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Exo-polygalacturonase production by *Bacillus subtilis* CM5 in solid state fermentation using cassava bagasse

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ABSTRACT

The purpose of this investigation was to study the effect of *Bacillus subtilis* CM5 in solid state fermentation using cassava bagasse for production of exo-polygalacturonase (exo-PG). Response surface methodology was used to evaluate the effect of four main variables, i.e. incubation period, initial medium pH, moisture holding capacity (MHC) and incubation temperature on enzyme production. A full factorial Central Composite Design was applied to study these main factors that affected exo-PG production. The experimental results showed that the optimum incubation period, pH, MHC and temperature were 6 days, 7.0, 70% and 50°C, respectively for optimum exo-PG production.

Key words: Exo-polygalacturonase, *Bacillus subtilis* CM5, response surface methodology, solid state fermentation, cassava bagasse

INTRODUCTION

In recent year the potential of using microorganism as a biotechnological source of industrially relevant food processing enzymes has stimulated renewed interest in the exploration of extracellular enzymatic activity. Enzymes that hydrolyze pectic substance are broadly known as pectinases that include polygalacturonase, pectine esterase, pectin lyase and pectate lyase on the basis of their mode of action. Bacteria, yeast, actinomycetes and filamentous fungi have been reported to produce pectinases (11, 28). Pectinase production from microorganisms has been reported under both submerged and solid state fermentations. Solid state fermentation (SSF) is defined as the cultivation of microorganisms on moist solid substrate, preferably on agricultural residues like wheat bran, rice husk, etc. that can in addition, be used as carbon and energy source. SSF takes place in the absence and near absence of free water thus being close to the natural environment to which microorganisms are adapted (13).

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Several agro – industrial wastes such as orange bagasse (17), sugarcane bagasse (22), wheat bran (5) and cassava (*Manihot esculent* Crantz.) bagasse (2) were found to be effective substrates for PG production by SSF. Among agricultural residues, cassava bagasse was found as the cheapest (US$ 10/ton) substrate for SSF in comparison to others [sugarcane (US$ 30/ton), rice and wheat bran (US$ 40/ton)], thus it has been successfully put to use for production of various end products such as animal feed after enriching the protein content using fungi (20), enzymes (26), organic acids (15) and aroma compounds (6).

The optimization process condition under SSF is generally done by varying one condition at a time approach (22). However, these strategies are laborious and time consuming especially for a large number of variables. Response surface methodology (RSM) is an experimental strategy for seeking the optimum conditions in a multivariable study (1, 2) and is used for optimization of culture conditions for production of primary and secondary metabolites (21), i.e. amino acid (29), ethanol (3) and enzymes (26). RSM can be used to study relations between one or more responses and number of independent variables (parameters). This experimental methodology generates a mathematical model that accurately describes the overall process (12) and less time consuming (26).

In our earlier study, it was found that *Bacillus subtilis* strains were the predominant bacteria isolated from culturable cow dung microflora (25). These strains exhibit several beneficial attributes such as production of growth regulator, i.e. indole-3 acetic acid (25) and thermostable α-amylase (24).The present study was carried out to investigate the production of exo - polygalacturonase (PG) (E.C., 3.2.1.67.) by *B. subtilis* in SSF using cassava bagasse as the substrate and optimization of the fermentation parameters [incubation period, medium pH, moisture holding capacity (MHC) and temperature] by applying RSM.

MATERIALS AND METHODS

Bacillus subtilis strain

Bacillus subtilis strain CM5 isolated earlier from cow dung microflora (27) was used in this study. The culture was maintained on nutrient agar (NA) slants at 4ºC.

Cassava bagasse

Cassava bagasse [(g/100g dry residue); moisture: 11.2; starch: 63.0; crude fibre: 10.8; crude protein: 0.9 and total ash: 1.2] was used as solid substrate (support and nutrient source) for SSF. Cassava bagasse was collected during starch extraction (October - November, 2007) from cassava using a mobile starch extraction plant, developed by our institute (7) and oven - dried at 80°C for 24 h. The dried cassava bagasse was stored in air- tight container until required.

Optimization of incubation period, initial medium pH, MHC and temperature by applying RSM

The characterization of different parameters for exo-PG production was optimized by applying RSM using Central Composite Design (CCD). The first step in this study was the identification of parameters likely to be effective on the response (enzyme production). Screening experiments were, therefore, performed to confirm the optimization of independent factors level by taking incubation period (A) (4 - 8 days), pH (B) (5.0 - 9.0), MHC (C) (50 - 90%) and temperature (D) (30 - 70°C) in this study. The level of independent factors (incubation period, pH, MHC and temperature) were optimized by studying each factor in the design at five different levels (-α, -1, 0, +1 and +α) (Table 1). The minimum [coded as (-1)] and maximum [coded as (+1)]

range of experimental values of each factor used. Here, "α" values are hypothetical values result in terms coded factor values which represented the distance how far out [(-α from -1) and (+α from +1); these star points will be placed in CCD (ver, 7.1; STATEASE INC; Minneapolis, MN, USA). A set of 30 experiments was performed. All variables were taken at a central coded value considered as zero. The minimum and maximum ranges of variables were used and the full experimental plan with respect to their values in coded form is listed in Table 2.

Table 1. Range of the values for the response surface methodology.

