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Enhanced Production of Thermostable Pullulanase Type 1 Using *Bacillus cereus* FDTA 13 and Its Mutant

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Summary

This paper describes optimization of fermentation conditions for the production of thermostable pullulanase from a new strain *Bacillus cereus* FDTA 13, isolated from tapioca soil. By using one-factor-at-a-time and orthogonal array method, a simple medium with effective and minimal components was optimized for maximal pullulanase production. The optimized medium containing (in g/L): starch 20, yeast extract 5, NaCl 2, $MgSO_4$ 0.1 and K_2HPO_4 0.17 gave nearly 3.6-fold increase in pullulanase activity. Addition of Mn^{2+} ions in the optimized medium further enhanced the production by 1.4-fold. Effects of proteolytic inhibitors on stability of pullulanase in the medium were studied. A mutant *Bacillus cereus* FDTA 13/NTG04-B4, obtained by chemical mutation of the parent strain, further increased the pullulanase production by 2-fold in optimized medium, and gave similar yield as the parent strain in a medium with lower levels of carbon source.

Key words: thermostable pullulanase, *Bacillus cereus*, orthogonal array, optimization, mutation, polymethylsulphonate fluoride

Introduction

Enzymes that hydrolyze pullulan are of following types: 1. glucoamylase – which acts on the non-reducing ends of pullulan to produce glucose, 2. pullulanase – which acts on α-(1,6)-glucosidic linkage in pullulan to produce maltotriose, 3. isopullulanase – which hydrolyzes α-(1,4)--linkage to produce isopanose, and 4. neopullulanase – which acts on α-(1,4)-linkage to produce panose (*1*). Pullulanase type 1 (E.C. 3. 2. 1. 41), also termed as α-dextrin 6-glucanohydrolase or true pullulanase or limit dextrinase (*2*), was first discovered in *Klebsiella* species (*3*). Apart from pullulan, they also hydrolyze the branch points in starch, and hence can be used with other starch degrading enzymes in industrial applications (*4*). They can be used in combination with glucoamylase to produce glucose from starch (*5*), and in combination with β-amylase for the production of maltose (*6*). In saccharification, the addition of pullulanase helps in increasing the substrate concentration, decreasing the reaction time and increasing the product yield (*7*). In combination with glucoamylases, it can be used to produce low calorie beer (*8*).

Pullulanases are used at 60 °C or above for saccarification process. Hence, thermal stability under operating conditions is commercially important. In industry, thermostable and acidophilic pullulanase from *Bacillus stearothermophilus* is used (*9*). Industries are constantly on the lookout for new sources of thermostable and more efficient enzymes by isolating new microbes, or by molecular recombination (*10*). Pullulanases are reported in several mesophiles (*11–14*) and thermophiles (*9,15–20*). Genes responsible for pullulanase production have been characterized and successfully cloned (*1*). The difficulties in construction and expression of recombinant enzymes by molecular techniques, and the economic constraints in using extremophiles in industry, make a mesophile with desirable characteristics advantageous (*21,22*). *Ba-*

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cillus has simple nutritional requirements and also exhibits considerably good enzyme yield (*23*). Hence, isolating a potential strain from this genus is industrially relevant.

It is also economically beneficial to design the fermentation process using a medium with minimum and/or low-cost components. Most reports on fermentation media for pullulanase production employ a large number of minerals, expensive amino acids and vitamin supplements (*11,15,17,18*). It would be ideal to eliminate many of these components and keep only the effective ones, without compromising the efficiency. Optimization of medium components by statistical techniques is less time consuming and laborious as compared to one--dimensional methods which are often error prone (*24*). Taguchi constructed a special set of arrays called orthogonal arrays that give full information about all the factors that affect the performance parameter with minimal number of experiments. The assumption of the Taguchi design implies that the individual or main effects of the independent variables on performance parameter are separable. The crux of the orthogonal array method lies in choosing the level combinations of the input design variables for each experiment. Strain improvement involving mutation or molecular techniques helps in the isolation of a mutant that not only produces high yield of product but is also relieved from induction-repression control (*25,26*).

The aim of this work was to optimize the fermentation conditions for the production of thermostable pullulanase from *Bacillus cereus* FDTA 13 by using one-factor--at-a-time and orthogonal array method. Enhancement of pullulanase production using manganese and improving the stability of pullulanase in media by using protease inhibitors are also described.

