Indian Journal of Biotechnology
Vol 13, October 2014, pp 502-507

Selection of nutrients for polygalacturonase production by *Aspergillus awamori* MTCC 9166 using Plackett-Burman design

K Anuradha[1], P Naga Padma[1], S Venkateshwar[2] and Gopal Reddy[3]*

[1]Department of Microbiology, BVB Vivekananda College, Secunderabad 500094, India
[2]College of Technology and [3]Department of Microbiology, Osmania University, Hyderabad 500 007, India

Received 15 February 2013; revised 31 May 2013; accepted 16 July 2013

Selection of the best nutrients is one of the most critical stage in media optimization for polygalacturonase production. Plackett-Burman design was used to screen various pectin substrates, nitrogen sources and mineral nutrients for polygalacturonase production by *Aspergillus awamori* MTCC 9166. Fifteen different pectin sources like crude pectin, polygalacturonic acid, orange peel, citrus peel, jackfruit peel, etc. were selected for polygalacturonase production using 16 experimental design of Plackett-Burman. Similarly, eleven nitrogen sources like yeast extract, tryptone, casein hydrolysate, sodium nitrate, ammonium chloride, etc. and eleven mineral nutrients like NaCl, $MgSO_4$, KH_2PO_4, $CaCl_2$, etc. were screened for polygalacturonase production using 12 experimental design of Plackett-Burman. The enzyme production was studied for 5 d, where the maximum production was observed on 3rd d and so this data was analyzed using Indostat software to obtain regression coefficients and t-values. Based on these values significant nutrients like seven pectin sources (orange peel, jack fruit rind, apple peel, pine apple peel, mango peel, banana peel & tomato pulp), four nitrogen sources (urea, yeast extract, casein hydrolysate & potassium nitrate) and four mineral nutrients (NaCl, KH_2PO_4, $CaCl_2$ & KH_2PO_4) were selected for second level screening of efficient nutrients for polygalacturonase production using 16 experimental design of Plackett-Burman. Orange peel as pectin source, casein hydrolysate as nitrogen source and NaCl showed maximum enzyme production and so were selected for further quantitative optimization.

Keywords: *Aspergillus awamori* MTCC 9166, mineral nutrients, nitrogen sources, pectin sources, Plackett-Burman design, polygalacturonase

Introduction

Pectinases are a group of enzymes that degrade pectins present in middle lamella and primary cell walls of plant tissues[1]. Pectinases produced by different microbes are divided into depolymerizing enzymes and saponifying enzymes. Depolymerizing enzymes are polymethylgalacturonases, pectin lyases, polygalacturonases and pectate lyases, and saponyifying enzymes are pectinesterases[2,3]. These enzymes have wide applications in food industry for clarification of fruit juices and wines[2,4], coffee and tea fermentations[2], extraction of essential oils[5], etc. They have significant commercial value and today 75% of the estimated sale of industrial enzymes is contributed by pectinases[6].

The production of pectinolytic enzymes has been widely reported in bacteria and filamentous fungi[7]. Fungal polygalacturonases are very significant for clarification of fruit juices and wines, and for extraction of vegetable oils[8]. Their significance in clarification of fruit juices is due to the fact that their optimal pH is closer to that of many fruit juices.

Nutritional factors like pectin, nitrogen, mineral nutrients are significant as components of low cost production media that is of great interest in the industry. It is also known that 30-40% of the production cost of industrial enzymes is accounted for the cost of growth or fermentation medium[6,9]. In our earlier study, a fungal polygalacturonase producer along with multienzyme production ability was screened[10]. Effective screening and selective isolation of pectinolytic microorganisms from different sources was done using enrichment culture technique. Different bacteria, yeasts and molds for enzyme production were screened. They were tested for pectinolytic activity and multienzyme production and one isolate characterized as *Aspergillus awamori* MTCC 9166 was selected for the study and it was deposited at Microbial Type Culture Collection and Gene Bank (MMTC), Institute of Microbial Technology, Chandigarh, India. The strain had a positive character of pellet formation, which could be very useful in product recovery.