Independent variables	Levels*				
	-α	-1	0	+1	+α
Incubation period (days)	2	4	6	8	10
Initial medium pH	3	5	7	9	11
Moisture holding capacity (%)	30	50	70	90	110
Temperature (°C)	10	30	50	70	90

* Here, "α" values are hypothetical values result in terms coded factor values which represented the distance how far out [(-α from -1) and (+α from +1); these star points will be placed in CCD (ver, 7.1; STATEASE INC; Minneapolis, MN, USA).

Statistical analysis and optimization

The data obtained from RSM of exo-PG production was subjected to a test for significant sequential models, which was performed by analysis of variance (ANOVA). In developing the regression equation, the results obtained from RSM were used to fit a second order polynomial equation given below.

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_4 D + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{44} D^2 + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC + \beta_{14} AD + \beta_{24} BD + \beta_{34} CD \quad (1)$$

Where Y was the response variable, β_0 was the intercept, β_1, β_2, β_3 and β_4 were the linear coefficients, β_{11}, β_{22}, β_{33} and β_{44} were the squared coefficient, β_{12}, β_{13}, β_{14}, β_{23}, β_{24} and β_{34} were the interaction coefficient and A, B, C, D, A^2, B^2, C^2, D^2, AB, AC, AD, BC, BD and CD were the level of independent variables. The statistical significance of this equation was determined by 'F' test. The quality of polynomial model was checked by coefficient of determination R^2 value. The optimization process searches for a combination of parameter levels that simultaneously satisfy the requirements placed (i.e. optimization criteria) on each one of process parameters and response. Numerical and graphical optimization methods were used in this work by selecting the desired goals for each parameter and response was used in this investigation. All the statistical analyses from experimental design to optimization were performed using statistical software Design Expert (ver, 7.1; STATEASE INC; Minneapolis, MN, USA).

Effect of incubation period on enzyme production

The inoculum was prepared in basal medium (BM) with following composition (g/l): pectin pure, 5.0; peptone 3.0; K_2HPO_4, 0.6; KH_2PO_4, 0.2, $MgSO_4$ $7H_2O$, 0.1 and distilled water, 1l (pH adjusted to 7.0 before autoclaving) by

transferring a loop full of organism (*B. subtilis* CM5) from a stock culture and incubated at 50°C and 120 rpm for 24 h in an orbital incubator shaker (Remi Pvt. Ltd, Bombay, India). The inoculum contained 1×10^8 colony forming units (CFU)/ml.

Cassava bagasse (20 g) was taken in Roux bottles (132 mm × 275 mm), moistened with 27 ml of distilled water containing 1% peptone to provide 70% moisture holding capacity (MHC) and were mixed thoroughly. The bottles were autoclaved at 15 lb pressure for 30 min. After autoclaving, the bottles were taken out and cooled at room temperature, 30 ± 2°C and inoculated with 15% (w/v) (determined by pre experiment) inoculum of *B. subtilis* (1×10^8 CFU/ml). Then the inoculated substrates were incubated under static condition at 50°C for 8 days in an incubator (Beautex instruments, New Delhi, India). Triplicate bottles were maintained for each treatment. The contents in the bottle were periodically mixed by gentle tapping. After 4 days of incubation, the bottles were taken out at interval of one day (24h). The enzyme was extracted with 25 ml of distilled water (1: 2.4 [Cassava bagasse: Water] ratio) and squeezed through wet cheese cloth. The pooled enzyme extract was centrifuged at 8000 rpm for 20 min in a refrigerated centrifuge (Remi India Pvt Ltd, Bombay, India) and the clear supernatant was used for enzyme assay.

Table 2. Experimental design and result of CCD of response surface methodology.

Observations	A: Incubation period (days)	B: pH	C: Moisture holding capacity (%)	D: Temperature (°C)	Enzyme production (U/gds)	
					Predicted	Experimental
1	-1	-1	-1	-1	170.64	172.45
2	1	-1	-1	-1	180.95	183.38
3	-1	1	-1	-1	183.04	185.63
4	1	1	-1	-1	196.18	195.96
5	-1	-1	1	-1	183.49	186.77
6	1	-1	1	-1	195.07	197.70
7	-1	1	1	-1	193.56	199.35
8	1	1	1	-1	207.18	210.28
9	-1	-1	-1	1	156.37	152.19
10	1	-1	-1	1	167.94	163.13
11	-1	1	-1	1	167.99	164.78
12	1	1	-1	1	181.54	175.71
13	-1	-1	1	1	165.00	166.51
14	1	-1	1	1	177.79	175.20
15	-1	1	1	1	174.92	172.45
16	1	1	1	1	189.00	190.02
17	-α	0	0	0	150.21	142.32
18	α	0	0	0	175.13	172.13
19	0	-α	0	0	138.05	145.23
20	0	α	0	0	165.21	170.00
21	0	0	-α	0	210.21	213.21
22	0	0	α	0	222.21	225.21
23	0	0	0	-α	192.32	185.23
24	0	0	0	α	189.32	175.21
25	0	0	0	0	228.95	229.62
26	0	0	0	0	228.95	230.40
27	0	0	0	0	228.95	228.00
28	0	0	0	0	228.95	231.00
29	0	0	0	0	228.95	227.42
30	0	0	0	0	228.95	226.80

Effect of MHC and initial medium pH on enzyme production

The influence of MHC on the enzyme production was evaluated by varying the moisture content of the substrate from 50 to 90%, and initial medium pHs were adjusted to 5.0- 9.0 by using 0.1N HCl or NaOH. The samples (n = 3) were incubated for 6 days at 50°C.