Materials and Methods

Microorganisms and growth conditions

A bacterial strain of *Bacillus cereus* FDTA 13 isolated from tapioca plantation soil, Kerala, India, was used in this work. Stock culture was maintained on nutrient agar slants through regular subculturing. Slants were incubated at (32±2) °C for 18 h and stored at 4 °C. An 18-hour--old actively growing culture was suspended in saline and its turbidity in terms of absorbance was adjusted to 0.800 at 650 nm (~10^8 cells/mL). The basal medium used for the experiments had the following minimal composition (in g/L): pullulan 10, tryptone 2, yeast extract 2 and NaCl 2. After the sterilization at 121 °C for 20 min, pH of the medium was adjusted to 6.5. Unless otherwise specified, fermentation was carried out in 250-mL flasks containing 50 mL of media at (32±2) °C for 48 h at 200 rpm and an initial pH=6.5 and inoculum 1 %. All experiments were performed at least in triplicates and mean values were reported.

Effect of process parameters on pullulanase production

Effect of pH was studied in the range of 3.5–9.5 by adjusting the initial pH of the medium. To study the effect of inoculum density, 1–7 % of inoculum was inoculated in fermentation medium. To study the effect of carbon source, 10 g/L of different starches and dextrins like soluble starch, potato starch, maltodextrin, potato dextrin, tapioca dextrin, amylopectin, glycogen and carbohydrate substrates like glucose, maltose and amylose were substituted in place of pullulan. The basal medium with 10 g/L of pullulan was used as control. To study the effect of nitrogen sources yeast extract and tryptone were substituted with 4 g/L of single organic nitrogen sources like peptone, corn steep liquor, soy flour, tryptone and yeast extract and inorganic sources like ammonium chloride and ammonium sulphate. The basal medium with yeast extract and tryptone at the concentration of 2 g/L of each was used as control. KH_2PO_4, K_2HPO_4, $MgSO_4 \cdot 7H_2O$, $CaCl_2$, $FeSO_4 \cdot 7H_2O$, $ZnSO_4 \cdot 7H_2O$, Na_2CO_3, $CuSO_4 \cdot 5H_2O$ and $BaCl_2$ at 0.5 mM were incorporated into basal medium to study the effects of mineral sources. The basal medium without any minerals was used as control. To study the effect of manganese in the optimized medium a sterile stock of $MnSO_4 \cdot 7H_2O$ was added to the medium so that the final concentration of manganese ranged from 0.1 to 0.7 mM at 0.2 mM intervals.

Effect of protease inhibitors

Sterilized stock of EDTA dissolved in sterile distilled water and PMSF dissolved in anhydrous ethanol were added at the onset and after 12 h of fermentation (final concentration in the medium ranged from 0.1 to 1 mM). Protease inhibitors were not added to the control.

Orthogonal matrix method

MINITAB 13.30 software was used to design and analyze an L_{16} orthogonal array. Table 1 shows the design of the same and the concentration of different components that are used in the present study. The concentration of 2 g/L of NaCl was kept constant in the medium.

Screening and isolation of the mutant

Cells from actively growing culture were suspended in saline and the absorbance adjusted to 1.000 at 650 nm (~10^{10} cells/mL). After centrifugation at 10 000 × *g* for 10 min at 35 °C, the precipitated cells were treated with sterile phosphate buffer (pH=6.0) containing nitrosoguanidine (1500 μg/mL) for 20 min. Treated cells were washed twice with saline and allowed to recover in nutrient broth for 3 h. Dilutions were spread out on a plate medium with red pullulan as sole source of carbon with other components (in %): yeast extract 0.1, tryptone 0.2, $CaCl_2$ 0.02, $MgSO_4$ 0.02, $FeSO_4$ 0.001, K_2HPO_4 0.003, except soluble starch and blue-amylose (*27*). The plates were incubated for 24 h at (32±2) °C. The zone of clearance around pullulanase producers was measured and the selected strains were subcultured for 20 generations to confirm phenotypic stability, after which they were grown in optimized medium for analysis of pullulanase production.

