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OPTIMIZATION OF PECTINASE PRODUCTION FROM *GEOTRICHUM CANDIDUM* AA15 USING RESPONSE SURFACE METHODOLOGY

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Abstract

Pectinolytic enzymes are used in industries involving in fruits and vegetables processing, wine making, retting and degumming of fiber crops, extraction of olive oil, treatment of pectin containing waste water and in the fermentation of coffee, tea and cocoa. Pectinase production from bacteria and fungi has been reported widely, however, very few reports are available for pectinolytic yeasts. This research was carried out to study the parameters affecting pectinase production from an indigenously isolated yeast strain, *Geotrichum candidum* AA15, using statistical tools. Plackett-Burman design revealed initial pH of the medium, inoculum size and substrate concentration as significant factors. Subsequently, optimum conditions for pectinase production were determined using Box-Behnken design. The data showed that the strain produced 0.250 IU/ml pectinase under optimized conditions using inoculum size, 3%; substrate concentration, 0.75%; and a starting pH of 5.3. Time course studies of the enzyme production revealed that the pectinase production was associated with the growth and optimum enzyme production was achieved after 48 h of cultivation. The presence of isozymes with approximate molecular weights of 60 and 66.2 kDa was indicated by SDS-PAGE and in-gel visualization of enzyme activity. These results provide a merit to study the strain further to explore its biotechnological potential.

Key words: Pectinase, *Geotrichum candidum*, Plackett-Burman Design, Box Behnken Design.

Introduction

Pectin is a heteropolysaccharide which is mostly present in the primary cell wall and middle lamella of higher plants and provide structural integrity and stickiness to plant tissues (Gummadi & Panda, 2003). It constitutes 0.5-4% wet weight of fruits (Jayani *et al.,* 2005). It is degraded by a complex set of proteins that are referred as pectinases, namely, pectate lyases (EC 4.2.2.2), lyases (EC 4.2.2.10), and polygalacturonases (EC 3.2.1.15 and EC 3.2.1.67). Polygalacturonases are further classified into two types which also differ in their technological applications. Exo-polygalacturonases act on distal groups of pectin molecule and slowly decrease chain length; while endo-polygalacturonases act on all bonds of pectin randomly and rapidly reduce the molecular dimension and viscosity (van Rensburg & Pretorius, 2000). Polygalacturonases work by hydrolyzing the glycosidic bonds of the polymer while, pectin-esterases act on ester bond between galacturonic acid and methanol (Fernandez-Gonzalez *et al.,* 2004).

Pectinases play significant roles in various industrial processes including clarification of wine and fruit juices, formulation of different pectin products, extraction of oil from seeds and pigments, and degumming of fiber crops (Kashyap *et al.,* 2001).

Pectinase production has been reported by plants, bacteria, and fungi (Singh *et al.,* 1999). Generally, molds produce a mixture of pectinolytic enzymes with cellulase and hemicellulase activities. Some commercial processes require activity of single type of pectinolytic enzyme, such as in the clarification process of orange juice, polygalacturonase activity is required without any accessory enzyme. Food industry utilizes commercial pectinases that are derived from *Aspergillus niger*, that also possesses pectin methyl-esterase activity that may cause the production of toxic alcohols. The cost of polygalacturonase from *A. niger* is increased if the pure enzyme is obtained. Therefore, alternate sources of pectinase have remained the subject of research.

Production of pectinase is not commonly carried out in yeasts. In spite of some reports about the production of a single type of pectinase, yeast pectinases have remained a neglected area of industrial microbiology. Pectinolytic yeasts offer numerous benefits over filamentous-fungi as their structure and growth is comparatively simple as well as their growth medium does not need any inducer (Jia & Wheals, 2000). Moreover, the enzyme can be obtained as a by-product of single cell protein. The few genera that are reported for this ability include *Candida, Kluyveromyces, Saccharomyces, Cryptococcus, Rhodotorula* and *Geotrichum* (Vaughn *et al.,* 1969; Winborne & Richard, 1978; Lim *et al.,* 1980; Federici, 1985; Barnby *et al.,* 1990). Various species of *Geotrichum* have been described for pectinase production including *G. candidum*, *G. lactis* and *G. klebahnii* (Pardo *et al.,* 1991; Cavalitto & Mignone, 2007; Piegza *et al.,* 2014). Polygalacturonase activity of *G. candidum* renders it to soften of fruits and vegetables tissues (Piegza *et al.,* 2014). Although, Illoka *et al.,* (2012) has employed solid state fermentation for polygalacturonase production from *G. candidum* using grape pomace, however, the production of enzyme has not been investigated on statistical ground.

Statistical tools are often employed to investigate the influence of various factors on a process and hence

the production cost can be reduced. The initial step in the optimization is to determine the significant variables affecting enzyme production then the optimal levels of significant factors are determined. The conventional method for the optimization of enzyme production is one factor at a time strategy in which single variable is changed at a time by keeping other factors at fixed level. Besides being intensive labor and time-consuming this method may generate unreliable results (Box *et al.,* 1978; Akhnazarova & Kafarov, 1982; Oh *et al.,* 1995) and hence is not preferred. Strategies based on Statistical tools provide better economical substitutes in which interaction between factors can also be studied. Plackett-Burman design (PBD) is one of the statistical tool that is well-known and extensively applied design for the evaluation of significant factors (Plackett & Burman, 1946). Subsequent to PBD, factorial designs in Response surface methodology (RSM) evaluate the effect of variables on response and determine optimum levels of significant factors (Quintavalla & Paralari, 1993; Sen & Swaminathan, 1997) by employing regression analysis. Earlier, RSM has been used for the optimization of different enzymes from various organisms (Sharma *et al.,* 2006). This study describes optimization of pectinase production from *G. candidum* AA15 by using PBD and RSM.