Effect of temperature on enzyme production

The effect of temperature was studied by incubating the organism at different temperatures (30 – 70°C) maintained in an incubator for 6 days at 70% MHC and 7.0 pH.

Exo-PG assay

Exo-PG production was measured by quantifying reducing groups expressed as D-galacturonic acid equivalents liberated during the incubation of 0.4 ml of 0.1% (w/v) citrus pectin prepared in 1 mM phosphate buffer (pH, 7.0) and 0.1 ml culture supernatant at 50°C for 30 min (14). After incubation, the reaction was stopped by adding 1 ml of Nelson's reagent (18). The mixture was then heated at 100°C for 20 min and 1 ml of arsino -molybdate reagent was added into the mixture for development of colour. The absorbance (Abs) was measured at 520 nm. One unit of exo-PG activity was defined as the amount of enzyme required to release 1 µmol of D-galacturonic acid / min / ml from citrus pectin under the assay condition. The enzyme yield was expressed as unit (U)/ gm dry substrate (gds) (i.e. U/gds).

Determination of moisture of the substrate

The moisture content of the substrate was analyzed by a Mettler LP16 Infra – Red analyser.

RESULTS

Effect of treatment conditions

The effect of four independent variables (i.e. incubation period, pH, MHC and temperature) for exo-PG production by *B. subtilis* CM5 on cassava bagasse were presented along with predicted and observed responses in Table 2. Regression analysis was performed to fit the response function with the experimental data. The statistical significance of the second order equation was checked by an F-test (ANOVA) and data were shown in Table 3. The regression model for exo-PG production was highly significant ($p < 0.001$) with a satisfactory value of determination coefficient ($R^2 = 0.9763$), indicating that 97.63% of the variability in the response could be explained by the following second order polynomial equation:

$$Y = 228.87 + 6.29\ A + 6.10\ B + 5.38\ C - 7.98\ D + 17.46\ A^2 - 17.46\ B^2 - 2.06\ C^2 - 11.80\ D^2 + 0.52\ AB - 0.31\ AC - 0.31\ BC - 0.31\ AD - 0.31\ BD - 0.52\ CD$$

Where Y was the enzyme production (U/gds), A was the incubation period (days), B was the initial medium pH, C was the MHC (%) and D was the temperature (°C).

The coefficient of determination R^2 value always lies between 0 and 1. The closer the value of R^2 is to 1.0, the stronger the model and the better it predicts the response. An adequate precision of 20.88 for Exo-PG production was recorded. The predicted R^2 value of 0.8665 was in reasonable agreement with the adjusted R^2 of 0.9542. Further, a high similarity was observed between the predicted and experimental result (Fig. 1).

The model F- value of 44.16 and values of prob > F less than 0.05 indicated that the model terms were significant. For exo-PG production, the coefficients of A, B, C, A^2, and AB were significant at 1% level but the interaction terms (AC, AD, BC, BD and CD) were not significant. The incubation

Table 3. ANOVA for exo-PG production in solid state fermentation.

Source	Sum of Squares	Degree of freedom	Mean Square	F-Value	p-value
Model	1474.40	14	200.15	44.16	0.001
Pure Error	14.58	5	2.92		
Total	1488.98	19			

R^2	=	0.9763
Adjusted R^2	=	0.9542
Predicted R^2	=	0.8665
Adequate Precision	=	20.88

