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Enhanced Production of Pectin Lyase and Pectate Lyase by *Debaryomyces nepalensis* in Submerged Fermentation by Statistical Methods

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Abstract: Production of pectic lyases (PL and PGL) from *Debaryomyces nepalensis* has been optimized in submerged fermentation using Plackett-Burman design and response surface methodology. Four of the eleven fermentation variables (yeast extract, galactose, lemon peel and temperature) tested by Plackett-Burman design showed significant effect on both pectin lyase and pectate lyase production. The four screened variables were further optimized by central composite design. The optimal values of yeast extract, galactose, lemon peel powder and temperature for PL production was found to be 1.885, 0.956, 2.39% and 31.5°C and for PGL was at 2.5, 0.55, 2.4% and 32.7°C, respectively. The optimum activities of PL and PGL were found to be 10.73 and 8.73 U mL^{-1} after optimization which shows a 2.5 and 2.9 fold increase in PL and PGL production, respectively. This is the first time an optimization was done for production of PL and PGL by yeast *Debaryomyces nepalensis*. This could be an alternative to fungal pectolytic enzymes.

Key words: Pectin lyase, pectate lyase, *Debaryomyces nepalensis*, submerged fermentation, Plackett-Burman design, central composite design

Introduction

Pectin has a linear backbone comprised of α-1,4-linked D-galacturonic acid residues, which are esterified by methanol at the carboxylic groups. Based on the degree of esterification, the different pectic substances, viz., protopectin, pectin, polygalacturonic acid and pectinic acid exist in nature. The carboxylic groups of galacturonic acid are partially or completely neutralized by Na^+, K^+ and NH_4^+ ions. The side chains of pectin molecule consist of rhamnose, galactose, arabinose and xylose (Whitaker, 1991). Pectinases are a complex group of enzymes capable of degrading pectic substances. The hydrolysis of pectin backbone is obtained by the synergistic action of several enzymes, including pectin methylesterase (EC.3.1.11.1), endo-polygalacturonase (EC. 3.2.1.15), exo-polygalacturonase (EC. 3.2.1.67), endo-pectate lyase (EC.4.2.2.2), exo-pectate lyase (EC. 4.2.2.9) and endo-pectin lyase (4.2.2.10) (Gummadi and Panda, 2003; Gummadi and Kumar, 2005).

Pectic transeliminases or pectic lyases are one among the complex group of pectinases, which degrade pectic substance by transelimination mechanism yielding unsaturated oligogalacturonates. Pectate Lyase (PGL), acting on polygalacturonic acid and Pectin Lyase (PL), acting on pectin are the two important transeliminases. PL is extensively used in fruit juice industries, in the degumming of ramie, hemp, flax and jute fibers (Hoondal *et al*., 2002; Naidu and Panda, 1998; Bruhlmann *et al*., 1994;

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Kapoor *et al.*, 2001). PGL is effective for scouring of crude cotton fiber (Hoondal *et al.*, 2002) and also used in tea and coffee fermentations (Carr, 1985). Pectinases also play an important role in nature by degrading plant wastes for carbon recycle. In general PL is mainly produced by fungi and PGL is produced by pathogenic bacteria. Since new applications for pectic transeliminases are emerging, the demand for production of these enzymes is increasing. Hence it is important to produce these two enzymes by a single organism is crucial.

In this context, we previously isolated a yeast strain *Debaryomyces nepalensis* from apple capable of utilizing pectin as the sole source of carbon and the isolate produced both pectic lyases. In this study, fermentation conditions were optimized for maximum production of these enzymes using statistical experimental design. The general practice of determining these optima is by varying one parameter while keeping the other at an unspecified constant level. The major disadvantage of this single variable optimization is that it does not include interactive effects among the variables; thus, it does not depict the net effects of various parameters on enzyme activity. An alternative and more effective approach is the use of statistical design. Response Surface Methodology (RSM) involves full factorial search by examining simultaneous, systematic and efficient variation of all components. It is useful for small number of variables (up to five), but impractical for a large number of variables, due to a high number of experimental runs required. Therefore, for screening more than five factors, Plackett-Burman design is recommended. In this study, the initial screening was performed by Plackett-Burman design to identify the critical variables and optimization of these critical variables was determined by RSM. This is the first report on optimization of both the transeliminases from a single microorganism and also the first report from a novel strain *Debaryomyces nepalensis*.

Materials and Methods

Materials and Chemicals

All the chemicals were of analytical grade procured in India. Pectin and polygalacturonic acid, substrates for PL and PGL was purchased from Sigma.

Source of Microorganism

The isolate was identified as *Debaryomyces nepalensis* (99.8% sequence identity) based on 26S rDNA D1/ D2 sequence analysis and is deposited in National Collection of Yeast Cultures (NCYC), Norwich, UK with accession number D3893. The isolate was maintained on YEPD agar plate at 4°C and subcultured for every two weeks.