Materials and Methods

The yeast strain: The yeast strain used in this study was isolated on SDA plate from mayonnaise and identified on cultural and biochemical basis according to Kurtzman and Fell (1998). For molecular identification, genomic DNA was extracted and amplified using primers ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3'). The band corresponding to 400bps was purified and sequenced. Alignment of the sequence was carried out by BLAST and the phylogenetic tree was created by Mega v.6 software. The sequence was submitted to Genbank and accession number was obtained.

The strain was cultured on Sabauraud's Dextrose Agar (SDA) slant at 28°C for 48 h and maintained at 4°C in a refrigerator and sub-cultured periodically.

Enzyme production and extraction of enzyme: The inoculum for pectinase production was prepared by transferring a single colony of the strain, AA15, from SDA slant to seed broth (Sabauraud's Dextrose broth) and incubated at 28°C for 24 h. For subsequent inoculation in the production medium, the cell concentration was standardized as Macfarlane Index 4.

The fermentation medium was prepared as suggested by PBD and RSM, and inoculated. After incubation, the yeast cells were separated by centrifugation at 3000 rpm for 15 min and cell-free culture supernatant (CFCS) was used for pectinase assay as a crude enzyme.

Pectinase activity: For the determination of pectinolytic activity, 25µl of CFCS was mixed with 25µl of 0.5% buffered citrus pectin (50mM Sodium citrate buffer; pH 4.8) and incubated at 35°C in a water bath for 30 min. Dinitrosalicylic acid method was used for the determination of reducing sugars using galacturonic acid as standard (Miller, 1959). One unit of pectinase was defined as the amount of the enzyme required to release one µmole of reducing sugar under standard assay conditions.

Experimental design and statistical analysis: In PBD, 7 factors including physical (temperature, incubation period), chemical (Medium, Substrate concentration, pH, Glucose concentration) and biological (inoculum size) were set at two levels. Minitab 17 software was used to create and analyze PBD. The variables having p-value < 0.05 were considered as significant and selected for further optimization. Each experiment was carried out in 100 ml Erlenmeyer flask (in triplicate) and mean value of IU/ml was used as a response.

After analysis, 3 factors showed p-value less than 0.05 and were selected for further optimization using RSM approach. Box-Behnken Design (BBD) was applied for optimization of significant variables. A design of 15 experiments for 3 variables with 3 levels were generated and performed and the pectinase activity was taken as the response. The regression equation for the factors was:

$$Y= \beta_o+\sum\beta_i x_i+ \sum\beta_{ij}X_iX_j+ \beta_{ii}X_i^2 \quad (1)$$

where Y= predicted response, β_o= a constant and β_i= linear coefficient, β_{ii}= squared coefficient, β_{ij}= interaction coefficient. Equation 1 was used to create surface plots of variables.

The model was validated for pectinase production by performing an experiment as predicted where the strain was cultivated at 25°C for 48 h by transferring 3% inoculum to mineral salt media of pH 5.3 containing 0.75% pectin and CFCS was assayed for pectinase activity.

Time course studies of pectinase production: Fermentation was carried out by maintaining the factors at the optimum level (as investigated earlier) in a shaking incubator at 150 rpm for 72 h. Aliquots were collected intermittently and OD_{600} was taken. After centrifugation, CFCS was analyzed for pectinase activity, reducing sugar and pH variations.

SDS-PAGE analysis: The enzyme sample was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Molecular weight markers (Biorad) ranging from 45-200 KDa were used and the gel was silver stained. For the development of zymogram, lyophilized enzyme sample was resolved under non-reducing conditions in 10% SDS-PAGE gel containing 0.25% pectin, stained with 0.02% ruthenium red solution and destained with distilled water until a clear zone of pectinase activity appeared against the purple background (Ried & Collmer, 1985).

Results and Discussions

Identification of the strain: The yeast strain was identified as *Geotrichum candidum* on the basis of morphological, biochemical and 18S rDNA sequence analysis. The strain was observed as rectangular-shaped under the microscope and all the biochemical tests performed according to Kutzman & Fell (1998) were also found to be similar as reported for the members of *G. candidum*. Internal Transcribed (ITS) regions are targeted frequently for the molecular identification of broad range of fungi (Kowalchuk *et al.,* 2006), whereas, ITS4/ITS5 regions have been reported for yeasts in general and for *Geotrichum* strains in particular (Garner *et al.,* 2010; Sacristan *et al.,* 2013). Therefore, similar regions of the strain AA15 was PCR amplified and sequenced. The sequence was submitted to GenBank with the Accession No. MG199188. The analysis of the sequence using BLAST and phylogenetic tree showed the similarity of the strain AA15 with *G. candidum* clade (Fig. 1).

Optimization of pectinase production: Several physical, chemical and biological factors significantly affect the fermentation process. The first step in optimization is to evaluate the significant parameters affecting the response then the optimum levels of these variables are determined. PBD is a well-established and efficient statistical tool for the evaluation of significant variables. Earlier, PBD was employed to optimize the conditions affecting immobilization of *G. candidum* AA-15 on corncob and for pectinase production from immobilized cells (Ejaz *et al.*, 2018). Usually, subsequent to PBD, factorial design and response surface methodology are applied for the determination of optimum levels of significant variables. These tools have been used by several researchers in order to optimize cultural conditions and to determine optimal values of significant parameters (Kalil *et al.,* 2000; Sunitha *et al.,* 2000; Vohra & Satyanarayana, 2002) for pectinase production from *Kluveromyces winkerhamii* and from *G. klebahnii* (Moyo *et al.,* 2003; Cavalitto & Mignone, 2007).