*Author for correspondence:
Tel: +91-40-27682246/27090661
Mobile: +91-9948693767
E-mail: gopalred@hotmail.com

Polygalacturonase is known to be produced by different fungal strains like those of *Aspergillus, Penicillium* and *Rhizopus* using pectin as substrate[11]. It is reported that *A. niger* produced 14.5 U/mL[12], *P. frequentans* produced 3 U/mL[13] and *A. awamori* produced 0.046 U/mL[14]. The strain under study, *A. awamori* MTCC 9166 produced 17.8 U/mL initially[10] and it improved after the media optimization. As a result, the enzyme yields increased to an average of 800 U/mL for pectin sources and 30 U/mL for nitrogen and mineral nutrients.

In the present study, locally available wastes or cheaper materials were used as pectic substrates. Different nitrogen and mineral nutrients were screened using Plackett-Burman[15] statistical design in an attempt to optimize suitable production medium. This design is a statistical methodology used to screen up to n-1 variables in just n number of experiments.

Materials and Methods

Microorganism

A. awamori MTCC 9166 strain was isolated from vegetable dump yard soil and maintained on PDA slants in a refrigerator[10].

Inoculum Preparation

Fungal spores were scrapped from PDA slants and added to water to get a concentration of 10^6 spores/mL spore suspension.

Pretreatment of Solid Substrates

Peels of various fruits like apple, banana, citrus (sweet lime), jackfruit rind, mango, pine apple, etc. were subjected to water treatment till sugar free. These were dried and powdered and their pectin content was determined by carbazole method[10].

Screening of Nutrients

Sixteen run Plackett-Burman (PB) design of 15 variables at two levels (+1 and -1) was used for screening of pectin rich sources (Table 1). Submerged fermentation was carried out in 250 mL Erlenmeyer flasks containing 50 mL Czapek broth with 1% commercial pectin (citrus peel pectin, S D Fine Chemicals) and other raw pectins. The flasks were inoculated with 1×10^6 spores/mL. The spore number was estimated by direct microscopic count using haemocytometer. Flasks were incubated for 5 d at 28°C in an Orbital shaker incubator at 200 rpm. Broth samples were collected after 48 h (3rd d) and assayed for the enzyme activity.

The fifteen pectin sources, namely, pectin crude (S D Fine Chemical), polygalacturonic acid, orange peel, sweet lime peel, jack fruit peel, apple pomace, sapota peel, pineapple peel, mango peel, banana peel, guava pulp, sugar cane bagasse, papaya peel, tomato pulp and lemon peel, were tested for their significant effect on production of polygalacturonase (PGUase) enzyme. Eleven nitrogen sources (including organic and inorganic), namely, urea, yeast extract, tryptone, casein hydrolysate, corn steep liquor, peptone, ammonium sulphate, sodium nitrate, ammonium chloride, ammonium nitrate and potassium nitrate, were tested for their significant effect on production of PGUase enzyme using 12 run Plackett-Burman design[15]. Based on

Table 1—Plackett-Burman 16 experimental design for 15 pectin sources for PGUase production *by A. awamori* MTCC 9166