Transeliminase Production

A loopful of the strain from YEPD agar plates was transferred to 5 ml sterile YEPD medium and incubated on rotary shaker at 180 rpm and 30°C. After 12 h, 2% (v/v) of the seed culture was transferred into a 100 mL Erlenmeyer flask containing 25 mL of pectic transeliminase medium (PT medium) and incubated at 180 rpm and 30°C. PT medium had the following composition (0.01% $MgS0_4.7H_20$, 0.2% NH_4Cl, 0.6% Na_2HPO_4, 0.3% K_2HPO_4, 0.5% NaCl, 0.5% lemon peel). The initial pH was adjusted to 7.0 before sterilization. The composition of the PT medium is changed according to the experimental requirement for Plackett Burman design and Central Composite Design (CCD).

Enzyme Assay

Supernatant was used as the source for enzyme assay. PL activity was assayed by measuring the formation of unsaturated oligogalacturonates at 235 nm (Albersheim, 1966). The reaction mixture

contained 0.19% (w/v) pectin in 100 mM citrate phosphate buffer (pH 6.4) and suitably diluted enzyme. The assay mixture was incubated for 3 min at 35°C for measuring PL activity and at 32°C for measuring PGL activity, the increase in absorbance at 235 nm was measured using Perkin Elmer UV-Visible spectrophotometer. To test PGL activity, pectin was replaced by 0.15% (w/v) polygalacturonic acid in 75 mM Tris-HCl buffer (pH-7.5) with 1 mM $CaCl_2$. One unit of enzyme activity was defined as an increase of 1.0 unit of absorbance at 235 nm of the reaction mixture per minute per ml of enzyme solution (Alana *et al*., 1990; Hayashi *et al*., 1997; Nakagawa *et al*., 2000).

Experimental Design and Data Analysis: Plackett-burman Design

Plackett-Burman, a two factorial design was used to screen and identify significant medium components and physical parameters that influence the enzyme productivity. The total number of experiments carried out for N variables in (N + 1) experiments (Plackett and Burman, 1946). Each variable is represented at two levels maximum (+) and minimum (-). The number of positive signs is equal to (N+1)/2 and the number of negative signs is equal to (N-1)/2 in a row and a column should contain equal number of positive and negative signs. The 11 variables, eight medium components ($MgSO_4.7H_2O$, Na_2HPO_4, K_2HPO_4, NaCl, lemon peel, galactose, Yeast extract and $FeCl_3$) and three physical parameters (pH, temperature and agitation) were analyzed; the experimental design and levels of each variable were shown in Table 2. The first row contains (N+1)/2 positive signs and (N-1)/2 negative signs and the choice of placing the signs is arbitrary. The next (N-1) rows were generated by shifting cyclically one place (N-1) times and the last row contains all negative signs.

The effect of each variable was calculated using the following equation

$$E_{xi} = (\sum M_{i+} - M_{i-})/N \quad (1)$$

where, E_{xi} is the effect of the tested variable, M_{i+} and M_{i-} are the PL and PGL production from trials in which the variables being measured were added to the medium at their maximum and minimum level, respectively and N is the number of experiments carried out.

The Standard Error (SE) of the variables was the square root of variance and the significance level (p-value) of each variable is calculated by using the student's *t*-test:

$$t = E_{xi}/SE \quad (2)$$

where, E_{xi} is the effect of the tested variable. The variables with higher confidence levels were considered to influence enzyme production.

Experimental Design and Data Analysis: Central Composite Design

The significant variables obtained by Plackett Burman design were optimized by using statistical experimental design technique called the response surface methodology and central composite design (circumscribed) was used. According to this design, the total number of treatment combinations is $2^k + 2k + n_0$, where 'k' is the number of independent variables and n_0 is the number of repetitions of the experiments at the center point. For statistical calculation, the variables X_i have been coded as x_i according to the following transformation:

$$x_i = (X_i - X_0)/\delta X \quad (3)$$

where x_i is dimensionless coded value of the variable X_i, X_o is the value of the X_i at the center point and X is the step change. A 2^k-factorial design with eight axial points and six replicates at the center point with a total number of 30 experiments was employed for optimizing the medium components. The coded and uncoded values of the variables at various levels are given in Table 5. The number of center point replications can also be chosen to verify any change in the estimation procedure, which will also be a measure of precision described by the following equation:

$$n_o = \lambda_4(\sqrt{F} + 2)^2 - F - 2k \quad (4)$$

where, F is the number of points in factorial portion, i.e., first four experiments in experimental design and λ_4 is the mixed fourth order moment (Box and Wilson, 1951).

