono-D-galacturonic acid (Merck) as a standard. The culture broth was centrifuged, and 0.5 mL of the culture supernatant and 0.5 mL of pectin were added. The tubes were incubated for 30 min at 50 °C in a water bath (JULABO GmbH, Seelbach, Germany). A volume of 1.5 mL of DNS was added and the tubes were incubated in boiling water bath for 5 min. Then the samples were diluted and the colour intensity was measured at 540 nm (spectrophotometer model UV-1601; Shimadzu, Kyoto, Japan). The pectinase activity (in (µM/mL)/min) was calculated by the following equation:

$$\text{Pectinase activity} = \frac{c\,(\text{monogalacturonic acid}) \cdot \text{dilution factor} \cdot 1000}{\frac{212.15}{t\,(\text{incubation})}} \qquad /1/$$

Identification of pectinase-producing bacterial isolate MPTD1

The pectinase-producing bacterial isolate was identified on the basis of 16S rRNA gene sequence analysis. The universal primers used for the amplification of 16S rDNA were: 1) 27F 5'AGAGTTTGATCCTGGCTCAG-3' and 2) 1492R 5'GGTTACCTTGTTACGACTT-3' and final concentrations of the reagents were: $MgCl_2$ 1 mM, dNTP 200 µM, primers 100 pmol and DNA 50 ng. The BioRad Thermal Cycler (Bio-Rad, San Francisco, CA, USA) was used for the amplification of DNA with the following PCR cycles: initial cycle at 94 °C for 3 min, followed by 35 cycles at 94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min, and final extension of 5 min at 72 °C. The amplified product was checked on 1 % agarose gel and the sample was purified using Gene JET-PCR purification kit (Thermo Scientific, Vilnius, Lithuania). Purified 16S rDNA product was sequenced at CSIR-NIIST sequencing facility centre, Trivandrum, India. The isolate was identified by comparing the similarity of amplified DNA sequence with other sequences in databases like National Centre for Biotechnology Information (NCBI) GenBank (*16*) and EzTaxon (*17*). Most similar sequences from a database were collected and a multiple sequence alignment was performed using ClustalW2 (*18*). A phylogenetic tree was constructed from the alignment file using MEGA5 (*19*). The phylogeny across the data was analysed by UPGMA (*20*) and the reliability of the phylogram was checked by Bootstrap analysis (1000 replicates) (*21*). 16S rDNA sequence data were used to identify the selected bacterial isolate from spoiled fruit and vegetable samples. A PCR product of approx. 1.5 kb was amplified and sequenced. The highly similar sequences were recovered from the database as reference sequences and aligned with the 16S rDNA sequences of the pectinase-producing isolate MPTD1. Phylogenetic analysis was conducted with pectinase-producing isolate MPTD1 and reference sequences collected from the database. Evolutionary distances were calculated using the maximum composite likelihood method and expressed in the units of the number of base substitutions per site.

Optimization of various process parameters for pectinase production

Plackett-Burman design

Optimization of various process parameters for pectinase production in submerged fermentation was done by adopting Plackett-Burman design (*22*), using different mass fractions of $NaNO_3$, K_2HPO_4, KCl, $MgSO_4$, pectin, yeast extract, pH and incubation time. These eight parameters were used at two levels (higher and lower) to choose the prime factors for

pectinase production by the selected strain (*23*). Details are presented in **Table 1**.

Response surface methodology (Box-Behnken design)

The prime factors for pectinase production were selected from the Placket-Burman design and optimized by adopting response surface methodology. The selected parameters were: $MgSO_4$, pectin mass fractions and pH. There were a total of 15 runs with three parameters (*24*). Details are presented in **Table 2**.

RESULTS AND DISCUSSION

Isolation and primary screening of pectinolytic organisms

Among 50 isolated microorganisms, about seven strains were found to have pectinolytic activity. Positive isolates were selected based on the clearance zone in Gram's iodine assay. **Fig. 1** shows pectinolytic microorganisms on pectin agar plates after flooding with iodine reagent.