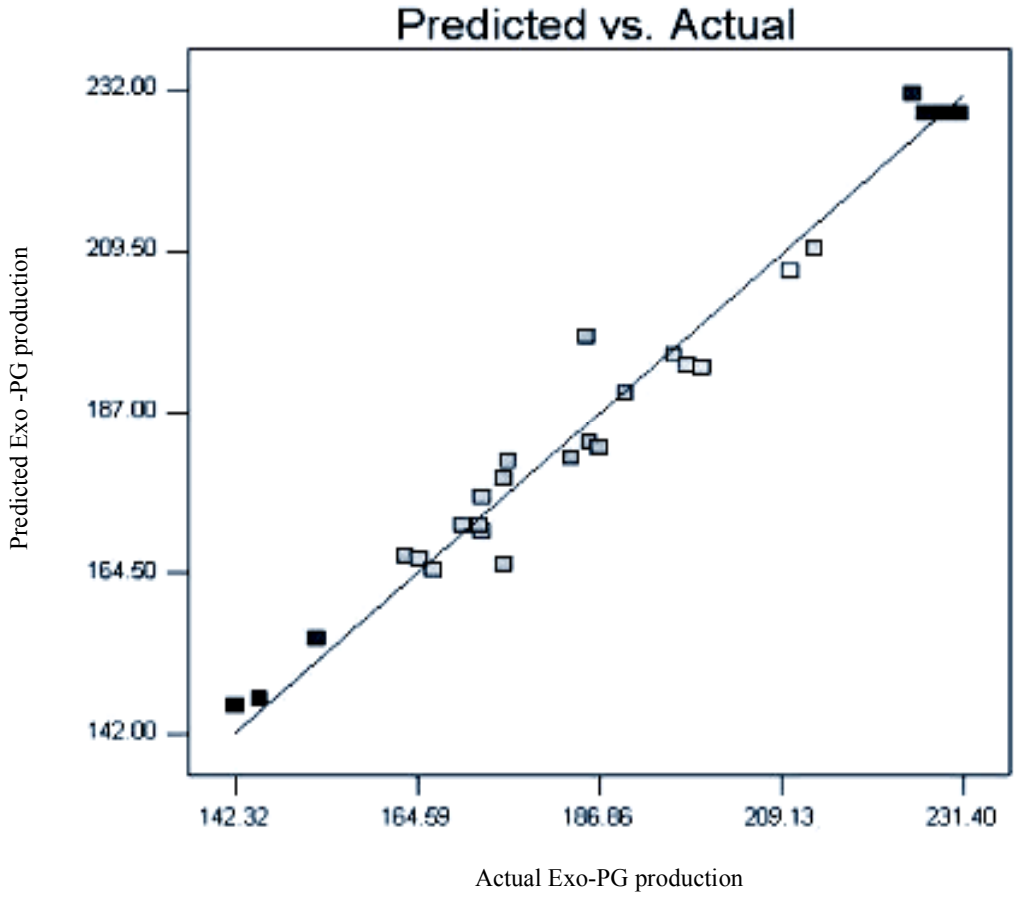


Figure 1. Plot of predicted versus actual exo-PG production

period, pH and MHC had significant positive effect on exo-PG production while temperature displayed negative effect.

Response surface estimation for maximum enzyme production

To investigate the interactive effect of variables on the exo-PG production, the response surface graphs were employed by plotting the effect of independent variables (incubation period, pH, MHC and temperature). Out of four variables, two were fixed at zero level while other two were varied.

Fig. 2A depicts three dimensional diagram and contour plot of calculated response surface from the interaction between incubation period and pH while keeping the other variables (MHC and temperature) at zero level. The result demonstrated that with increase in incubation period and pH up to 6 days and 7.0, respectively, the enzyme production had increased up to 229.87 U/gds and thereafter, it declined.

Fig. 2B shows the effect of incubation period and MHC on enzyme production, keeping pH and temperature at zero level. The graph showed that the maximum exo-PG production (229.65 U/gds) occurred at MHC of 70% and incubation period of 6 days, which was in conformity with the model. An interaction between incubation period and temperature on enzyme production was studied by keeping pH and MHC at zero level (Fig. 2C). The graph shows that the maximum exo-PG production (229.52 U/gds) occurred at temperature of 50°C and incubation period of 6 days, which was in conformity with the model. The response surface was mainly used to find out the optima of the variables for which the response was maximized. An interaction between the remaining two parameters (MHC and temperature) (Fig. 2D) suggested little difference with the earlier responses. Fig. 2E and F represented the three dimensional diagram and contour plots of calculated response surface from the interaction between MHC and pH, and temperature and pH, respectively. Thus, incubation period (6 days), initial medium pH (7.0), MHC (70%) and temperature (50°C) were adequate for attaining maximum enzyme titre (231.0 U/gds) as shown in Table 2.

Optimization

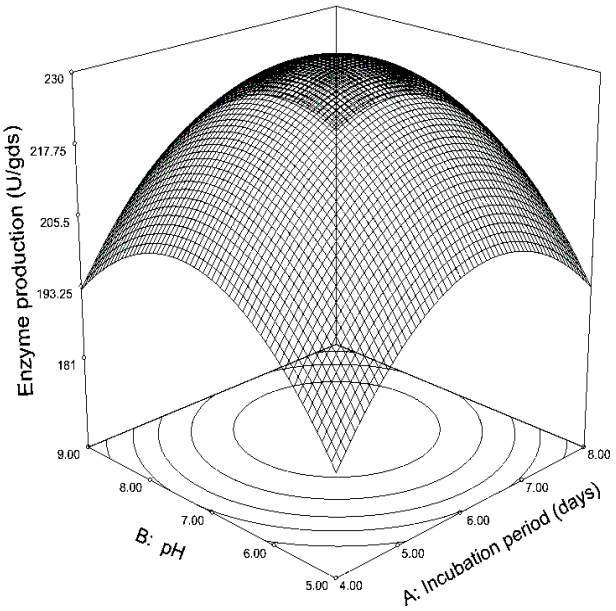
To find out optimum level of process parameters for maximizing the response, the criteria were set, as given in Table 4. The optimization criteria were used to get maximum yield of exo-PG by minimizing incubation period as well as pH and maximizing MHC and temperature.

Testing of model adequacy

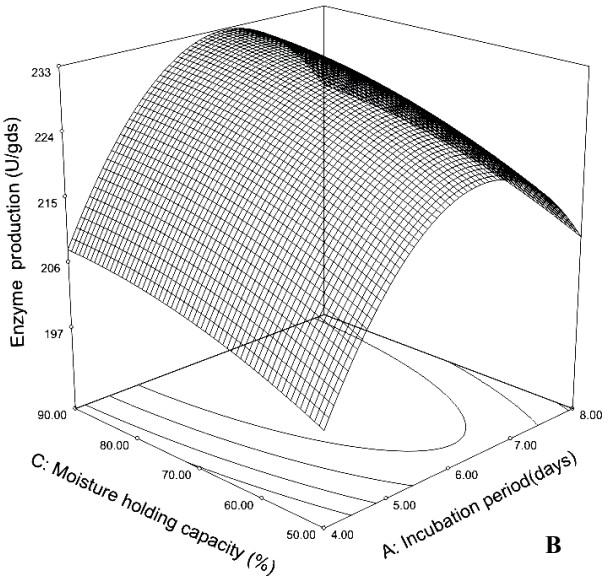
Usually, it is necessary to check the fitted model to ensure that it provides an adequate approximation to the real system. Unless the model shows an adequate fit, processing with investigation and optimization of the fitted response surface likely give poor or misleading results. By constructing a normal probability plot of the residuals, a check was made for the normality assumption, as given in Fig. 3. The normality assumption was satisfied as the residuals are approximated along a straight line.