In this study, optimization of pectinase production from *G. candidum* AA15 was performed in two stages. During the initial step, Plackett-Burman design (PBD) was performed with two levels in order to determine the significant variables influencing pectinase production. A set of 20 experimental runs (Table 1) was executed. After analysis, three out of seven factors were found significant having Prob. < F less than 0.05 (Table 2). Other model terms with p-values > 0.05 were not significant and were kept at constant level in further steps. These three important factors including pH, inoculum size and substrate concentration were selected for further optimization. In a previous study, Li *et al.,* (2014) screened eight variables for exo-pectinase production by *Penicillium oxalicum* PJ02 using 12 runs PBD and found two significant factors including NH_4Cl and temperature. Interestingly, temperature was not appeared as a significant factor for the production of pectinase by the strain AA-15 that might be due to the fact that the strain was able to grow under a wide range of temperature and hence pectinase production was not influenced by this other-wise an important factor.

Subsequent to PBD, RSM was applied to evaluate the optimum levels of three selected variables for maximum response using Box-Behnken design. A design with 15 runs was generated (Table 3) and performed with three equidistant levels of pH (5-7), Inoculum size (3-5%) and substrate concentration (0.25-0.75%).

The following regression equation was derived after the analysis of variance.

$$Y = 0.155 + 0.0144A - 0.0294B + 0.035C - 0.0182AA + 0.0328BB + 0.0065CC + 0.0032AB - 0.036AC + 0.0145BC$$

where Y is response (IU/ml), A is pH, B is inoculum size (%), and C is substrate concentration (%)

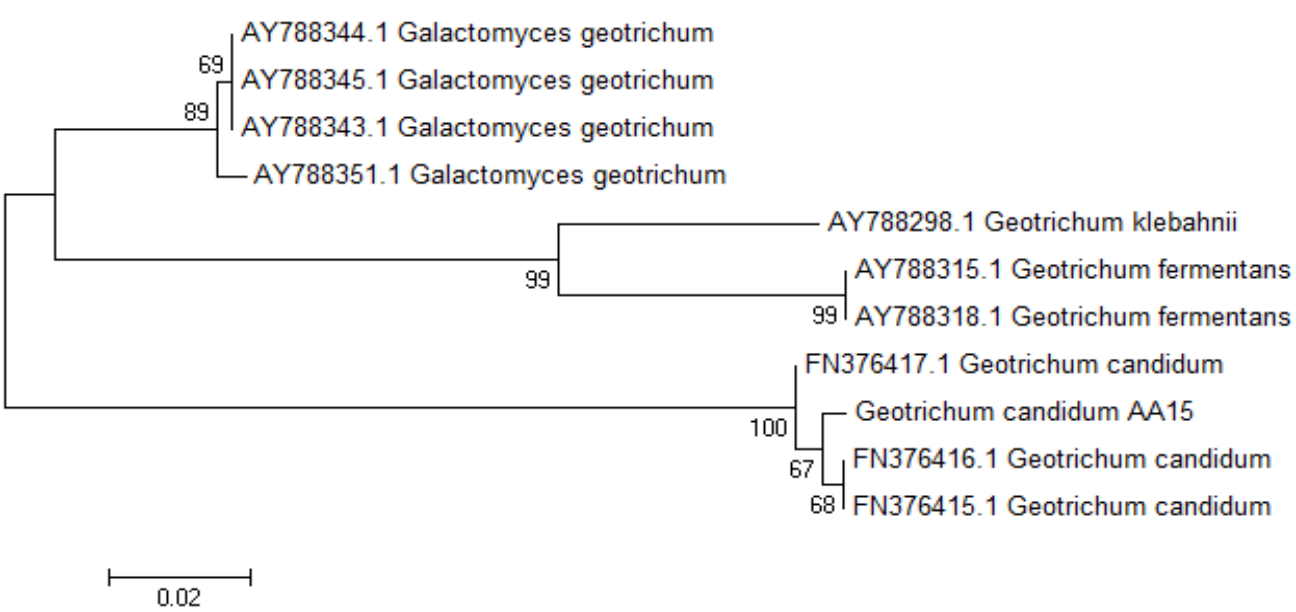

Fig. 1. Phylogenetic tree of *G. candidum* AA15. The Phylogenetic tree was constructed using neighbor-joining method with 1000 bootstraps.

Table 1. Plackett-Burman design for the screening of significant factors for pectinase production using *G. candidum* AA15.

Runs	Variables							Pectinase production (IU/ml)
	A Temp. (°C)	B pH	C Inoculum size (%v/v)	D Inc. period (h)	E Glucose conc. (%w/v)	F Substrate conc. (%w/v)	G Medium	
1	30	5	4	72	0	0.5	MSM	0.0493
2	30	7	3	72	0.5	0.5	MSM	0.0530
3	25	7	4	48	0.5	1	MSM	0.1360
4	25	5	4	72	0	1	YPM	0.0800
5	30	5	3	73	0.5	0.5	YPM	0.0490
6	30	7	3	48	0.5	1	MSM	0.1110
7	30	7	4	48	0	1	YPM	0.0920
8	30	7	4	72	0	0.5	YPM	0.1220
9	25	7	4	72	0.5	0.5	MSM	0.0800
10	30	5	4	72	0.5	1	MSM	0.1110
11	25	7	3	72	0.5	1	YPM	0.0800
12	30	5	4	48	0.5	1	YPM	0.1440
13	25	7	3	72	0	1	YPM	0.0890
14	25	5	4	48	0.5	0.5	YPM	0.0860
15	25	5	3	72	0	1	MSM	0.0520
16	25	5	3	48	0.5	0.5	YPM	0.0400
17	30	5	3	48	0	1	MSM	0.0500
18	30	7	3	48	0	0.5	YPM	0.0980
19	25	7	4	48	0	0.5	MSM	0.1220
20	25	5	3	48	0	0.5	MSM	0.0100

Table 2. Significance of variables screened in Platkett-Burman design for the production of pectinase from *G. candidum* AA15.