Table 1. Plackett-Burman design for optimization of various process parameters affecting pectinase production

Run number	*w*/%						pH	*t*(incubation)	Enzyme activity	*N*(colony)
	KCl	K_2HPO_4	$MgSO_4$	YE	$NaNO_3$	Pectin		h	(μM/mL)/min	CFU/mL
1	2	0.5	0.25	0.5	2	15	8	24	1.30	$1.05 \cdot 10^5$
2	0.5	0.5	0.25	0.5	0.5	5	6	24	0.94	$3 \cdot 10^5$
3	2	0.5	0.75	0.5	0.5	5	8	60	1.43	$2.5 \cdot 10^7$
4	0.5	2	0.75	0.5	2	5	6	24	1.02	$2.5 \cdot 10^6$
5	0.5	0.5	0.25	2	2	15	6	60	1.77	$4.52 \cdot 10^4$
6	0.5	2	0.25	0.5	0.5	15	8	60	1.69	$3.5 \cdot 10^7$
7	2	2	0.25	2	2	5	8	24	1.68	$6.55 \cdot 10^8$
8	2	2	0.25	2	0.5	5	6	60	0.74	$4.21 \cdot 10^8$
9	0.5	2	0.75	2	0.5	15	8	24	2.44	$6.9 \cdot 10^8$
10	2	2	0.75	0.5	2	15	6	60	2.33	$6 \cdot 10^6$
11	2	0.5	0.75	2	0.5	15	6	24	1.03	$1.5 \cdot 10^7$
12	0.5	0.5	0.75	2	2	5	8	60	2.13	$3.65 \cdot 10^7$

YE=yeast extract

Table 2. Box-Behnken design for optimization of various process parameters affecting pectinase production

Run number	Point type	Block	pH	*w*/%		Enzyme activity	*N*(colony)
				Pectin	$MgSO_4$	(μM/mL)/min	CFU/mL
1	2	1	8	25	1	1.22	$5 \cdot 10^7$
2	2	1	9	20	0.75	1.42	$3 \cdot 10^7$
3	2	1	7	20	0.75	1.53	$1 \cdot 10^8$
4	0	1	8	20	0.88	1.48	$91 \cdot 10^6$
5	2	1	8	25	0.75	1.32	$1 \cdot 10^7$
6	2	1	9	20	1	1.54	$46 \cdot 10^6$
7	0	1	8	20	0.88	1.82	$3 \cdot 10^7$
8	2	1	7	25	0.88	2.16	$61 \cdot 10^6$
9	2	1	7	20	1	1.30	$79 \cdot 10^7$
10	0	1	8	20	0.88	1.32	$67 \cdot 10^6$
11	2	1	7	15	0.88	1.34	$11 \cdot 10^6$
12	2	1	8	15	0.75	1.55	$8 \cdot 10^7$
13	2	1	9	25	0.88	1.29	$11 \cdot 10^8$
14	2	1	9	15	0.88	1.12	$8 \cdot 10^7$
15	2	1	8	15	1	1.14	$125 \cdot 10^7$

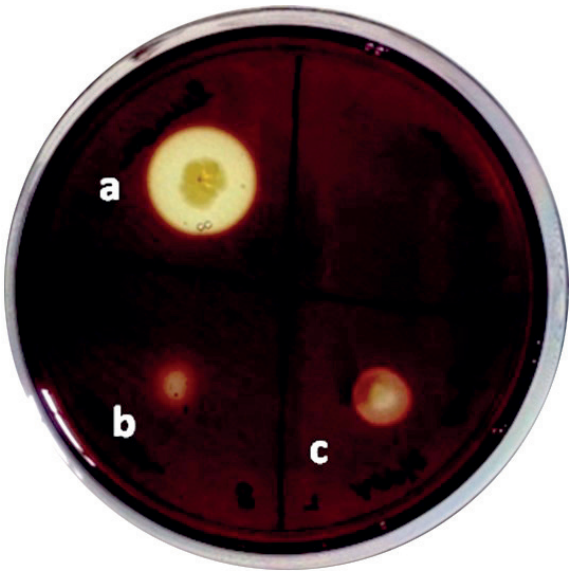

Fig. 1. Pectinase-producing microorganisms showing clear zones on pectin agar plates after Gram's iodine staining: MPTD1 (a), MPTD4 (b) and MPTD6 (c)

Secondary screening of pectinolytic microorganisms based on DNS assay

Seven strains showed pectinolytic activity on pectin agar medium. Submerged fermentation was done to select the potent strain. High pectinase activity in short time was obtained with MPTD1. Maximum enzyme activity obtained was 2.1 (μM/mL)/min of MPTD1, so it was selected for further optimization studies. Enzyme activity of other isolates (MPTD2, MPTD3, MPTD4, MPTD5, MPTD6 and MPTD7) was 1.2, 1.1, 1.5, 1.3, 1.4 and 1.3 (μM/mL)/min, respectively.