Practical verification of theoretical results

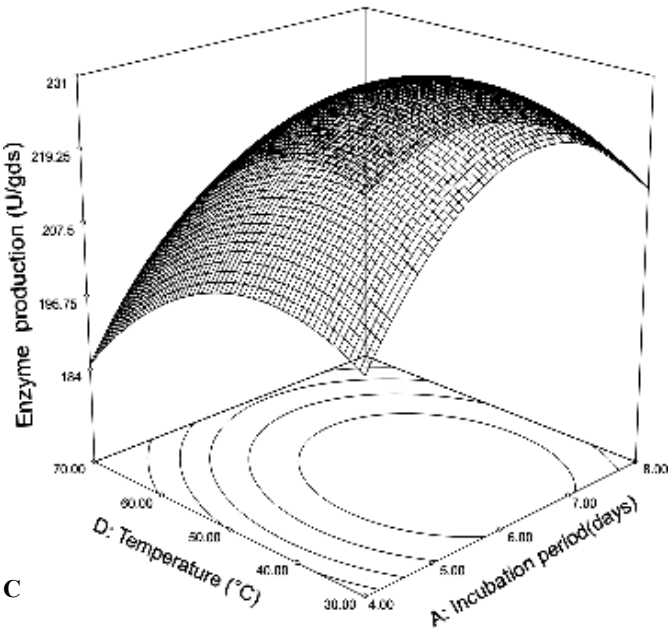
Further to support the optimized data as given by statistical modeling under optimized condition, the confirmatory experiments were conducted with the parameters as suggested by the model (incubation period, 6 days; pH, 7.0; MHC, 70% and temperature, 50°C). The optimized process condition yielded exo-PG production (231 U/gds) which was closer to the predicted exo-PG production (228.95 U/gds) at same optimal point.


Enzyme production (U/gds)
230
217.75
205.5
193.25
181
9.00
8.00
7.00
6.00
5.00
B: pH
4.00
5.00
6.00
7.00
8.00
A: Incubation period (days)

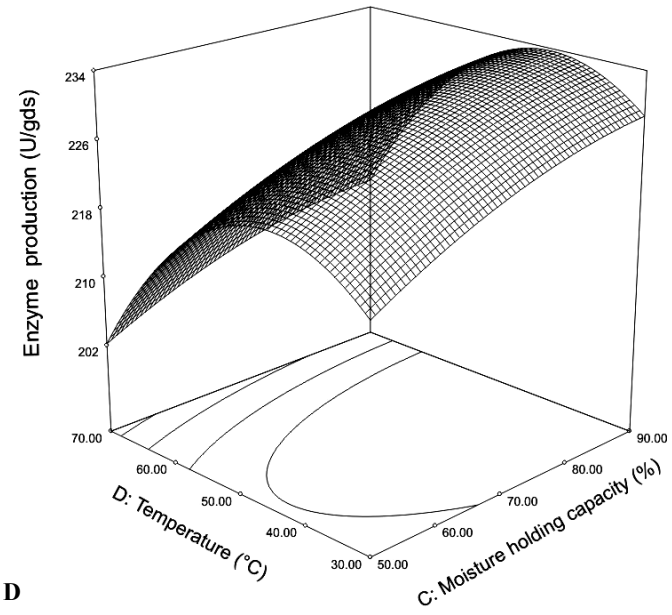
A


Enzyme production (U/gds)
233
224
215
206
197
90.00
80.00
70.00
60.00
50.00
C: Moisture holding capacity (%)
4.00
5.00
6.00
7.00
8.00
A: Incubation period(days)

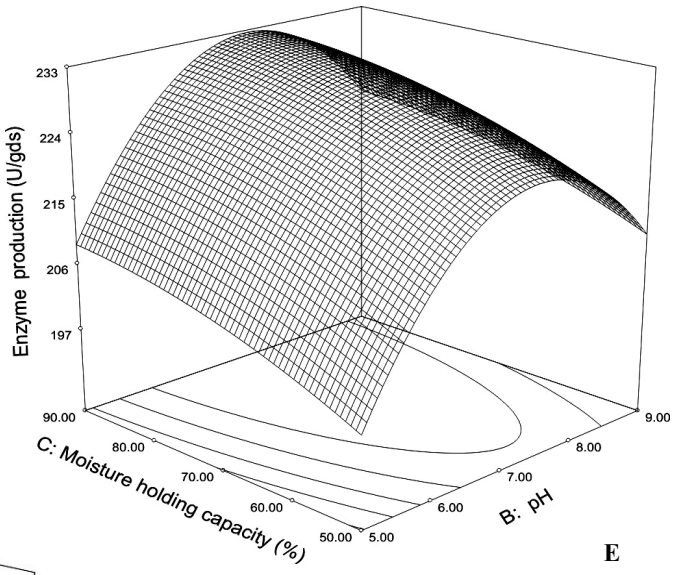
B


Enzyme production (U/gds)
231
219.25
207.5
195.75
184
70.00
60.00
50.00
40.00
30.00
D: Temperature (°C)
4.00
5.00
6.00
7.00
8.00
A: Incubation period(days)