Analytical methods

Samples were collected at various intervals from shake flasks, centrifuged at 10 000 × *g* for 20 min at 4 °C and the resulting supernatant was used as crude source of pullulanase enzyme. In liquid media, growth of the

Table 1. L_{16} (4^4) orthogonal array design for pullulanase production by *Bacillus cereus* FDTA13

RUN	A	B	C	D	(A) γ(soluble starch)	(B) γ(yeast extract)	(C) $c(MgSO_4)$	(D) $c(K_2PHO_4)$	Pullulanase activity as V(PUN)
					g/L	g/L	mM	mM	mL
1	1	1	1	1	5	1	0.5	0.5	0.223±0.009
2	1	2	2	2	5	3	1.0	1.0	0.248±0.022
3	1	3	3	3	5	5	1.5	1.5	0.285±0.017
4	1	4	4	4	5	7	2.0	2.0	0.264±0.039
5	2	1	2	3	10	1	1.0	1.5	0.497±0.024
6	2	2	1	4	10	3	0.5	2.0	0.663±0.054
7	2	3	4	1	10	5	2.0	0.5	0.601±0.051
8	2	4	3	2	10	7	1.5	1.0	0.635±0.007
9	3	1	3	4	20	1	1.5	2.0	0.599±0.033
10	3	2	4	3	20	3	2.0	1.5	0.759±0.039
11	3	3	1	2	20	5	0.5	1.0	0.895±0.013
12	3	4	2	1	20	7	1.0	0.5	0.782±0.032
13	4	1	4	2	30	1	2.0	1.0	0.457±0.011
14	4	2	3	1	30	3	1.5	0.5	0.587±0.009
15	4	3	2	4	30	5	1.0	2.5	0.554±0.014
16	4	4	1	3	30	7	0.5	1.5	0.477±0.016

organism was determined as absorbance at 650 nm in spectrophotometer (Helios, Thermos Co., USA) using distilled water as blank. Pullulanase activity was defined as the amount of enzyme required to produce reducing sugar equivalent to one μmol of glucose per minute under assay condition from pullulan, which was used as the substrate. The reaction mixture contained 500 μL of 1 % pullulan (mass per volume) in 200 mM phosphate buffer (pH=6.8) with 500 μL of culture filtrate, incubated for 20 min at 50 °C. The reducing sugar released was measured using dinitrosalicylic acid method (*28*). Pullulanase activity was expressed in U/mL.

Results and Discussion

One–factor-at-a-time method

Medium optimization by one-factor-at-time method involves changing one variable while fixing the others at certain arbitrary levels (*29*). Before determining the effect of carbon and nitrogen sources, effect of pH on pullulanase production was studied. Maximum pullulanase production was observed at pH=6.5, although growth of *Bacillus cereus* FDTA 13 was observed at pH=4.5–7.5 (data not shown). These results are in agreement with the growth pattern of a typical *Bacillus* sp. (*30*).

Microorganisms behave differently under stress conditions, such as elevated cell numbers in the medium, which may influence the yield of a product. Hence, the effect of percentage of inoculum on pullulanase production was studied. Pullulanase production increased slightly with 3 % inoculum, beyond which no further increase was seen (data not shown).

Selection of appropriate carbon source is critical in pullulanase production, since it is reportedly controlled by substrate induction and catabolite repression (*31*). Most of the saccharides such as soluble starch, potato starch, amylopectin, potato dextrin, maltodextrin, and maltose enhanced pullulanase synthesis (Fig. 1). This could be attributed to the presence of α-(1,6)-linkages, present in complex polysaccharides that can induce the production of pullulanase (*9,31*). Both maltose and pullulan induced pullulanase in the medium. Contradictory reports are available in literature, wherein maltose is reported to repress pullulanase synthesis in some cases (*16*). Maximum production of pullulanase was observed with soluble starch, which increased the yield 1.8-fold as compared to control. Soluble starch is a good source of carbon for production of pullulanase, since it can induce its release in case of cell associated pullulanase (*11,12,22,32,33*). Glucose, amylose and glycogen severely repressed pullulanase production by approximately 70–80 %. Except pullulan, all other carbon sources gave maximum pullulanase activity in the 12th hour of fermentation, which coincides with the late log phase in *Bacillus cereus* FDTA 13. In all cases, pullulanase production paralleled the growth phase. But with pullulan as control, the maximum production was prolonged to the late stationary phase of growth, *i.e.* after 24 h of fermentation.