Strain identification based on 16S rDNA sequence data

Comparing the 16s rDNA sequence data of isolated MPTD1 with those of the organisms presented in the databases GenBank and EzTaxon, it was established that it has 99 % similarity with *Bacillus sonorensis* sp. In the dendrogram, all *Bacillus sonorensis* isolates were clustered together with very high bootstrap value, where the sequence of *Bacillus subtilis* was used as an outgroup (**Fig. 2**). The 16S rDNA sequence of pectinase-producing *Bacillus sonorensis* MPTD1 was deposited in NCBI with GenBank ID MG774437.

Optimization of pectinase production

Determination of significant process parameters by Plackett-Burman design

Estimation of monogalacturonic acid yield based on Plackett-Burman design indicates that higher pH, $MgSO_4$ and pectin mass fractions have a stronger positive effect, while K_2HPO_4, $NaNO_3$ and yeast extract mass fractions and incubation time have a weaker impact on pectinase production by MPTD1 strain. High KCl mass fraction had a negative effect on pectinase production. An identical observation was reported earlier by Thiagarajan *et al.* (*25*) for xylanase production by *Aspergillus fumigatus* MKU1. Exact molecular mechanism for this effect is not known. These positive factors were found to have stronger effect on pectinase production and were selected for further optimization. Maximum pectinase activity of 240 (μM/mL)/min was observed in run number 9 (**Table 1**).

The results depicted in Pareto chart (**Fig. 3**) indicate that $MgSO_4$ and pectin mass fractions and pH were found to have a stronger effect on pectinase production and were further optimized using Box-Behnken design.

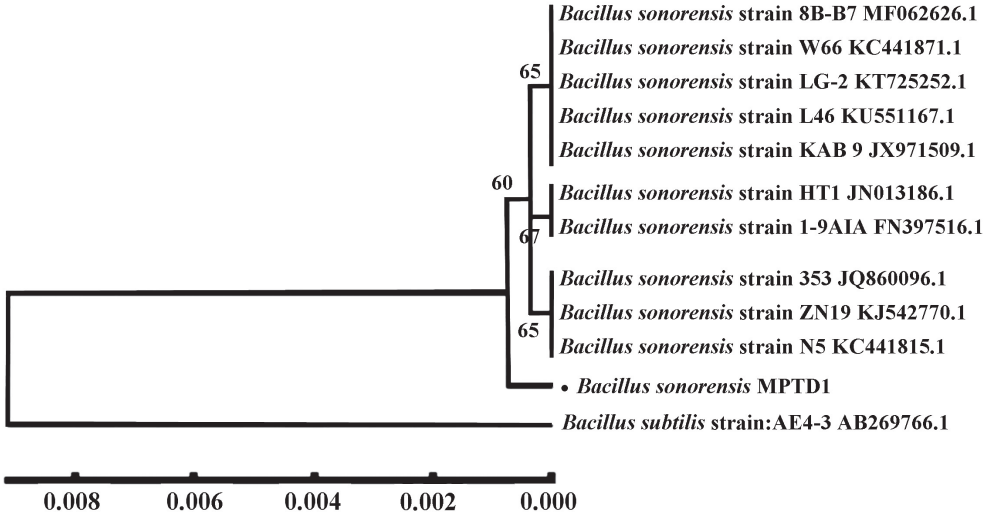

Fig. 2. Phylogenetic tree expressing the relationship between pectinase-producing bacterial strain MPTD1 with reference strains based on the 16S rDNA sequences. *Bacillus subtilis* is used as an outgroup. Numbers shown in each node are confidence level generated from 1000 bootstraps. Scale bar is nucleotide substitution per sequence position

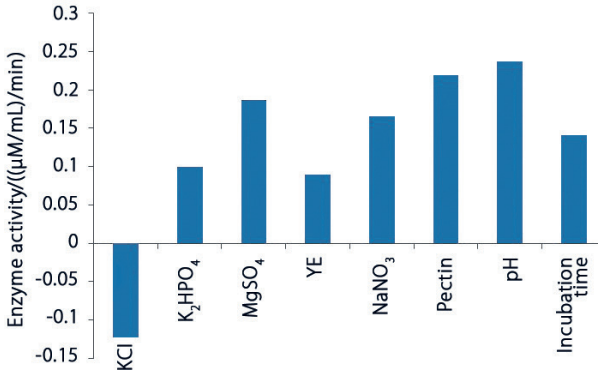

Fig. 3. Pareto chart showing significant parameters affecting pectinase activity

Box-Behnken design experiment to find the interactions between the significant process parameters

The results indicate that the maximum enzyme activity of 2.16 (μM/mL)/min was observed in run number 8 (**Table 2**). The maximum pectinase activity was 2.16 (μM/mL)/min (*26*).