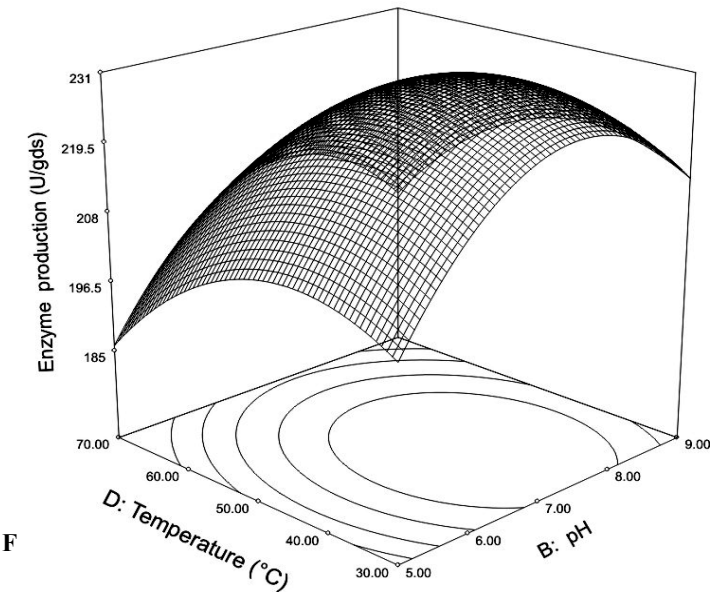
C



D



E



F

Figure 2. Statistical optimization of enzyme production using RSM, A: incubation period and pH; B: incubation period and MHC; C: incubation period and temperature; D: MHC and temperature; E: pH and MHC; F: pH and temperature

Table 4. Optimization criteria used in this study.

Parameter or Response	Limits		Importance	Criterion
	Lower	Upper		
Incubation period (days)	4	8	3	Minimize
pH	5.0	9.0	3	Minimize
Moisture holding capacity (%)	40	90	3	Maximize
Temperature (°C)	30	70	5	Maximize
Enzyme production	1372	3828	5	Maximize

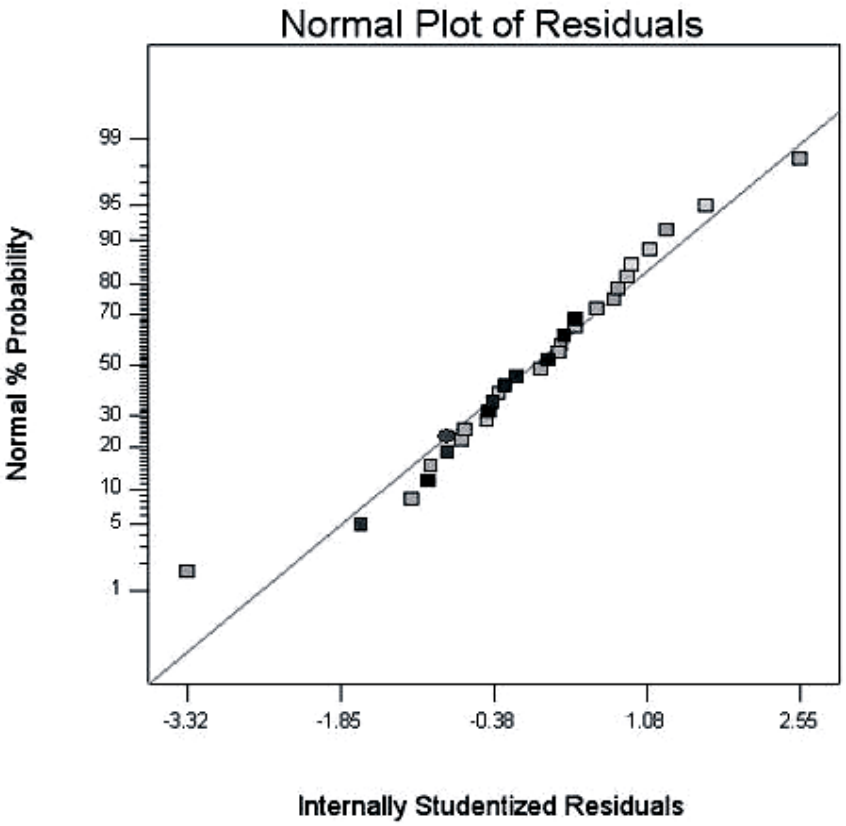


Figure 3. Normal probability plot of studentized residuals

DISCUSSION

RSM used in this investigation suggested the importance of various fermentation parameters at different levels. In the study, high similarities were observed between the predicted and experimental results, which reflected the accuracy and applicability of RSM to optimize enzyme production in SSF. In this study, an incubation period of 6 days, initial medium pH of 7.0, MHC of 70% and temperature of 50°C were the major factors that influenced the enzyme production. The production of PG reached a pick at 6 days (229.0 U/gds) and thereafter, it declined. This could be due to loss of moisture with prolonged incubation at 50°C and/or interaction with other components in the culture medium (9). In most cases, the optimum incubation period for PG production in SSF varied from 3 to 6 days, depending on the environmental conditions (4,16). In contrast, PG production from *Penicillum* sp. EGC5 was reported maximum at 8 days using a mixture of substrates (16).