Earlier reports on polypeptone and yeast extracts inducing the production of pullulanase are available (*12*). In our work, peptone did not increase pullulanase production in the medium (Fig. 2). However, pullulanase activity seemed to be considerably induced by yeast extract, since the activity with yeast extract as sole source of nitrogen paralleled that of the medium with combination of tryptone and yeast extract. Moreover, medium with tryptone showed lower levels of pullulanase. This indicated the possibility of yeast extract to slightly induce pullulanase in *Bacillus cereus* FDTA 13. The inorganic sources not only shifted pullulanase production to lower levels, but also slowed the growth of *Bacillus ce-*

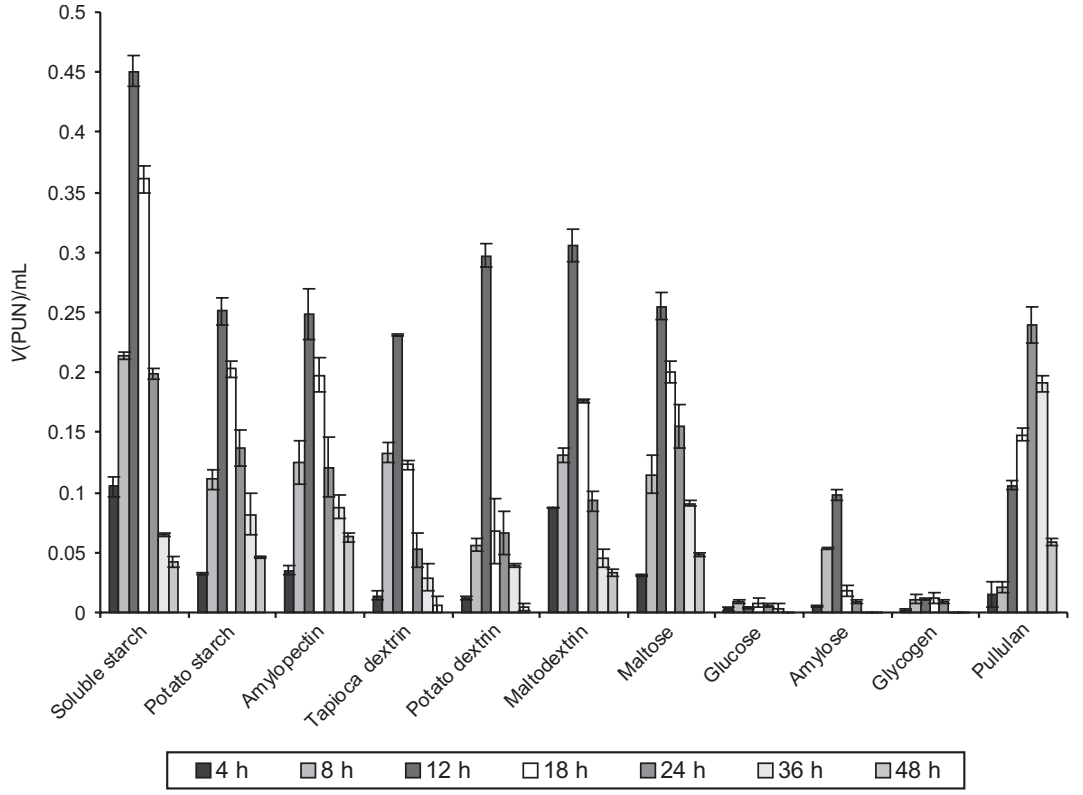

Fig. 1. Effect of different carbon sources on pullulanase production. Basal medium with yeast extract and tryptone (2 g/L of each) as nitrogen source and varying carbon source (10 g/L); agitation: 200 rpm; pH=6.5; cultivation period: 48 h; temperature: (32±2) °C