The behavior of the system was explained by the following quadratic equation:

$$Y = \beta_0 + \sum\beta_i x_i + \sum\beta_{ii} x_i^2 + \sum\beta_{ij} x_i x_j \quad (5)$$

where, Y is the predicted response, β_0 is the intercept term, β_i is the linear effect, β_{ii} is the squared effect and β_{ij} is the interaction effect. The regression equation was optimized for maximum value to obtain the optimum conditions using MATLAB® version 7.0 (Mathworks Inc., Natick, Massachusetts, USA).

Results

The preliminary experiments carried out to find out the best carbon source, nitrogen source and metal ions on transeliminases production. These experiments were carried out by adding one variable and keeping others at a constant level to the PT medium (Table 1). The effect of carbon sources were performed by adding different carbon source at 0.5 % (w/v) to the PT medium, the control for this experiment is PT medium without any carbon source which contains ammonium chloride at 0.2%. Galactose produces the maximum amount of PL and PGL (Table 1) and hence galactose has been kept constant in the medium to see the effect of nitrogen source. The effect of nitrogen sources was studied by varying the nitrogen source in the medium and with galactose at 0.5% (w/v) in the PT medium. The different nitrogen sources tested were yeast extract, peptone, tryptone, malt extract and beef extract at an initial concentration of 0.2% (w/v). Among the tested nitrogen source, yeast extract showed maximum production of PL and PGL (Table 1). The control for this experiment is in PT medium containing galactose at 0.5% (w/v) and ammonium chloride at 0.2% (w/v). The effect of metal ions was studied by varying different metal ions in the medium containing galactose (0.5% w/v) and yeast extract (0.2% w/v). Among all metal ions, Fe^{3+} in the medium enhanced the production of both PL and PGL (Table 1). The addition of other metal ions reduced the production of PL and PGL than control (Table 1). Hence the medium considered for screening and optimization had galactose, yeast extract (instead of ammonium chloride) and Fe^{3+} along with other nutrients in the PT medium.

The initial screening was done to screen out the significant components effecting transeliminases production by Plackett-Burman method. The design matrix along with the experimental values is shown in Table 2. Of the 11 variables tested by Plackett- Burman design yeast extract (E), lemon peel

Table 1: The effect of carbon, nitrogen and metal ions on production of PL and PGL by *Debaryomyces nepalensis*

	Pectic transeliminase activity (U mL^{-1})[$]	
Medium component	PL	PGL
Control[a]	4.32	3.04
Carbon source[b]		
Glucose	4.36	2.76
Fructose	4.84	2.95
Galactose	5.14	3.1
Lactose	4.81	2.9
Sucrose	4.54	2.65
Xylose	4.56	3.32
Arabinose	4.2	3.37
Maltose	4.3	2.86
Cellobiose	3.8	2.81
Nitrogen source[c]		
Yeast extract	5.82	3.95
Peptone	4.3	3.25
Tryptone	4.74	3.18
Malt extract	4.64	3.0
Beef extract	2.77	2.51
Metal ions[d]		
Cobalt	4.43	3.46
Iron	6.13	4.16
Manganese	3.69	3.42
Nickel	4.26	3.36
Zinc	4.08	3.15
Calcium	3.93	3.5

[$] The experiments were performed in triplicates and the values reported are mean with an error varying between ±5 to 8%, [a]Experiments were performed with PT medium composition, [b]PL and PGL activities in PT medium using different carbon sources at 0.5% (w/v), [c]PL and PGL activities in PT medium containing galactose at 0.5% (w/v) and different nitrogen source at 0.2% (w/v), [d]PL and PGL activities in PT medium containing galactose at 0.5% (w/v), yeast extract at 0.2% (w/v) along with metal ions at 0.1% (w/v)

Table 2: Plackett-Burman experimental design matrix and the corresponding PL and PGL activities produced by *Debaryomyces nepalensis*[a]

												Pectic transeliminases activity (U mL^{-1})	
Run No.	A	B	C	D	E	F	G	H	I	J	K	PL	PGL
1	-	-	+	+	+	-	+	+	-	+	-	6.2	4.74
2	-	-	-	+	+	+	-	+	+	-	+	5.68	4.25
3	+	-	-	-	+	+	+	-	+	+	-	5.8	4.68
4	-	+	-	-	-	+	+	+	-	+	+	5.35	4.95
5	+	-	+	-	-	-	+	+	+	-	+	6.04	4.64
6	+	+	-	+	-	-	-	+	+	+	-	3.7	4.65
7	-	+	+	-	+	-	-	-	+	+	+	3.04	4.95
8	+	-	+	+	-	+	-	-	-	+	+	3.92	4.15
9	+	+	-	+	+	-	+	-	-	-	+	6.84	4.55
10	+	+	+	-	+	+	-	+	-	-	-	5.87	3.9
11	-	+	+	+	-	+	+	-	+	-	-	5.62	4.15
12	-	-	-	-	-	-	-	-	-	-	-	4.88	4.12