Moisture is one of the most important parameter in SSF that influences the growth of the organism and thereby enzyme production (30). In general, the moisture level in SSF process varies between 70-80% for bacteria (9, 19). PG production from *B. subtilis* RCK was reported at 70% MHC using wheat bran as the solid substrate. Beyond 70% MHC, the enzyme production declined; the decline might be due to low porosity, lower O_2 transfer, poor aeration and adsorption of enzyme to the substrate particles (10, 13). Moreover, the optimum PG production for *B. subtilis* RCK on wheat bran was found to be at 60% MHC (11).

The influence of temperature is related to the growth of the organism. The isolate *B. subtilis* CM5 showed optimum PG production at 50°C. Further increase in the temperature led to a decrease in enzyme production. The optimum PG production for other *Bacillus* spp. was found in the range of 40 - 50°C in SSF using wheat bran as solid substrate (23).

Among physico-chemical parameters, pH plays an important role including morphological changes on organism in enzyme production. In this study, maximum PG production was achieved at pH 7.0. Further increase in pH, a reduction in enzyme production was observed. Freitas et al. (8) reported a pH of 5.5 to be the best for the production of PG by *Monasus* sp. N8 and *Aspergillus* sp. NIZ in SSF. Moreover, *Bacillus* sp. is reportedly produces PG at an optimum pH of 6.0 - 7.0 in SSF using wheat bran (23).

To sum of, cassava bagasse, an inexpensive agro-residue can serve as a suitable substrate for production of PG by *B. subtilis* by optimizing process parameters like incubation period (6 days), pH (7.0), temperature (50^0C) and MHC (70%), Further research is being carried out in our laboratory to study the application of *B. subtilis* PG in extraction of vegetable juice and degumming of jute fibre.

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RESUMO

Produção de exo-poligalacturonase por *Bacillus subtilis* CM5 por fermentação em estado sólido empregando bagaço de mandioca

O objetivo desta investigação foi estudar a produção de exo-poligalacturonase (exo-PG) por *Bacillus subtilis* CM5 por fermentação em estado sólido empregando bagaço de mandioca. Empregou-se a metodologia de superfície de resposta para avaliar o efeito de quatro variáveis na produção da enzima: período de incubação, pH inicial do meio, MHC e temperatura de incubação. Os resultados experimentais

mostraram que os ótimos de temperatura, período de incubação, MHC e temperatura para produção de exo-PG foram seis dias, 7,0, 70% e 50^{o}C, respectivamente.

Palavras-chave: exo-poligalacturonase, *Bacillus subtilis* CM5, metodologia de superfície de resposta, fermentação em estado sólido, bagaço de mandioca