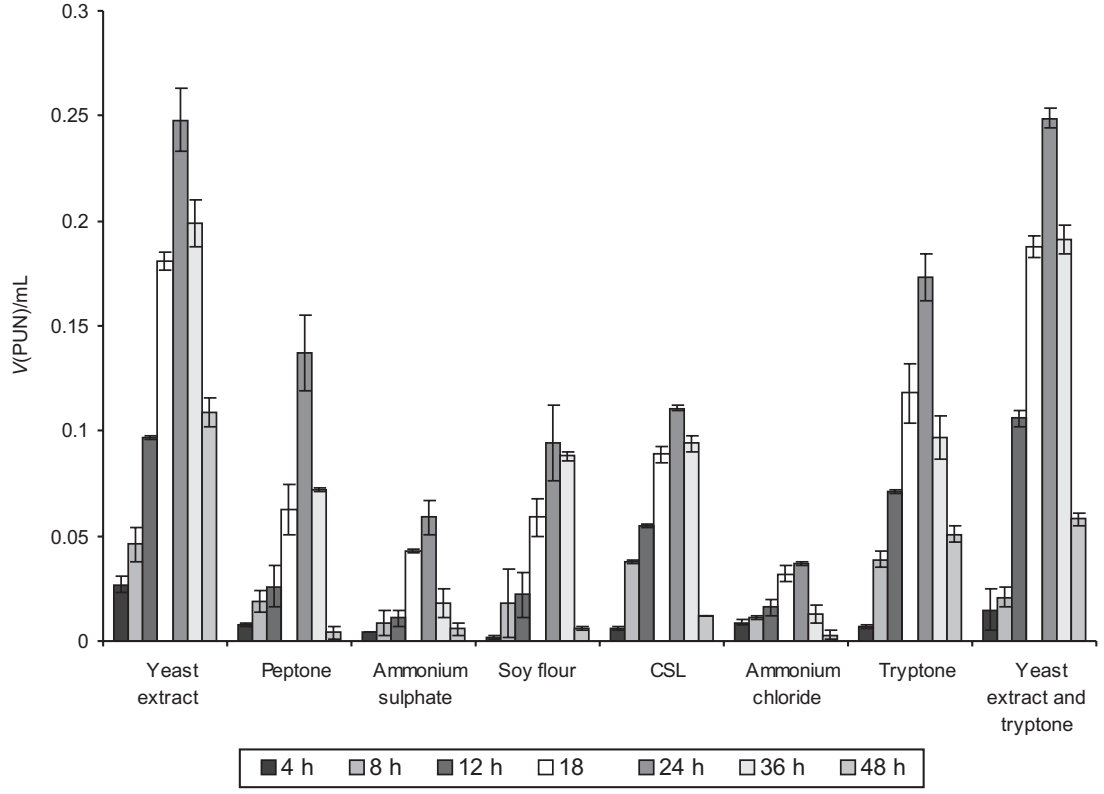

Fig. 2. Effect of different nitrogen sources on pullulanase production. Basal medium with pullulan (10 g/L) as carbon source and varying nitrogen source (4 g/L); agitation: 200 rpm; pH=6.5; cultivation period: 48 h; temperature: (32±2) °C

reus FDTA 13. Soy flour and corn steep liquor did not enhance pullulanase production as compared to control. In all cases, nitrogen sources caused fluctuations in growth pattern of the cell that was in proportion to the yield of pullulanase production.

To investigate the effects of minerals, the basal medium devoid of any mineral sources was supplemented with 0.5 mM of different minerals. It can be seen that only K_2HPO_4 enhanced the pullulanase production (Fig. 3). It was also observed that in the medium with $MgSO_4$, pullulanase activity was stable until the 48th hour as compared to control and medium with other mineral source. Though phosphates are reported to aid the release of cell associated pullulanase in the medium (*31*), an increase with the addition of potassium phosphate was not observed in this case. Also, unlike earlier reports, pullulanase production was not enhanced in presence of sodium, calcium, copper, barium and zinc (*12*), although the decrease in pullulanase production with incorporation of ferrous salts is in accordance with the published data (*31*).

Orthogonal matrix method

To evaluate the importance of various nutrients and to optimize their levels of improved pullulanase production, orthogonal matrix method was employed. This method was applied in improving biopolymer production and secondary metabolites (*24*,*29*).

The optimized parameters involved concentrations of soluble starch, yeast extract, $MgSO_4 \cdot 5H_2O$ and K_2HPO_4. The medium was incorporated with constant 2 g/L of NaCl. Table 2 represents the response table for means (larger is better) with L_{16} orthogonal array. The last two rows show delta and rank order that help to assess which factors have the greatest effect on the response characteristic of interest. Higher delta value indicates greater effect of that component, while rank orders the factors from the greatest effect (based on the delta values) to the least effect on the response characteristic. According to the magnitude, the order of effect of factors on pullulanase activity was soluble starch > yeast extract > K_2HPO_4 > $MgSO_4$. This suggests that soluble starch has the maximum effect, while $MgSO_4$ had the minimum effect on pullulanase production.