Factors	Coefficient estimate	P-value	Significance
A	0.00522	0.319	*
B	0.01559	0.009	Significant
C	0.01951	0.002	Significant
D	-0.00619	0.241	*
E	0.00629	0.234	*
F	0.01179	0.037	Significant
G	0.00528	0.321	*

The enzyme activity was used as a response and the equation was analyzed. Significance of the model (p<0.05) was determined by Analysis of variance (ANOVA) that showed an F-value of 8.22 (Table 4). The coefficient of determination (R^2) value was calculated as 0.93 indicating 93.67% of variation in response is demonstrated explained by this model. The R^2 value adjacent to 1 indicates the better relationship between predicted and actual values suggesting goodness of the model (Li *et al.,* 2008). Removal of non-significant model terms yielded a corrected R^2 value or adjusted R^2 value which was 0.8228 for this model that further supported the significance of the model (Bibi *et al.,* 2016).

A model exhibits lack of fit value when it fails to explain the correlation between independent factors and response. The Lack of fit F-value of this model was 0.33 with a p-value <0.05 (Table 4). Non-significant lack of fit indicates fitness of model (Bibi *et al.,* 2016).

Further ANOVA for the model terms demonstrated that the linear effect of inoculum size and substrate concentration was significant (p<0.05) while the effect of pH was not significant. The interaction between BB and AC had significant effect on pectinase production while the interactions between other variables were not significant (Table 4).

The interaction between two factors can be described clearly by visualizing contour plots because the shape of the contour plots indicates nature and extent of interaction. The elliptical shape indicates good interaction while the circular nature of contour plots shows the negligible interaction between two factors (Moyo *et al.,* 2003). In case of response surface plots, a flat surface plot suggests a less prominent interaction whereas a pointed surface plot reflects a prominent interaction between two variables (Montgomery, 1997).

As shown in Fig. 2, increase in the substrate concentration had positive effect on pectinase production while the pectinase production was negatively affected with an increasing inoculum size. The interaction between these two factors was not significant. Decrease in pectinase activity with increase in inoculum size was probably due to the limitation of getting sufficient nutrient in controlled environment of experimental flasks for metabolite production (Bibi *et al.,* 2016). pH and inoculum size did not exhibit any significant interaction (Fig. 3), however, pectinase titers increased with the increase in pH. Whereas, pH had significant effect with substrate concentration with a p-value of 0.016 (Fig. 4). The optimum pH for the production of *G. candidum* AA15 was in accordance with the reports for fungal pectinases (Luh & Phaf 1954; Schwan *et al.,* 1997).

Table 3. Experimental design of Box-Behnken for optimization of pectinase production from *G. candidum* AA15 with experimental and predicted responses

Runs	Factors			Observed response (IU/ml)	Predicted response (IU/ml)
	A pH	B Inoculum size (%v/v)	C Substrate concentration (%w/v)		
1	5	3	0.5	0.1780	0.1875
2	7	3	0.5	0.2150	0.2097
3	5	5	0.5	0.1170	0.1223
4	7	5	0.5	0.1670	0.1575
5	5	4	0.25	0.0700	0.0576
6	7	4	0.25	0.1560	0.1584
7	5	4	0.75	0.2020	0.1996
8	7	4	0.75	0.1440	0.1564
9	6	3	0.25	0.2000	0.2029
10	6	5	0.25	0.1080	0.1151
11	6	3	0.75	0.2510	0.2439
12	6	5	0.75	0.2170	0.2141
13	6	4	0.5	0.1460	0.1547
14	6	4	0.5	0.1840	0.1547
15	6	4	0.5	0.1340	0.1547

Table 4. Analysis of variance (ANOVA) for regression model.

Source	Sum of squares	*Df**	Mean square	F-value*	P-value*	status
Model	0.030103	9	0.003345	8.22	0.016	Significant
A-initial pH	0.001653	1	0.001653	4.06	0.100	Not significant
B-Inoculum size	0.006903	1	0.006903	16.97	0.009	Significant
C-Substrate concentration	0.009800	1	0.009800	24.09	0.004	Significant
AA	0.001649	1	0.001224	3.01	0.143	Not significant
BB	0.003872	1	0.003970	9.76	0.026	Significant
CC	0.000158	1	0.00158	0.39	0.560	Not significant
AB	0.000042	1	0.000042	0.10	0.760	Not significant
AC	0.005184	1	0.005184	12.74	0.016	Significant
BC	0.000841	1	0.000841	2.07	0.210	Not significant
Lack of fit	0.000671	3	0.000224	0.33	0.810	Not significant
Pure error	0.001363	2	0.000681			
Total	0.032137	14				
R^2	0.93					
Adjusted R^2	0.82					
Predicted R	0.57					
S	0.0201689					
Press	0.013806					

df= Degree of freedom; F-value= Fishers's function; P-value= level of confidence

Validation of experimental model: All the analysis showed that the optimum pectinase production was attained when 3% inoculum was transferred to the medium containing 0.75% pectin with an initial pH 5.3 and was incubated at 25 °C for 48 h. The model predicted 0.252 IU/ml of pectinase production while experimentally 0.250 IU/ml of the enzyme was obtained that indicated fitness of the model. The amount of pectinase produced by this indigenous yeast is comparable with the study of Hang & Woodam (1992) which reported 0.290 U/ml of polygalacturonase from *G. candidum* ATCC 34614 in brine supplemented with 0.3% polygalacturonic acid. Likewise, a citrus race of *G. candidum* was found to produce 0.370 U/ml of endopolygalacturonase (Nakamura *et al.*, 2001).