Figs. 4a and **4b** show surface and contour plots of the effect of interactions between $MgSO_4$ and pectin mass fractions on pectinase activity. At low mass fractions of pectin (15-18 %) and $MgSO_4$ (0.75-0.8 %), the pectinase activity was low. It increased with an increase of pectin and $MgSO_4$ mass fractions. Maximum pectinase activity of 1.8 (μM/mL)/min was observed at medium mass fractions of pectin (19-23 %) and $MgSO_4$ (0.08-0.09 %). Pectinase production decreased at higher mass fractions of pectin and $MgSO_4$. Different results were reported by Mehta *et al.* (*27*) where maximum enzyme production was observed with 0.5 % of pectin.

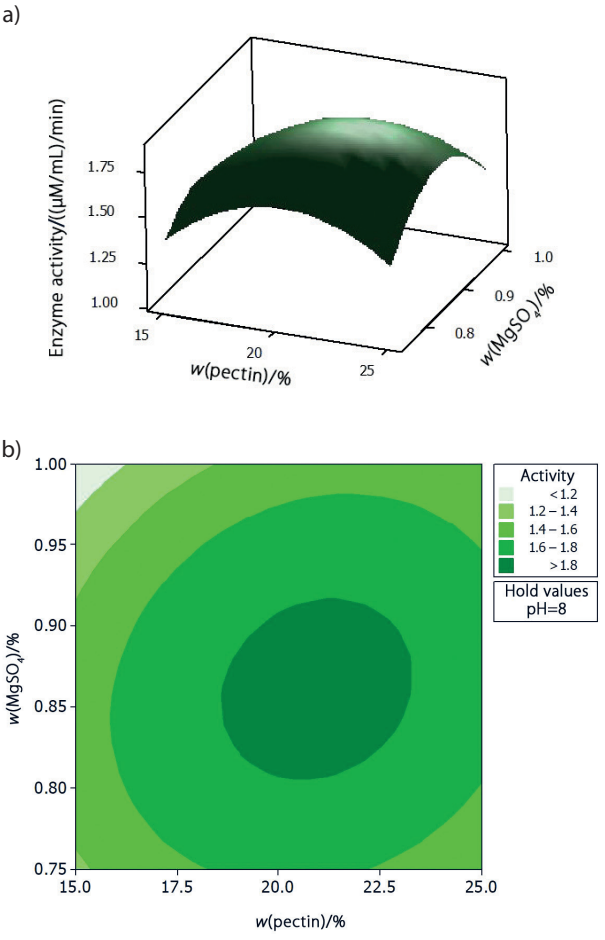

Fig. 4. Surface (a) and contour (b) plots showing the effect of interactions of $MgSO_4$ and pectin mass fractions on pectinase activity

Figs. 5a and **5b** show surface and contour plots of the effect of interactions between pH and pectin mass fractions on pectinase activity. At low mass fractions of pectin, the pectinase activity was low. It increased with the increase of pectin mass fraction and maximum pectinase production of 1.8 (μM/mL)/min was observed at high mass fractions of pectin (20-25 %). At low to middle value of pH (2.5-8.0), pectinase production was high and it decreased with an increase of pH (8.0-9.0). Akinyemi *et al.* (*28*) reported maximum pectinase activity at pH=8.0 when using *Bacillus megaterium* and *Bacillus bataviensis*.

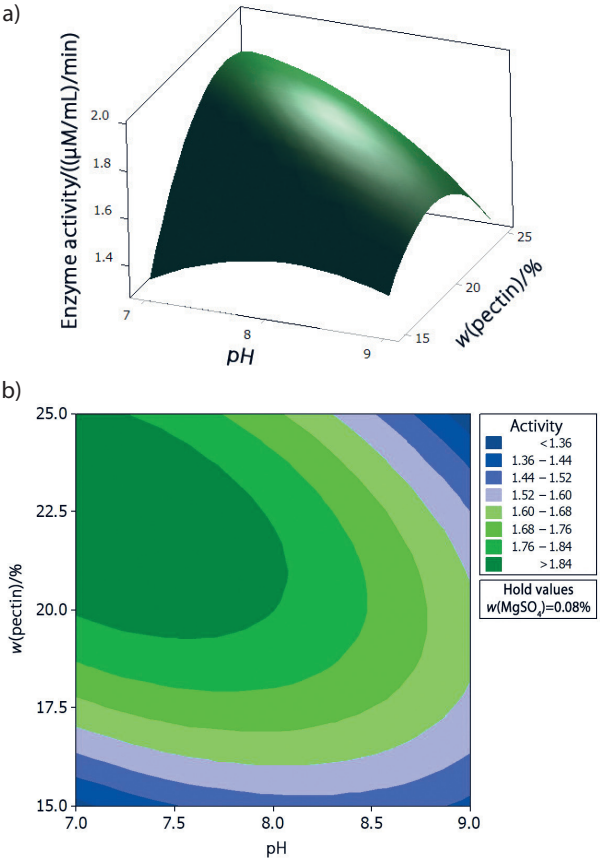

Fig. 5. Surface (a) and contour (b) plots showing the effect of interactions of pH and pectin mass fraction on enzyme production