Table 2. Response table for mean with L_{16} (4^4) orthogonal array design for pullulanase production by *Bacillus cereus* FDTA13

Level	(A) Soluble starch	(B) Yeast extract	(C) $MgSO_4$	(D) K_2PHO_4
1	0.25	0.44	0.56	0.54
2	0.59	0.56	0.52	0.55
3	0.75	0.58	0.52	0.50
4	0.51	0.53	0.52	0.52
Delta	0.50	0.13	0.04	0.05
Rank	**1**	**2**	**4**	**3**

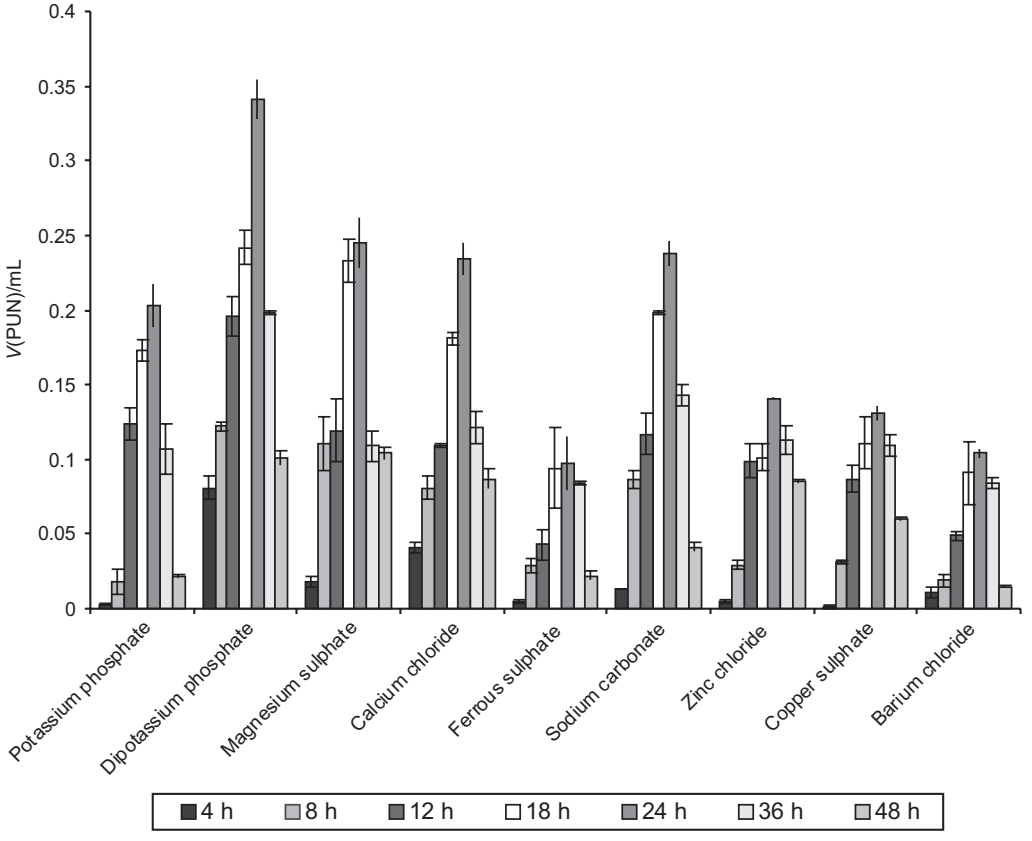

Fig. 3. Effect of different mineral sources on pullulanase production. Basal medium with pullulan (10 g/L) as carbon source and yeast extract and tryptone (2 g/L of each) as nitrogen source and different mineral sources at 0.5 mM; agitation: 200 rpm; pH=6.5; cultivation period: 48 h; temperature: (32±2) °C

Fig. 4 represents the main effect plots for the system. This plot was created by the software MINITAB by plotting the average for each factor level as per the averages in Table 2. The line which connects the points of each factor, if parallel to X-axis, it indicated lower effects, and if parallel to Y-axis, it indicated the main effect. Hence, greater the difference in the vertical position of the plotted points (the less the line is parallel to the X-axis), greater the magnitude of the main effect. In the present study, it can be seen that for each of the four variables at four levels, we found that one level increased the mean value compared to the other level. The difference is the main effect, *i.e.* starch at level 3, yeast extract at level 3, K_2HPO_4 at level 2 and $MgSO_4$ at level 1. These levels along with the response table can also be used to predict the optimal levels of each component used in the study. An increase in concentration of soluble starch considerably increased pullulanase activity, beyond which, the yield did not increase. This is due to the substrate repression regulation that prevents the cell from secreting enzymes into the medium (*16*). The software predicted a value of 0.867 U/mL of pullulanase. To confirm these results, experiments were carried out using the optimized medium. The final optimized medium produced 0.891 U/mL after 12 h as compared to 0.246 U/mL in the unoptimized medium (Fig. 5). Thus a simple medium with minimal components was optimized, which gave approximately 3.6-fold increased pullulanase activity.