Studies on yeast growth and enzyme production kinetics: Time course study of fermentation showed that the pectinase activity was increased with incubation time until 48 h while the maximum biomass was produced after 30 h. A similar trend of increase in the reducing sugar was also noted. Initial pH was slightly increased from 5.3 to 6 and then declined to 4 at the end of fermentation (Fig. 5). These results are comparable with the previous study reported by Moyo *et al.,* (2003) where optimum pectinase production was obtained at 28.5-35.5°C and a pH range of 3.8-4.5 by *Kluyveromyces wickerhamii*. Growth-linked production of pectinase was observed for polygalcturonase synthesis by *A. kawachii* and *G. klebahnii* (Contreras-Esquivel *et al.*, 1999; Cavalitto *et al.,* 2000). Previously, *G. candidum* ATCC 34614 was found to produce small quantity of polygalacturonase during initial 40 h of fermentation followed by a rapid increase in activity that reached to its maximum after 64 h (Hang & Woodams, 1992). Decrease in the pectinase activity with extended cultivation time may be due to activity of protease produced by the same organism (Malvessi & Silveira, 2004) or denaturation of pectinase.

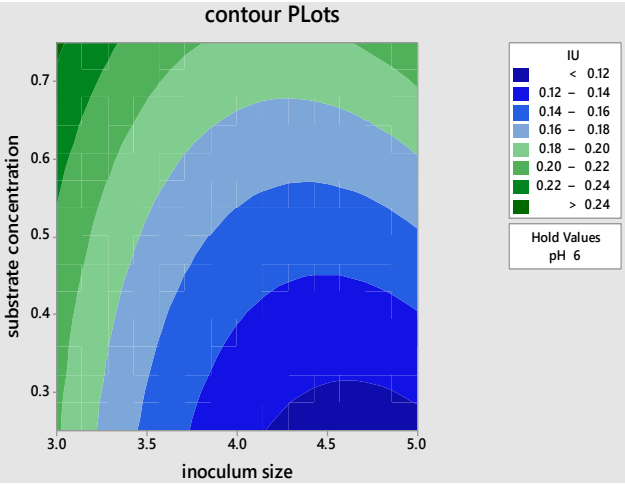

Fig. 2. Contour plot showing the effect of substrate concentration and inoculum size on pectinase production by *G. candidum* AA15.

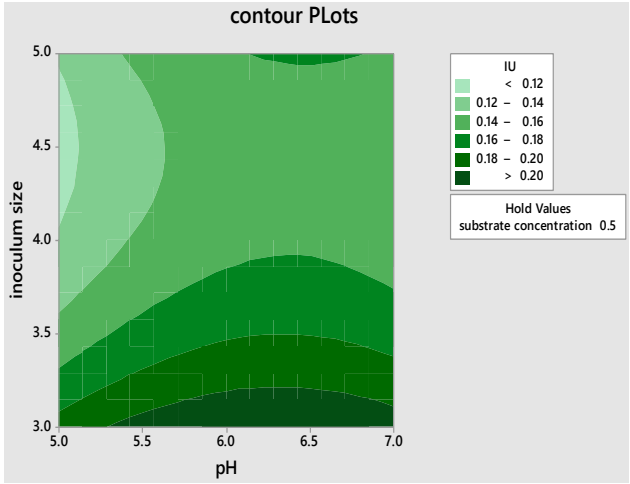

Fig. 3. Contour plots showing the effect of inoculum size and pH on pectinase production by *G. candidum* AA15.

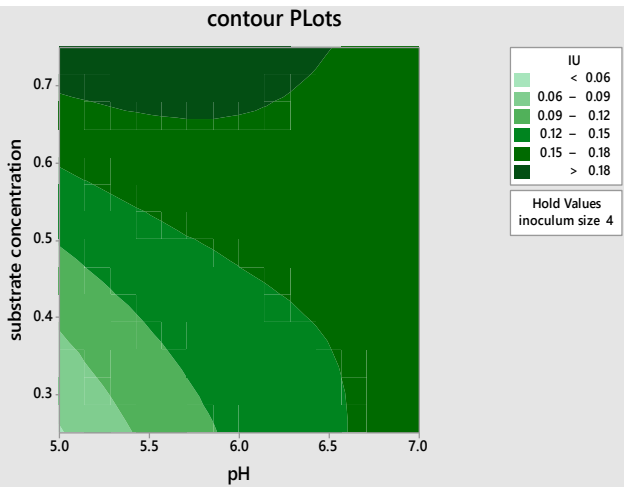

Fig. 4. Contour plot to understand the interaction between substrate concentration and pH for pectinase production by *G. candidum* AA15.

SDS-PAGE analysis and zymography: SDS-PAGE and zymographic analysis revealed the presence of isozymes of pectinase produced by the yeast strain (Fig. 6). The approximate molecular weight of isozymes was found to be 60 and 66.2 kDa respectively. Generally, the molecular weight of polygalacturonases from different sources range from 40-60 kDa (Jayani *et al.*, 2005). *Wickerhamomyces anomalus*, *Cystofilobasidium capitatum* and *Kluyveromyces marxianus* were reported to produce polygalacturonase of 43, 44 and 41.7 KDa, respectively, as determined by SDS-PAGE (Nakawa *et al.*, 2004; Serrat *et al.*, 2002; Martos *et al.*, 2014). Whereas, an extracellular polygalacturonase was produced by *G. lactis* ATCC 48590 with a molecular weight of 53 KDa as determined by gel filtration (Pardo *et al.*, 1991). Moreover, *G. candidum* CCY 16-1-29 was reported to produce various forms of polygalacturonase (Illkova *et al.*, 2012).