Runs	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	Set1	Set2
1	+	-	-	-	+	-	-	+	+	-	+	-	+	+	+	854*	825*
2	+	+	-	-	-	+	-	-	+	+	-	+	-	+	+	657	647
3	+	+	+	-	-	-	+	-	-	+	+	-	+	-	+	484	474
4	+	+	+	+	-	-	-	+	-	-	+	+	-	+	-	647	637
5	-	+	+	+	+	-	-	-	+	-	-	+	+	-	+	555	567
6	+	-	+	+	+	+	-	-	-	+	-	-	+	+	+	927	935
7	-	+	-	+	+	+	+	-	-	-	+	-	-	+	+	628	658
8	+	-	+	-	+	+	+	+	-	-	-	+	-	-	+	620	630
9	+	+	-	+	-	+	+	+	+	-	-	-	+	-	-	825	840
10	-	+	+	-	+	-	+	+	+	+	+	-	-	+	-	925	900
11	-	-	+	+	-	+	+	+	+	+	+	-	-	-	+	880	870
12	+	-	-	+	+	-	+	-	+	+	+	+	-	-	-	850	840
13	-	+	-	-	+	+	-	+	-	+	+	+	+	-	-	650	640
14	-	-	+	-	-	+	+	-	+	-	+	+	+	+	-	680	670
15	-	-	-	+	-	-	+	+	-	+	-	+	+	+	+	780	760
16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	620	630

*PGUase activity (U/mL)

[A, Pectin crude; B, Polygalacturonic acid; C, Orange peel; D, Sweet lime peel; E, Jack fruit peel; F, Apple pomace; G, Sapota peel; H, Pineapple peel; I, Mango peel; J, Banana peel; K, Guava pulp; L, Sugar cane bagasse; M, Papaya peel; N, Tomato pulp; & O, Lemon peel]

unidimensional experiments, concentrations of variables were fixed. The lower and upper levels fixed for the selected carbon sources were 0.1 and 1% and for the selected nitrogen sources were 0.02 and 0.2%, respectively. Similarly eleven mineral nutrients, namely, NaCl, $MgSO_4$, KH_2PO_4, $CaCl_2$, $CuSO_4$, $FeCl_3$, $MnSO_4$, $ZnSO_4$, $CoCl_2$, $ZnCl_2$ and K_2HPO_4, were selected for PGUase production and their lower and upper levels were 0.005 and 0.05%, respectively. The physical parameters like temperature, pH and agitation were held constant at 28°C, 5.5 and 200 rpm, respectively.

Enzyme Assay

Fermented broth was cold centrifuged at 4°C, 5000 rpm for 10 min and supernatant was taken as enzyme source. Sodium acetate buffer (0.05 M, pH 5.5) was used for assaying PGUase. One mL of enzyme with 1 mL substrate (1%) was incubated at 50°C temperature and the D-galacturonic acid released from polygalacturonic acid as substrate was measured[16,17]. One unit of enzyme activity is defined as the amount of enzyme required to produce 1 µ mole of galacturonic acid per minute at 50°C[8,16].

Statistical Analysis

All experiments were conducted in triplicates and repeated thrice. Statistical analysis of the data was done using INDOSTAT software. The enzyme production was studied for 5 d, but maximum production was recorded on 3rd d and this data was analyzed using Indostat software to obtain regression coefficients and t-values. The most important nutrients under different categories were selected after statistical analysis based on regression coefficients and highest t-values. Those with p-values less than 0.005 were considered to be significant and shortlisted for further optimization studies.

Results and Discussion

Screening of fifteen pectin sources was done for PGUase production by *A. awamori* MTCC 9166 using the sixteen run Plackett-Burman design. PGUase production was found to be maximum in combination 6 followed by 10 (Table 1). Seven variables, namely, orange peel, jack fruit peel, apple pomace, pine apple peel, mango peel, banana peel and tomato pulp, were found to be significantly affecting PGUase production as their P values were equal to zero with positive regression coefficients (Table 2). These were selected for further screening.