[a]A, $MgSO_4.7H_2O$ at a high concentration of 0.05% and low concentration of 0.01%; B, Na_2HPO_4 at a high concentration of 0.12% and low concentration of 0.6%; C, K_2HPO_4 at a high concentration of 0.6% and low concentration of 0.3%; D, NaCl at a high concentration of 0.8% and low concentration of 0.5%; E, Yeast extract at a high concentration of 0.5% and low concentration of 0.2%; F, Galactose at a high concentration of 0.1% and low concentration of 0.5%; G, Lemon peel at a high concentration of 0.1% and low concentration of 0.5%; H, $FeCl_3$ at a high concentration of 0.3% and low concentration of 0.1%; I, pH at higher range of 9 and lower range of 7; J, Temperature at higher range of 40°C and lower range of 30°C; K, Agitation at higher value of 220 rpm and a lower value of 180 rpm, + and -, the higher and lower concentration of variable, respectively

Table 3: Statistical analysis of fermentation conditions on PL production by *Debaryomyces nepalensis*

Variable	Effect	t-value	Probability>t	Confidence (%)
A	0.234	23.4	0.0153	98.5
B	-0.35	-35	0.0103	98.9
C	-0.26	-26	0.0153	98.5
D	0.163	16.32	0.022	97.8
E	0.652	65.2	0.0052	99.5
F	0.256	25.6	0.0153	98.5
G	1.46	146	0.0018	99.8
H	0.456	45.6	0.0068	99.3
I	-0.53	-53	0.0067	99.3
J	-1.152	-115.2	0.0018	99.8
K	-0.2	-20	0.018	98.2

Table 4: Statistical analysis of fermentation conditions on PGL production by *Debaryomyces nepalensis*

Variable	Effect	t-value	Probability>t	Confidence (%)
A	-0.098	-6.93	0.0505	94.9
B	0.95	6.72	0.0505	94.9
C	-0.112	-7.92	0.0401	95.9
D	-0.125	-8.84	0.0300	97.0
E	0.068	4.81	0.0601	93.9
F	-0.252	-17.8	0.02	98.0
G	0.282	19.9	0.0174	98.3
H	0.088	6.22	0.0307	96.9
I	0.152	10.75	0.0256	97.4
J	0.418	29.56	0.0117	98.8
K	0.208	14.71	0.023	97.7

powder (G) and temperature (J) were found to influence PL production significantly with % confidence levels 95% and above, whereas in case of PGL galactose (F), lemon peel powder (G) and temperature (J) shows significant effects with % confidence levels 98% and above (Table 3 and 4). As it has been found from results that lemon peel powder (G) and temperature (J) both has significant effect in common on PL and PGL production. In case of PL production yeast extract (E) shows significant effect and galactose (F) for PGL showed significant effect. In order to optimize both PL and PGL combinely, both yeast extract (E) and galactose (F) were chosen as critical component for further optimization along with lemon peel powder (G) and temperature (J).

The optimization was done by RSM using CCD and the results obtained were analyzed by ANOVA. Experiments were performed at different combinations of the variables in order to study the combined effect of these variables. A central composite design was used for studying the interaction of these variables within a range of -2 to +2 in relation to transeliminase production. The CCD experimental design along with the experimental values is shown in Table 5. The experimental activities of PL and PGL obtained were reported in Table 5. By applying multiple regression analysis on the experimental data, the following second order polynomial equations were obtained for PL and PGL production.

$$\text{PL production} = 9.92 + 0.458x_1 - 0.051x_2 + 0.259x_3 - 0.847x_4 - 0.539x_1^2 - 0.196x_2^2 - 0.344x_3^2 - 0.98x_4^2 + 0.278x_1x_2 - 0.018x_1x_3 - 0.144x_1x_4 + 0.123x_2x_3 - 0.203x_2x_4 - 0.237x_3x_4 \quad (6)$$

$$\text{PGL production} = 8.09 + 0.3345x_1 - 0.036x_2 + 0.165x_3 - 0.253x_4 - 0.231x_1^2 - 0.362x_2^2 - 0.589x_3^2 - 1.235x_4^2 - 0.285x_1x_2 + 0.387x_1x_3 - 0.295x_1x_4 + 0.157x_2x_3 - 0.065x_2x_4 - 0.355x_3x_4 \quad (7)$$