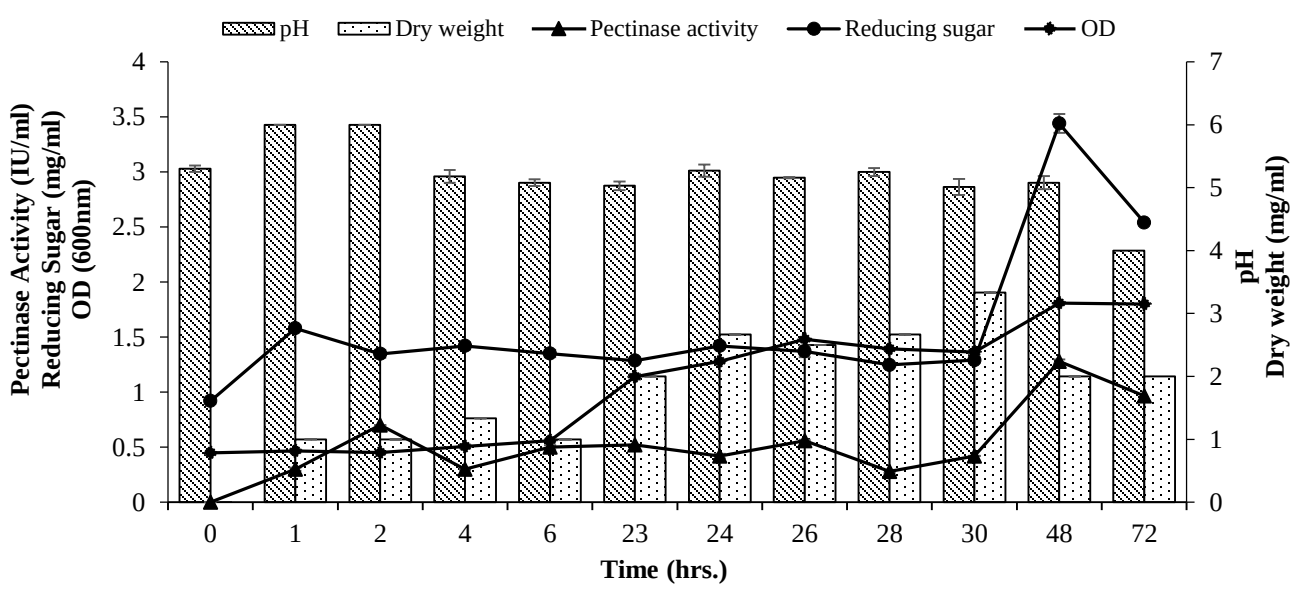

Fig. 5. Time course study of pectinase production with liberation of reducing sugars and variation in pH and growth profile of yeast strain using pectin as a sole source of carbon in submerged fermentation (The values of Pectinase activity and OD were multiplied by 10 in order to fit in graph).

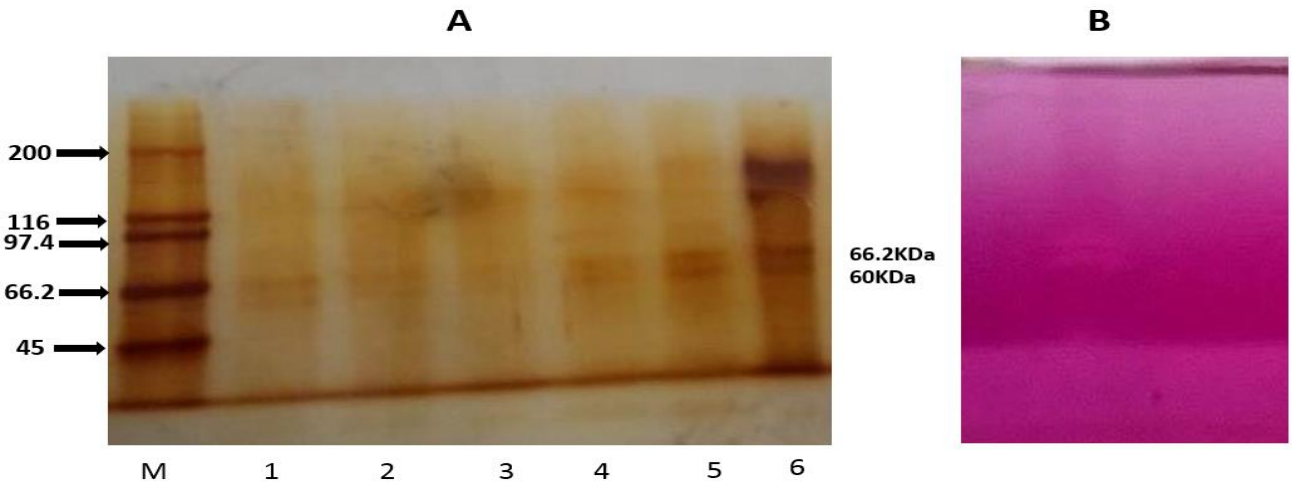

Fig. 6. Cell-free culture supernatant obtained after cultivation of AA-15 was lyophilized and subjected to (a) SDS-PAGE and (b) Zymography. Lane M contains molecular weight markers while lane 1-6 contain different loadings of lyolphilized enzymes.