REFERENCES

1. Boyaci, I.H. (2005). A new approach of determination of enzyme kinetic constants using response surface methodology. *Biochem. Eng. J.* 25, 55-62.
2. Budiatman, S.; Lonsane, B.K. (1987). Cassava Fibrous waste residues: a substitute to wheat bran in solid state fermentation. *Biotech. Letter*. 9, 597-600.
3. Carvalho, J.C.M.; Vitolo, M.; Sato, S.; Aquarone, E. (2003). Ethanol production by *Saccharomyces cerevisiae* grown in sugarcane blackstrap molasses through a feed batch process: optimization by response surface methodology. *Appl. Biochem. Biotechnol*. 110, 151-164.
4. Castilho, L.R.; Medronho, R.A.; Alves, T.L.M. (2000). Production and extraction of pectinases obtained by solid state fermentation of agro-industrial residues with *Aspergillus niger*. *Biores. Technol*. 71, 45-50.
5. Cavalitto, S.F.; Arcas, J.A.; Hours, R.A. (1996). Pectinase production profile of *Aspergillus foedidus* in solid state cultures at different acidities. *Biotechnol. Lett*. 18, 251-256.
6. Christen, P.; Meza, J.C.; Revah, S. (1997). Fruity aroma production in solid state fermentation by *Ceratocystis fimbriata*: influence of the substrate type and the presence of precursors. *Mycol. Res*. 101, 911-919.
7. Edison, S.; Anantharaman, M.; Srinivas, T. (2006). Status of cassava in India – an overall view. Tech. Bull. Ser. 46, Central Tuber Crops Research Institute, Thiruvanathapuram, India, p.79.
8. Freitas, P.M.; Martin, N.; Silva, D.; Silva, R.; Gomes, E. (2006). Production and partial characterization of polygalacturonases produced by thermophilic *Monascus* sp. N8 and by thermotolerant *Aspergillus* sp. N12 in solid-state fermentation. *Braz. J. Microbiol*. 37, 302-306.
9. Gangadharan, D.; Sivaramakrishnan, S.; Nampoothiri, K.M.; Panday, A. (2006). Solid culture of *Bacillus amyloliquefaciens* for alpha amylase production. *Food Technol. Biotechnol*. 44, 269-274.
10. Gessesse, A.; Mamo, G. (1999). High-level xylanase production by an alkalophilic *Bacillus* sp. by using solid-state fermentation. *Enzyme Microb. Technol*. 25, 68–72.
11. Gupta, S.; Kapoor, M.; Sharma, K.K.; Nair, L.M.; Kuhad, R.C. (2007). Production and recovery of an alkaline exo-polygalacturonase from *Bacillus subtilis* RCK under solid state fermentation using statistical approach. *Biores. Technol.* doi:10.1016/j.biotech.2007.03.009.
12. He, G.Q.; Kong, Q.; Dingm, L.X. (2004). Response surface methodology for optimizing the fermentation medium of *Clostridium butyricum*. *Lett. Appl. Microbiol*. 39, 363-368.
13. Holker, U.; Hofer, M.; Lenz, J. (2004). Biotechnological advantages of laboratory-scale solid state fermentation with fungi. *Appl. Microbiol. Biotechnol*. 64, 175-186.
14. Kapoor, M.; Beg, Q.K.; Bhushan, B.; Dadhich, K.S.; Hoondal, G.S. (2000). Production and partial purification and characterization of a thermo-alkali stable polygalacturonase from *Bacillus* sp. MG-cp-2. *Process Biochem*. 36, 467–473.
15. Kolicheski, M. B.; Soccol, C. R.; Marin, B.; Medeiros, E.; Raimbault, M. (1995). Citric acid production on three cellulosic supports in solid state fermentation, *In*: Roussos, S. (ed.). *Advance in Solid State Fermentation*, Kluwer Academic Publisher, Dordrecht, pp. 447-460.
16. Martin, N.; De Souza, S.R.; Da Silva, R.; Gomes, E. (2004). Pectinase production by fungal strains in solid-state fermentation using agro-industrial byproducts. *Braz. Arch. Biol. Technol*. 47, 813–819.
17. Martins, E.S.; Silva, D.; Da Silva, R.; Gomes, E. (2002). Solid state production of thermostable pectinases from thermophilic *Thermoascus aurantiacus*. *Preoess Biochem*. 37**,** 949-954.
18. Nelson, N. (1944). A photometric adaptation of Somogyi method for the determination of glucose. *J. Biol. Chem*. 153, 375-380.
19. Pandey, A.; Soccol, C.R.; Mitchell, W. (2000). New development in solid state fermentation: I.- bioprocess and products. *Process Biochem*. 35, 1153-1169.
20. Ray, R. C., Sahoo, A.K., Asana, K.; Tomita. F. (2007). Microbial processing of agricultural residues for production of food, feed and food-additives *In*: Ray, R C., (ed). *Microbial Biotechnology in Agriculture and Aquaculture,* vol II. Science Publishers, Inc, Enfield, New Hampshire, pp.511-552.
21. Shirai, K.; Guerrero, I.; Huerta, S.; Saucedo, G.; Castillo, A.; Gorzalez, R.O.; Hall, G.M. (2001). Effect of initial glucose concentration and inoculum level of lactic acid bacteria in shrimp waste ensilation. *Enzyme Microb. Technol*. 28, 446-452.
22. Silva, D.; Tokuioshi, K.; Martins, E.D.S.; Silva, R.D.; Gomes, E. (2005). Production of pectinase by solid-state fermentation with *Penicillium viridicatum* RFC3. *Process Biochem*. 40, 2885–2889.
23. Soares, M.M.C.N.; Silva R.D.; Gomes, E. (1999). Screening of bacterial strains for pectinolytic activity: characterization of the

polygalacturonase produced by *Bacillus* sp. *Rev de Microbiol*. 30, 299-303.

24. Swain, M.R.; Kar, S.; Padmaja, G.; Ray, R.C., (2006). Partial characterization and optimization of production of extracellular α-amylase from *Bacillus subtilis* isolated from culturable cowdung microflora. *Polish J. Microbiol*. 55, 289-296.
25. Swain, M.R.; Naskar, S.K.; Ray, R.C. (2007). Indole-3-acetic acid production and effect on sprouting of yam (*Dioscorea rotundata* L.) minisetts by *Bacillus subtilis* isolated from culturable cowdung microflora. *Polish J. Microbiol*. 56, 102-110.
26. Swain, M. R.; Ray, R.C. (2007a). Statistical optimization for α-amylase production by *Bacillus subtilis* CM3 isolated from cowdung microflora in solid-state fermentation using cassava fibrous residue. *J. Basic Microbiol*. 47, 417-128.
27. Swain, M.R.; Ray, R.C. (2007b). Biocontrol and other beneficial activities of *Bacillus subtilis* isolated from cowdung microflora. *Microbiol. Res*. doi:10.1016/j.micres.2006.10.009
28. Torres, E.F; Sepulveda, T.V.; Gonzalez, G.V. (2006). Production of hydrolytic depolymerising pectinases. *Food Technol. Biotechnol*. 44, 221-227.
29. Xiong, C.; Shouwen, C.; Ming, S.; Ziniu, Y. (2005). Medium optimization by response surface methodology for poly-Y- glutamic acid production using dairy manure as the basis of a solid substrate. *Appl. Microbiol. Biotechnol*. 69, 390-396.
30. Yang. S. S.; Wang, J. Y. (1999). Protease and amylase production of *Streptomyces rimosus* in submerged and solid state cultivations. *Bot. Bull. Acad. Sin.* 40, 259-265.