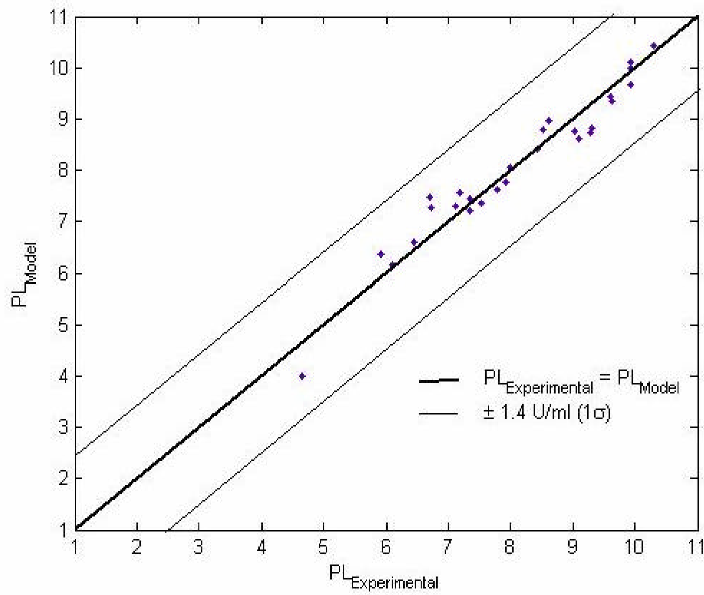
Table 5: Experimental design matrix for CCD and showing the corresponding activities of PL and PGL produced by *Debaryomyces nepalensis*

Design No.1	x_1 (=X_1) (Yeast extract)	x_2 (=X_2) (Galactose)	x_3 (=X_3) (Lemon peel)	x_4 (=X_4) (Temp.)	Experimental values (U mL^{-1}) PL	PGL
1	-1(=10)	+1(=10)	+1(=25)	+1(=40)	5.93	5.34
2	0(=15)	0(=7.5)	0(=20)	0(=35)	9.92	8.09
3	0(=15)	0(=7.5)	0(=20)	0(=35)	9.92	8.09
4	+1(=20)	-1(=5)	-1(=15)	-1(=30)	9.1	6.49
5	-1(=10)	-1(=5)	+1(=25)	-1(=30)	9.29	5.35
6	+1(=20)	-1(=5)	+1(=25)	+1(=40)	7.35	6.33
7	-1(=10)	-1(=5)	-1(=15)	+1(=40)	7.53	5.68
8	+1(=20)	+1(=10)	-1(=15)	+1(=40)	6.73	4.98
9	+1(=20)	+1(=10)	+1(=25)	-1(=30)	10.3	8.24
10	-1(=10)	+1(=10)	-1(=15)	-1(=30)	7.35	5.5
11	+1(=20)	+1(=10)	-1(=15)	-1(=30)	9.63	4.57
12	-1(=10)	-1(=5)	-1(=15)	-1(=30)	8.0	4.65
13	0(=15)	0(=7.5)	0(=20)	0(=35)	9.92	8.09
14	-1(=10)	+1(=10)	-1(=15)	+1(=40)	6.1	5.38
45	-1(=10)	-1(=5)	+1(=25)	+1(=40)	7.12	4.84
16	+1(=20)	+1(=10)	+1(=25)	+1(=40)	7.78	5.49
17	-1(=10)	+1(=10)	+1(=25)	-1(=30)	9.31	6.44
18	+1(=20)	-1(=5)	-1(=15)	+1(=40)	7.19	4.98
19	+1(=20)	-1(=5)	+1(=25)	-1(=30)	9.6	7.88
20	0(=15)	0(=7.5)	0(=20)	0(=35)	9.92	8.09
21	0(=15)	0(=7.5)	0(=20)	0(=35)	9.92	8.09
22	0(=15)	0(=7.5)	0(=20)	0(=35)	9.92	8.09
23	0(=15)	0(=7.5)	0(=20)	+2(=45)	4.65	2.98
24	-2(=5)	0(=7.5)	0(=20)	0(=35)	6.46	6.44
25	0(=15)	+2(=12.5)	0(=20)	0(=35)	9.02	6.31
26	0(=15)	0(=7.5)	-2(=10)	0(=35)	7.93	6.48
27	0(=15)	-2(=2.5)	0(=20)	0(=35)	8.61	6.63
28	+2(=25)	0(=7.5)	0(=20)	0(=35)	8.43	7.55
29	0(=15)	0(=7.5)	0(=20)	-2(=25)	6.71	3.26
30	0(=15)	0(=7.5)	+2(=30)	0(=35)	8.52	4.64