Conclusion

Pectinase production from an indigenously isolated yeast, *G. candidum* AA15, was optimized using statistical methods. The strain was identified on molecular basis by amplifying ITS region. Under optimum conditions, the strain exhibited >0.2 IU/ml of pectinase that was comparable with many of the yeasts strains reported earlier. Pectinase production was found to be growth-linked and maximal enzyme activity was reached at 48h of cultivation. Two isozymes were observed under zymography and SDS-PAGE. Studies to explore the potential of the strain to produce pectinase on crude pectic substrates and suitability of the enzyme for its utilization in fruit juice clarification are required to determine candidature of the strain for future biotechnological applications.

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References

Akhnazarova, S. and V. Kafarov. 1982. Experimental optimization in chemistry and chemical engineering. *Appl. & Environ. Microbiol.,* Moscow: Mir Publishers, 61(11), pp. 3809-3814.

Barnby, F.M., F.F. Morpeth and D.L. Pyle. 1990. Endopolygalacturonase production from *Kluyveromyces marxinuns*: I. Resolution and purification and partial characterization of the enzyme. *Enzyme Microb. Technol.,* 12: 891-897.

Bibi, N., S. Ali and R. Tabassum. 2016. Statistical Optimization of Pectinase Biosynthesis from Orange Peel by *Bacillus licheniformis* Using Submerged Fermentation. *Waste Biomass Valor.,* DOI 10.1007/s12649-015-9470-4.

Box, G.E.F., W.G. Hunter and J.S. Hunter. 1978. *Statistics for experimenters*. New York: John Wiley and Sons.

Cavalitto, S.F. and C.F. Mignone. 2007. Application of factorial and Doehlert designs for optimization of protopectinase production by a *Geotrichum klebahnii* strain. *Process Biochem.,* 42(2): 175-179.

Cavalitto, S.F., R.A. Hours and C.F. Mignone. 2000. Growth and protopectinase production of *Geotrichum klebahnii* in batch and continuous cultures with synthetic media. *J. Ind. Microbiol. Biotechnol.,* 25: 260-265.

Contreras-Esquivel, J.C., R.A. Hours, C.E. Voget and C.F. Mignone. 1999. *Aspergillus kawachii* produces an acidic pectin releasing enzyme activity. *J. Biosci. Bioeng.,* 88(1): 48-52.

Ejaz, U., A. Ahmed and M. Sohail. 2018. Statistical optimization of immobilization of yeast cells on corncob for pectinase production. *Biocatalysis Agric Biotechnol.,* 14: 450-456.

Federici, F. 1985. Production, purification and partial characterization of an endopolygalacturonase from *Cryptococcus albidus* var. *albidus*. *Antonie Leeuwenhoek.,* 51: 139-150.

Fernandez-Gonzalez, M., J.F. Ubeda, T.G. Vasudewan, R.R. Cordero Otero and A.I. Briones. 2004. Evaluation of polygalacturonase activity in *Saccharomyces cerevisiae* wine strains. *FEMS Microbiol. Lett.,* 237: 261-266.

Garner, C.D., K. Jennifer, P.L. Starr, McDonough and A. Craig. 2010. Molecular Identification of Veterinary Yeast Isolates by Use of Sequence-Based Analysis of the D1/D2 Region of the Large Ribosomal Subunit. *J. Clin. Microbial.,* 48(6): 2140-2146.

Gummadi, S.N. and T. Panda. 2003. Purification and biochemical properties of microbial pectinases-A review. *Process Biochem.,* 38: 987-996.

Hang, Y.D. and E.E. Woodams. 1992. Production and characterization of polygalacturonase from *Geotrichum candidum*. *World J. Microbiol. Biotechnol.,* 8: 480-482.

Illkova, K., Z. Zuzana, F. Dana and J. Jakub. 2012. Production of *Geotrichum candidum* polygalacturonase via solid state fermentation of grape pomace. *Chem. Pap.,* 66(9): 852-860.

Jayani, R.S., S. Saxena and R. Gupta. 2005. Microbial pectinolytic enzymes: A review. *Process Biochem.,* 40: 2931-2944.

Jia, J.H. and A. Wheals. 2000. Endopolygalacturonase genes and enzymes from *Saccharomyces cerevisiae* and *Kluyveromyces marxianus*. *Curr. Genet.,* 38: 264-270.

Kalil, S.J., F. Maugeri and M.I. Rodrigues. 2000. Response surface analysis and simulation as a tool for bioprocess design and optimization. *Process Biochem.,* 35: 539-550.

Kashyap, D.R., P.K. Vohra, S. Chopra and R. Tewari. 2001. Applications of pectinases in the commercial sector: a review. *Bioresource Technol.,* 77: 217-227.

Kowalchuk, G.A., B. Drigo, E. Yergeau and J.A. Van Veen. 2006. Assessing bacterial and fungal community structure in soil using ribosomal RNA and other structural gene

markers. In: (Eds.): Nannipieri, P. and K. Smalla. *Nucleic Acids and Proteins in Soil*, Berlin Heidelberg, Germany: Springer-Verlag, ISBN 978-3-540-29448-1. pp. 159-188.

Kurtzman, C.P. and J.W. Fell. 1998. The yeast, a taxonomic study. Elsevier Science, 1-1075.

Li, P.J., J.L. Xia, Y. Shan, Z.Y. Nie, D.L. Su, Q.R. Gao, C. Zhang and Y.L. Ma. 2014. Optimizing production of pectinase from orange peel by *Penicillium oxalicum* PJ02 using response surface methodology. *Waste Biomass Valor.,* DOI 10.1007/s12649-014-9317-4.

Li, X.Y., Z.Q. Liu and Z.M. Chi. 2008. Production of phytase by a marine yeast *Kodamaea ohmeri* BG3 in an oats medium: Optimization by response surface methodology. *Biores. Technol.,* 99: 6386-6390.

Lim. J., Y.Y. Suzuki and J. Ozawa. 1980. Multiple forms of endo-polygalacturonase from *Saccharomyces fragilis*. *Agri. Biol. Chem.,* 44: 473-480.