Similarly when 11 nitrogen sources were screened using 12 run design and PGUase production was found to be maximum in combination 5 (Table 3). Among the 11 variables, 4 variables were found to be significantly effecting PGUase production with P values <0.001 (Table 4). These were urea, yeast extract, casein hydrolysate and potassium nitrate. They had positive coefficients over enzyme production and they could be selected for further screening. Other variables, ammonium chloride, ammonium nitrate, sodium nitrate, tryptone, potassium nitrate, peptone and corn steep liquor were found to be insignificant. Among 11 variables screened, NaCl, K_2HPO_4, $CaCl_2$ and KH_2PO_4 were comparatively more significant in affecting PGUase production (Table 5) as their regression

Table 2—Estimated effects and coefficients of pectin sources for PGUase production by *A. awamori* MTCC 9166 using Plackett-Burman design

Variable	Term	Effect	Regression coefficient	Standard error coefficient	t	p	Significance
	Constant	363.91	727.81	6.31	115.34	0.000	
A	Pectin Crude	4.33	8.65	1.99	4.33	0.001	*
B	Polygalacturonic acid	-25.55	-51.09	1.99	25.6	0.000	*
C	Sweet lime peel	-4.70	-9.40	1.99	4.71	0.000	*
D	Orange peel	20.34	40.68	1.99	20.28	0.000	Significant
E	Jack Fruit peel	14.14	28.28	1.99	14.17	0.000	Significant
F	Apple pomace	6.35	12.71	1.99	6.37	0.000	Significant
G	Sapota peel	0.33	0.65	1.99	0.32	0.747	*
H	Pine apple peel	22.79	45.59	1.99	22.80	0.000	Significant
I	Mango peel	26.04	52.09	1.99	26.10	0.000	Significant
J	Banana peel	20.79	41.59	1.99	20.84	0.000	Significant
K	Guava pulp	-8.30	-16.60	1.99	8.34	0.000	*
L	Sugar cane bagasse	-22.54	-45.09	1.99	22.59	0.000	*
M	Papaya peel	-2.67	-5.34	1.99	2.67	0.017	*
N	Tomato pulp	18.01	36.03	1.99	18.05	0.000	Significant
O	Lemon peel	-20.7	-41.4	1.99	20.75	0.000	*

*Insignificant

Table 3—Plackett-Burman 12 experimental design for 11 nitrogen sources (NS) and 11 mineral nutrients (MNs) for PGUase production by *A. awamori* MTCC 9166

Runs	A	B	C	D	E	F	G	H	I	J	K	Set 1 NS (U/mL)*	Set 2 NS (U/mL)*	Set 1 MNs (U/mL)*	Set 2 MNs (U/mL)*
1	+	-	+	-	+	-	+	+	+	-	-	26.38	26.38	32.5	31.5
2	+	+	-	+	-	-	-	+	+	+	-	27.42	27.42	38.6	38.2
3	-	+	+	-	+	-	-	-	+	+	+	10.53	10.53	20.2	20.2
4	+	-	+	+	-	+	-	-	-	+	+	40.18	40.18	60.4	60.3
5	+	+	-	+	+	-	+	-	-	-	+	55.65	55.65	45.2	45.1
6	+	+	+	-	+	+	-	+	-	-	-	23.50	23.50	23.5	23.5
7	-	+	+	+	-	+	+	-	+	-	-	20.40	20.40	27.2	27.2
8	-	-	+	+	+	-	+	+	-	+	-	18.50	18.50	19.5	19.5
9	-	-	-	+	+	+	-	+	+	-	+	23.50	23.50	23.5	23.5
10	+	-	-	-	+	+	+	-	+	+	-	14.30	14.30	18.5	18.6
11	-	+	-	-	-	+	+	+	-	+	+	13.50	13.50	21.5	21.3
12	-	-	-	-	-	-	-	-	-	-	-	12.20	12.20	20.2	20.2

*PGUase activity

Nitrogen sources (NS): A, Urea; B, Yeast extract; C, Tryptone; D, Casein hydrolysate; E, Corn steep liquor; F, Peptone; G, Ammonium sulphate; H, Sodium nitrate; I, Ammonium chloride; J, Ammonium nitrate; & K, Potassium nitrate

Mineral Nutrients (MNs): A, NaCl; B, $MgSO_4$; C, KH_2PO_4; D, $CaCl_2$; E, $CuSO_4$; F, $FeCl_3$; G, $MnSO_4$; H, $ZnSO_4$; I, $CoCl_2$; J, $ZnCl_2$; & K, K_2HPO_4