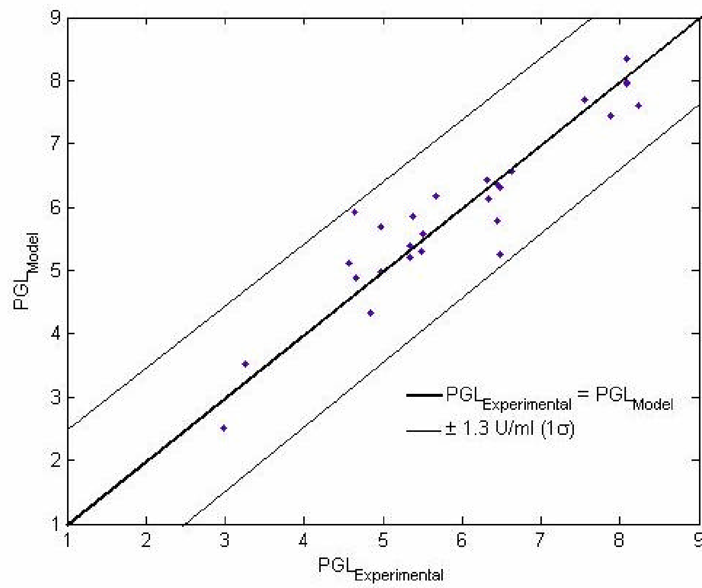
[a]PL, R^2 = 0.95; R = 0.97; PGL, R^2 = 0.9; R = 0.95, Values in the parenthesis indicate un-coded values. Experimental values are mean of triplicates within ±5 to 8% standard error

where, x_1 is the coded value of X_1 (yeast extract), x_2 is the coded value of X_2 (galactose), x_3 is the coded value of X_3 (lemon peel powder) and x_4 is the coded value of X_4 (temperature).

ANOVA for PL and PGL suggested that the model is very significant as indicated by a low probability value for PL [(P_{model}>F) = 0.0001] and for PGL [(P_{model}>F) = 0.0002] by Fisher's statistical test. The value of R (coefficient of linear regression) for PL and PGL were found to be 0.97 and 0.95 suggesting that the experimental values are in good agreement with the model predicted values (Fig. 1a and b). The model equation was solved using MATLAB software to generate the optimal values for the above four variables. The optimal values of yeast extract, galactose, lemon peel powder and temperature for PL production were found to be 1.885, 0.956, 2.39% and 31.5°C, respectively. Similarly, for maximum production of PGL the optimal conditions of yeast extract, galactose, lemon peel powder and temperature were 2.5, 0.55, 2.4% and 32.7°C, respectively. Response surface plots showing the dependence of PL and PGL production on the variables considered for optimization is shown in Fig. 2 and 3. Maximum activities of PL and PGL were indicated by the peak of the response surface plots. PL and PGL production were carried out at their respective model optimum conditions. A maximum activity of PL (10.7 U mL^{-1}) and PGL (8.8 U mL^{-1}) was obtained when the isolate was

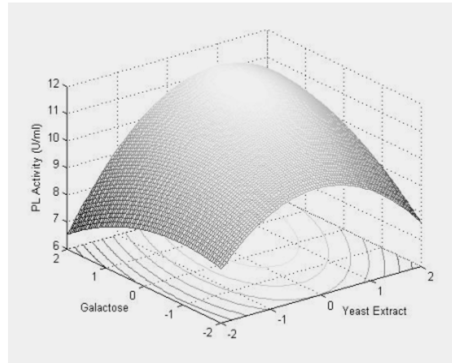


(a)

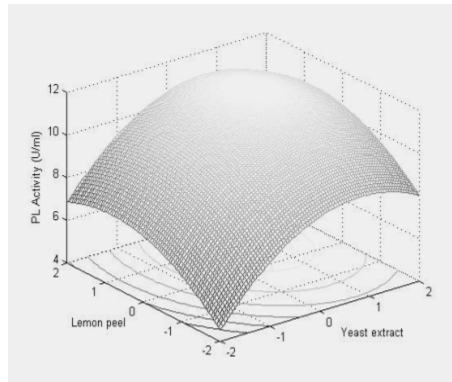


(b)

Fig. 1: Plot showing experimental values of activity (U mL^{-1}) vs model predicted values of activity (a) Pectin lyase (b) Pectate lyase



(a)



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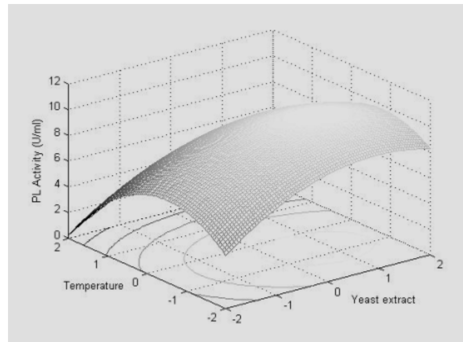
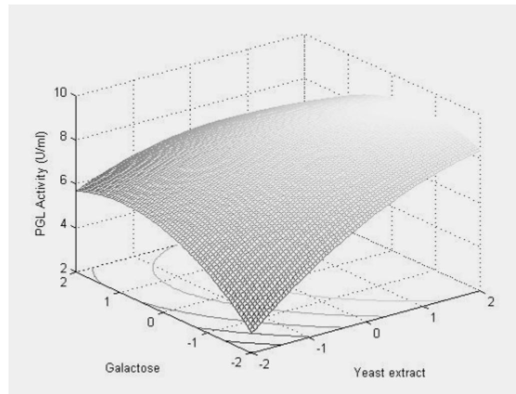
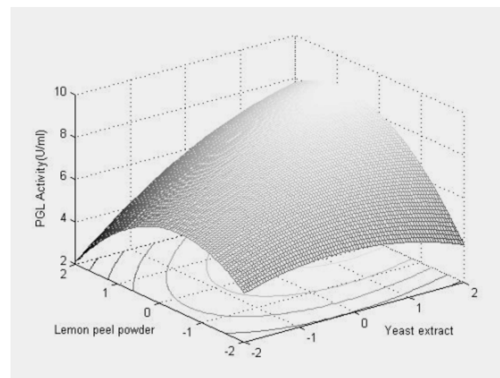