Figs. 6a and **6b** show surface and contour plots of the effect of interactions between pH and $MgSO_4$ mass fraction on pectinase activity. At low mass fractions of $MgSO_4$ (0.75-0.9 %) and low pH (7.0-8.0) the pectinase production was high (1.8 (μM/mL)/min). It decreased with the increase of pH and $MgSO_4$ mass fraction. At higher pH values (8.0-9.0) and higher mass fractions of $MgSO_4$ (0.9-1.0 %) the pectinase production

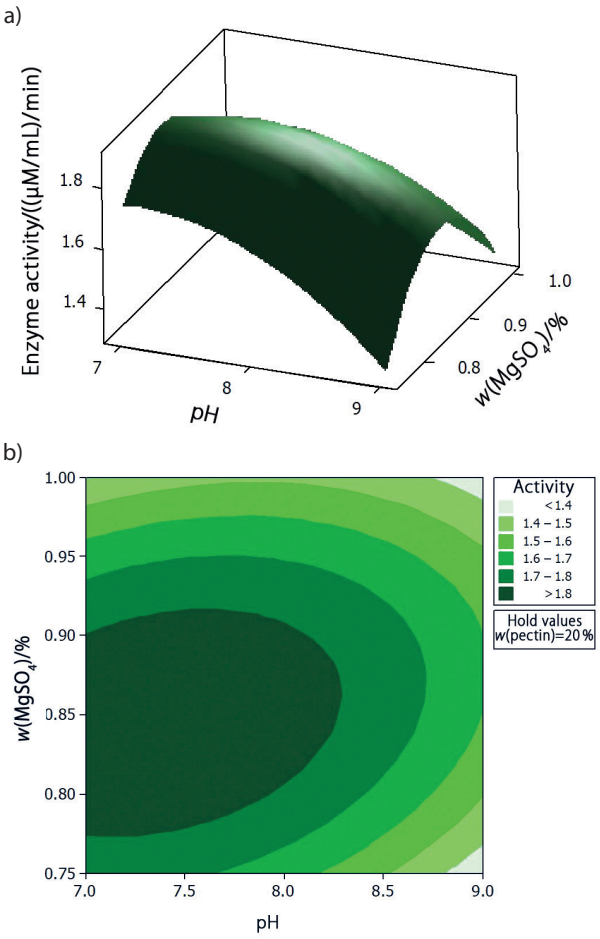

Fig. 6. Surface (a) and contour (b) plots showing the effect of interactions of $MgSO_4$ mass fraction and pH on enzyme production

was low (1.4 (μM/mL)/min). An identical observation was reported earlier by Mehta *et al.* (*27*) for pectinase production from a soil isolate where maximum pectinase activity was observed at pH=6.0-8.0.

CONCLUSIONS

Identifying the significant factors affecting pectinase production using experimental design is essential for the production of enzymes on a large scale. *Bacillus sonorensis* MPTD1, isolated from decayed fruit and vegetables, showed highest pectinase activity, so it was explored for optimisation of various process parameters affecting pectinase production by adopting a Plackett-Burman and Box-Behnken designs. Optimization studies revealed that pH and $MgSO_4$ and pectin mass fractions have a significant role in pectinase production. It is clear from Pareto chart that parameters like yeast extract, dipotassium hydrogen phosphate, incubation time and sodium nitrate have a lower impact on pectinase production, while potassium chloride has a negative impact on pectinase production by *Bacillus sonorensis*. Maximum pectinase production of 2.434 (μM/mL)/min was observed with 2 % K_2HPO_4, 0.75 % $MgSO_4$, 2 % yeast extract, 0.5 % $NaNO_3$ and 15 % pectin, at pH=8.0 and incubation time of 24 h. Optimization using Box-Behnken design revealed that maximum pectinase production was observed under the following conditions: pH=7.0, 25 % pectin and 0.88 % $MgSO_4$ mass fractions. Fine tuning can improve the process. This is the first report on pectinase production by *Bacillus sonorensis*.

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