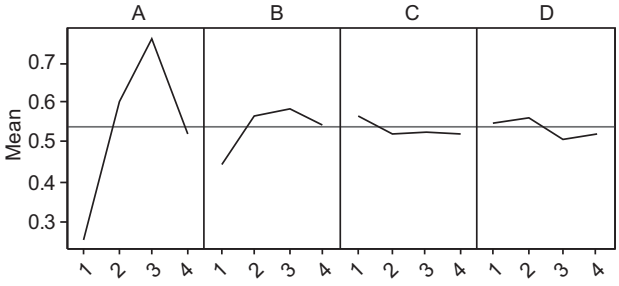

Fig. 4. Main effect plot for pullulanase production by *Bacillus cereus* FDTA 13. Medium with different concentrations of (A) starch, (B) yeast extract, (C) $MgSO_4$ and (D) K_2HPO_4 and NaCl 2 g/L; agitation: 200 rpm; fermentation period: 12 h; pH=6.5; temperature: (32±2) °C

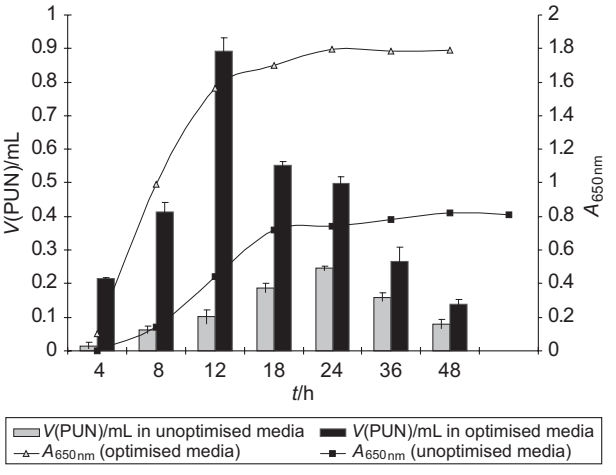

Fig. 5. Growth curve and pullulanase production of *Bacillus cereus* FDTA 13 in optimized and unoptimized medium; agitation: 200 rpm; fermentation period: 48 h; pH=6.5; temperature: (32±2) °C

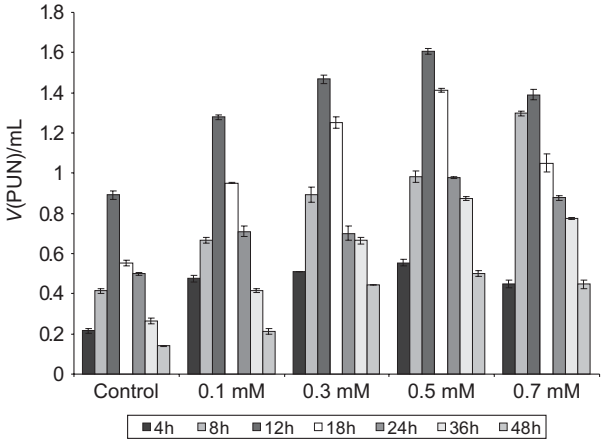

Fig. 6. Effect of manganese concentration on pullulanase production. Agitation: 200 rpm; fermentation period: 48 h; pH=6.5; temperature: (32±2) °C

Enhancement of pullulanase production using manganese

Manganese has a pronounced effect on pullulanase activity in the medium. Manganese is reported to remarkably stimulate the production of pullulanase in *Bacillus* species (*12*). Hence, investigating the effect of $MnSO_4$ at different concentrations on pullulanase production was undertaken. Manganese at 0.5 mM gave maximum pullulanase activity beyond which no further enhancement in the production of pullulanase was observed (Fig. 6). The production of pullulanase increased 1.8-fold than that in the optimized medium.

Effect of protease inhibitors on pullulanase production

The pullulanase activity in the medium decreased as fermentation was continued for 48 h or in the late stationary phase. The possible reason could be the action of proteolytic enzymes that may degrade the pullulanase in the medium. Hence, proteolytic inhibitors at different levels were added at the beginning, late log phase or after 12 h of fermentation. EDTA, a broad-spectrum inhibitor of metalloproteinase, and PMSF, a broad-spectrum serine protease inhibitor, were used. Addition of EDTA during the onset and late log phase did not have any effect on pullulanase production (data not shown). Although addition of different levels of PMSF at onset of fermentation did not have any effect on pullulanase production, its addition during the late log phase considerably stabilized pullulanase activity in the medium during late stationary phase (Fig. 7).