(c)

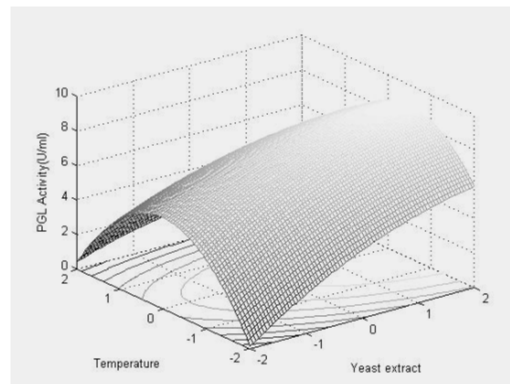
Fig. 2: Response surface plot (a) PL activity at different levels of yeast extract and galactose at optimum model predicted lemon peel powder and temperature, (b) PL activity at different levels of yeast extract and lemon peel powder at optimum model predicted galactose and temperature, (c) PL activity at different levels of yeast extract and temperature at optimum model predicted lemon peel powder and temperature in relation to production of pectin lyase (PL)



(a)



(b)



(c)

Fig. 3: Response surface plot (a) PGL activity at different levels of yeast extract and galactose at optimal model predicted lemon peel powder and temperature, (b) PGL activity at different levels of yeast extract and lemon peel powder at optimal model predicted galactose and temperature, (c) PGL activity at different levels of yeast extract and temperature at optimal model predicted lemon peel powder and temperature in relation to production of pectate lyase (PGL)

grown in optimized conditions for PL. Similarly a maximum of 9.75 U mL^{-1} of PL and 8.7 U mL^{-1} of PGL were obtained when grown at optimal conditions for PGL. Among the two optimal conditions, maximum enzyme activities were obtained when the isolate was grown in medium under optimal conditions obtained for PL. The maximum experimental activities of PL and PGL obtained under optimal condition of PL matched with model predicted activities of PL and PGL (10.5 U mL^{-1} for PL and 8.6 U mL^{-1} for PGL). Hence, the optimal conditions obtained for maximum PL production can be successfully used for the maximum production of both PL and PGL by *Debaryomyces nepalensis*.

Discussion

PL has been primarily produced by fungi belonging to genus *Aspergillus*, *Fusarium* and *Penicillium* sp. (Panda and Naidu, 2000; Piccoli-Valli *et al*., 2003) whereas PGL by bacteria (Hayashi *et al*., 1997; McCarthy *et al*., 1985). Yeasts like S*accharomyces cerevisiae* and *Candida* have been reported to produce pectinolytic enzymes (Gainvors *et al*., 2004; Nakagawa *et al*., 2000). However, a couple of reports were available where both the pectic lyases being produced by a single microorganism (Nakagawa *et al*., 2000; Soriano *et al*., 2005) but there has been no report on production of PL and PGL by *Debaryomyces nepalensis*. Carbon source in the medium enhances the production of PL (Alana *et al*., 1989) and PGL (Nakajima *et al*., 1999). In order to study the effect of carbon source on PL and PGL production by *Debaryomyces nepalensis* different carbon sources were added to the PT medium. From experimental data it has been found that galactose at 0.5% in the medium produces maximum amounts of both the transeliminases. It has also been reported that the presence of complex nitrogen source in the medium increases the production of pectic transeliminases. For e.g., yeast extract in the medium enhances PL production by *Paenibacillus amylolyticus* (Sakiyama *et al*., 2001) whereas presence of peptone in the medium increases PGL production by *Bacillus* sp. (Kelly and Fogarty, 1978). In the present study both synthetic and complex nitrogen sources were added to the medium (PT medium containing galactose) to see the effect on PL and PGL production. Yeast extract in the medium as the sole nitrogen source enhanced both PL and PGL productivity. Among all the tested metal ions, the addition of ironin the medium enhanced the production of both PL and PGL. From the preliminary experiments it has been evident that the presence of galactose, yeast extract and iron in the PT medium enhanced both PL and PGL production.