Luh, B.S. and H.J. Phaf. 1954. Properties of yeast polygalacturonase. *Arch. Biochem. Biophys.,* 48: 23-37.

Malvessi, E. and M.M. Silveira. 2004. Influence of medium composition and pH on the production of polygalacturonases by *Aspergillus oryzae*. *Braz. Arch. Biol. Technol.,* 47: 693-702.

Martos, A.M., A.P. Butiuk, N.A. Rojas and R.A. Hours. 2014. Purification and Characterization of a Polygalacturonase Produced by *Wickerhamomyces anomalus. Braz. Arch. Boil. Technol.,* 57: 587-594.

Miller, G.L. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.,* 31: 426-428.

Montgomery, D.C. 1997. *Experimental Designs.* (4th Ed) Wiley, New York.

Moyo, S., B.A. Gashe, E.K. Collison and S. Mpuchane. 2003. Optimizing growth conditions for the pectinolytic activity of *Kluyveromyces wickerhamii* by using response surface methodology. *Int. J. Food Microbiol.,* 85: 87-100.

Nakamura, M., N.S. Dewa, Hisashii and A. Kei. 2001. Comparison of endo-polygalacturonase activities of citrus and Non-citrus races of *Geotrichum candidum*, and cloning and expression of the corresponding genes. *Mol. Plant Pathol.,* 2(5): 265-274.

Nakawa, T., T. Nagaoka, T. Miaji and N. Tomizuka. 2004. Cold-active polygalacturonase from psychrophilic-basidiomycetous yeast *Cystofilobasidium capitatum* strain PPY-1. *Biosci. Biotechnol. Biochem.,* 69(2): 419-421.

Oh, S., S. Rheem, J. Sim, J. Kim and Y. Baek. 1995. Optimising the conditions of *Lactobacillus casei* YIT 9018 in tyrtone yeast extract-glucose medium by using response surface methodology. *Appl. Environ. Microbial.,* 61(11): 3909-14.

Pardo, C., M.A. Lapeña and M. Gacto. 1991. Purification and characterization of an extracellular exopolygalacturonase from *Geotrichum lactis. Can. J. Microbiol.,* 37(12): 974-977.

Piegza, M, W. Danuta and S. Regina. 2014. Enzymatic and molecular characteristics of *Geotrichum candidum* strains as a starter culture for malting. *J. Inst. Brew.,* 120: 341-346.

Plackett, R.L. and J.P. Burman. 1946. The design of optimum multifactorial experiments. *Biometrica.,* 33: 305-325.

Quintavalla, S. and G. Paralari. 1993. Effects of temperature, aw and pH on the growth of *Bacillus* cells and spores: a response surface methodology study. *Int. J. Food Microbiol.,* 19: 207-216.

Ried, J. and A. Collmer. 1985. Activity stain of rapid characterization of pectin enzymes in isoelectric focusing and sodium dodecyl sulfate-polyacrylamide gels. *Appl. Environ. Microbiol.,* 50: 615-622.

Sacristan, M., B. Mayo, E. Fernández, J.M. Fresno, M.E. Tornadijo and J.M. Castro. 2013. Molecular study of *Geotrichum* strains isolated from Armada cheese. *Food Microbiol.,* 33: 481-487.

Schwan, R.F., R.M. Cooper and A.E. Wheals. 1997. Endopolygalacturonases secretion by *Kluyveromyces marxinus* and other co-coa pulp degrading yeasts. *Enzyme Microb. Technol.,* 21: 243-244.

Sen, R. and T. Swaminathan. 1997. Application of response surface methodology to evaluate the optimum environmental conditions for the enhanced production of surfactin. *Appl. Microbiol. Biotechnol.,* 47: 358-363.

Serrat, N., C.R. Bermudez and T.G. Villa. 2002. Production, Purification, and Characterization of a Polygalacturonase from a New Strain of *Kluyveromyces marxianus* Isolated from Coffee Wet-Processing Wastewater. *Appl. Biochem. Biotechnol.,* 97: 193-208.

Sharma, D. and T. Satyanarayana T. 2006. A marked enhancement in the production of a highly alkaline and thermostable pectinase by *Bacillus pumilus* dcsr1 in submerged fermentation by using statistical methods. *Bioresoure Technol.,* 97(5): 727-733.

Singh, S.A., M. Ramakrishna and A.G. Appu Rao. 1999. Optimization of downstream processing parameters for the recovery of Pectinase from the fermented bran of *Aspergillus carbonarius*. *Process Biochem.,* 35: 411-417.

Sunitha, K., Y.O. Kim, J.K. Lee and T.K. Oh. 2000. Statistical optimization of seed and induction conditions to enhance phytase production by recombinant *Escherichia coli*. *Biochem. Eng. J.,* 5: 51-56.

Van Rensburg, P. and I.S. Pretorius. 2000. Enzymes in wine making; harnessing natural catalyst for efficient biotransformations – a review. *S. Afr. J. Enol. Vitic.,* 21: 52-63.

Vaughn, R.H., T. Jakubczyl, J.D. McMillian, T.E. Haggins, B.A. Dave and V.M. Crampton. 1969. Some pink yeasts associated with softening of olives. *Appl. Microbiol.,* 18: 771-775.

Vohra, A. and T. Satyanarayana. 2002. Statistical optimization of the media components by response surface methodology to enhance phytase production by *Pichia anomala*. *Process Biochem.,* 37: 999-1004.

Winborne, M.P. and P.A.D. Richard. 1978. Pectinolytic activity of *Saccharomyces fragilis* cultured in controlled environments. *Biotechnol. Bioeng.,* 20: 231-242.

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