Screening and isolation of a mutant

Bacillus cereus FDTA 13 strain was subjected to treatment with nitrosoguanidine (10–100 μL) for 30 min resulting in (10±5) % survival ratio. About 40 strains that

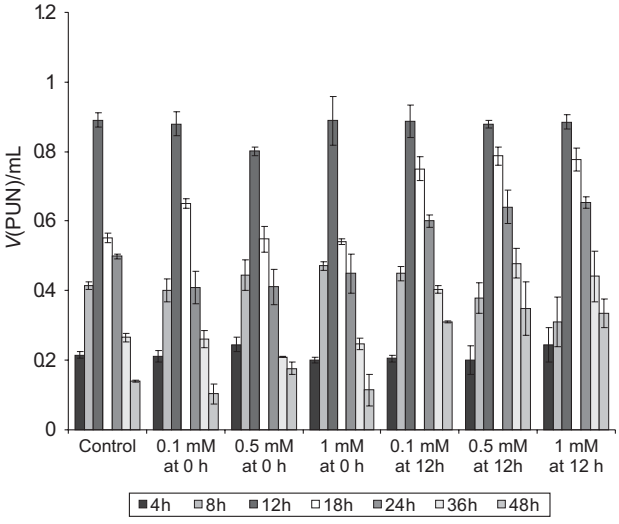

Fig. 7. Effect of concentration of phenylmethylsulphonyl flouride (PMSF) added at varying times of fermentation; agitation: 200 rpm; fermentation period: 48 h; pH=6.5; temperature: (32±2) °C

showed comparatively better zone of clearance than parent strain on red pullulan plate were selected. One mutant designated *Bacillus cereus* FDTA 13/NTG04 showed the largest zone of clearance among the selected strains. This strain was again subjected to mutagenesis using 50 μL of nitrosoguanidine for 30 min. One mutant *Bacillus cereus* FDTA 13/NTG04-B4 was selected on the basis of the zone of clearance. The pullulanase production of this strain was compared to the parent strain at varying levels of carbon source. The new mutant produced pullulanase up to 1.788 U/mL in the optimized medium (Fig. 8).

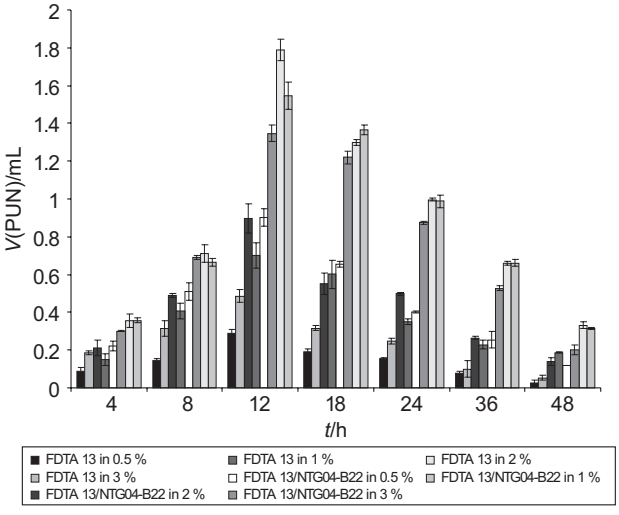

Fig. 8. Pullulanase production by *Bacillus cereus* FDTA 13 and mutant *Bacillus cereus* FDTA 13/NTG04-B4; agitation: 200 rpm; fermentation period: 48 h; pH=6.5; temperature: (32±2) °C

The mutant gave approximately the same amount of pullulanase as the parent strain at lower level, *i.e.* 5 g/L of soluble starch as carbon source. In both cases, pullulanase activity in the medium decreased towards the end of fermentation cycle.

Conclusions

Using one-factor-at-a-time and orthogonal array, complex mineral sources and other supplements were eliminated and most effective components were selected to design a simple yet very effective medium for remarkably improved pullulanase production (nearly 5.4 times) from the new strain *Bacillus cereus* FDTA 13. A new mutant *Bacillus cereus* FDTA 13/NTG04-B4 gave approximately 2-fold increase in pullulanase activity and comparatively same activity as the parent strain with low levels of carbon source. Thus, the parent isolate and its mutant could be considered as efficient producers of thermostable pullulanase and should be explored further as a potential candidate for commercial scale production.

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