Apart from that physical parameters like temperature, agitation, pH were some of the major factors that effects enzyme production. Temperature strongly influences the production of PL by *Erwinia caratavora* subsp. *Caratavora* (Nguyen *et al*., 2002) and PGL by *Chryseomonas leteola* (Laurent *et al*., 2000). Process conditions like pH and agitation also influences production of PL and PGL (Piccoli-Valli *et al*., 2001; Ding *et al*., 2001). PL and PGL are mainly inducible enzymes and are influenced by the presence of other nutrients in the medium. Pectin is the best inducer for both PL and PGL but naturally available pectic substrates can also be used, orange bagasse shows the maximum production of PL by *Penicillin veridicatum* (Silva *et al*., 2002) and lemon peel for polygalacturonase production (Maldonado *et al*., 1986). Taking into account the above factors an attempt was made to optimize the medium components and fermentation variables for production of PL and PGL using statistical optimization techniques. Plackett-Burman method was used to sort out the significant variables and optimize the same using CCD. As reported in literature analysis of Plackett-Burman design also establishes yeast extract, galactose, lemon peel powder and temperature as the significant factors affecting PL and PGL production. Hence these four variables were taken into consideration for CCD.

Optimization studies have already been carried out for pectinase production using RSM but till date there have been no reports on optimization of both the transeliminases by a single microorganism. This is the first report on optimization of pectic transeliminases by *Debaryomyces nepalensis*. Microbiological parameters like slant age, inoculum age and amount of inoculum have been studied to optimize production of pectolytic enzymes like polymethylgalacturonase, polygalacturonase and PL, using response surface methodology (Panda *et al*., 1999). Under the optimum conditions, the fermentation time was reduced from 144 to 120 h. Nair and Panda have studied pectinase production by optimizing carbon and nitrogen source present in the medium using central composite design and found 40% increase in enzyme synthesis (Nair and Panda, 1997). Sharma and Satyanarayana have reported an almost 34 fold increase in pectinase production under optimum conditions in shake flask when compared to unoptimized conditions (Sharma and Satyanarayana, 2006). In this study, the production of PL and PGL was enhanced by 2.5 and 2.9 fold after screening and optimization of critical parameters using response surface methodology.

In addition to optimization, RSM can be successfully used to study the interaction effect among the variables. The interaction between the variables in relation to enzyme production is clear from Table 5. The highest value for PL production was noted in Run # 9 where yeast extract, galactose, lemon peel powder were at +1 level and temperature at -1 level. As previous studies have indicated that temperature is a crucial factor in enzyme production, the effect of other variables at higher level is enhanced by temperature at an optimum level. Consequently the lowest rate of enzyme production (4.65 U mL^{-1})) is noted in Run # 23 where other factors being at 0 levels, temperature is at an extreme higher end. Run # 12 clearly shows that even though all variables are at lower levels enzyme production was not the least shows a strong interaction between the variables. The values of responses clearly indicate the ranges selected were appropriate. The values of coefficients obtained by the model equation reflect the interactions of the chosen parameters in relation to enzyme production. For e.g., the coefficient of x_1x_4 is greater than x_3x_4 indicating that the interaction between the variable x_1 and x_4 is more important than x_3 and x_4 (Eq. 6). Similarly the individual effect of x_2 is very less than the interaction effect between x_1 and x_2 (Eq. 6) suggesting that the interaction effect among the variables is very significant and can not be neglected.

For production of PGL also the interaction between the variables in relation to enzyme production is clearly indicated from the Table 5). Similar to PL, PGL production also followed the same trend with the highest value (8.24 U mL^{-1}) obtained in Run # 9 and the lowest in Run # 23 (2.98 U mL^{-1}). Temperature is one of the important factors affecting PGL production as seen from Run # 29 (3.26 U mL^{-1}) and Run # 23 (2.98 U mL^{-1}) where it is kept at the extreme conditions (-2 and +2 level). The centre points (Run # 2, 3, 13, 20, 21 and 22) with enzyme activity of 8.09 clearly indicates that the ranges selected for optimization best fits the model. Similarly, the interaction effect between the variables can be studied by examining the coefficients of equation 7. For e.g., the effect of linear terms for x_1 and x_3 is less than the interaction between x_1 and x_3. Hence, interaction effect among the variables is very crucial for the production of PL and PGL by *Debaryomyces nepalensis*. In all the cases, the maximum production of PL and PGL occurred between 24-36 h of fermentation.

Conclusions

After optimization using Plackett-Burman design and RSM it was found that the enzyme productivities increased by 2.5 and 2.9 fold for PL and PGL, respectively. This is the first report on production of both the transeliminases from a novel strain *Debaryomyces nepalensis* and first report

on optimization studies involving both PL and PGL from a single microorganism. PGL as reported in literature is mainly produced by pathogenic microorganisms. PL and PGL produced by *Debaryomyces nepalensis* with less fermentation time can be advantageous and alternative to the fungal enzymes available in the market. Response surface methodology can be successfully used to optimize and study the interaction effect among the fermentation conditions for maximum production of PL and PGL.

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