Table 4—Estimated effects and coefficients of nitrogen sources for PGUase production by *A. awamori* MTCC 9166 using Plackett- Burman design

Variable	Term	Effect	Regression coefficient	Standard error coefficient	t	p	Significance
	Constant	11.11	22.22	1.29	17.10	0.000	
A	Urea	3.83	7.66	0.41	18.65	0.000	Significant
B	Yeast Extract	1.07	2.14	0.41	5.21	0.000	Significant
C	Tryptone	-0.07	-0.14	0.41	0.34	0.735	*
D	Casein hydrolysate	3.62	7.24	0.41	17.64	0.000	Significant
E	Corn steep liquor	0.385	0.77	0.41	1.88	0.087	*
F	Peptone	-0.76	-1.52	0.41	3.70	0.003	*
G	Ammonium sulphate	0.695	1.39	0.41	3.40	0.006	*
H	Sodium nitrate	-0.805	-1.61	0.41	3.92	0.002	*
I	Ammonium chloride	-1.81	-3.62	0.41	8.81	0.000	*
J	Ammonium nitrate	-1.68	-3.36	0.41	8.18	0.000	*
K	Potassium nitrate	1.89	3.78	0.41	9.21	0.000	Significant

*Insignificant

Table 5—Estimated effects and coefficients of mineral nutrients for PGUase production by *A. awamori* MTCC 9166 using Plackett- Burman design

Variable	Term	Effect	Regression coefficient	Standard error coefficient	t	p	Significance
	Constant	14.555	29.11	1.06	27.32	0.000	
A	NaCl	3.790	7.58	0.33	22.50	0.000	Significant
B	$MgSO_4$	-0.030	-0.06	0.33	0.19	0.847	*
C	KH_2PO_4	0.830	1.66	0.33	4.94	0.000	Significant
D	$CaCl_2$	3.240	6.48	0.33	19.24	0.000	Significant
E	$CuSO_4$	-1.965	-3.93	0.33	11.60	0.000	*
F	$FeCl_3$	0.100	0.20	0.33	0.59	0.565	*
G	$MnSO_4$	-0.885	-1.77	0.33	5.26	0.000	*
H	$ZnSO_4$	-1.685	-3.37	0.33	10.01	0.000	*
I	$CoCl_2$	-1.465	-2.93	0.33	8.70	0.000	*
J	$ZnCl_2$	0.465	0.93	0.33	2.77	0.018	*
K	K_2HPO_4	2.325	4.65	0.33	13.82	0.000	Significant

*Insignificant

coefficients were positive with P values ≤ 0.001. However $CuSO_4$, $MnSO_4$, $ZnSO_4$ and $CoCl_2$ had negative coefficients and found to be insignificant for PGUase production.

Nutritional parameters play vital role in enzyme production. The important nutrient substrates are mostly carbon and energy sources, a nitrogen source and mineral nutrients. With interest to screen the efficient nutrients for PGUase production, the effect of carbon sources, nitrogen sources and mineral nutrients were studied. Media optimization studies are generally done using unidimensional approach, which is both laborious and time consuming, especially for large number of variables. Statistical methods give scope for study of different nutrients at different levels by performing minimum experiments, which saves both time and materials. In the present study, the Plackett-Burman design[15] was selected for screening nutrients as n-1 variables in n experiments. The statistical approach also gives scope for interactive and collective effect of nutrients on production of a product. There are reports of use of Plackett-Burman design in production of pectin lyases[6] and protopectinase[18].

Selection of pectin rich raw substrates for enzyme production is important as they not only serve as carbon and energy sources but also induce the inducible enzymes like PGUase. Earlier, studies in *A. awamori* on effect of glucose and pectin on PGUase production reported that addition of 0.4% pectin gave maximum enzyme production[6]. Similarly Nair *et al*[19] also reported that *A. foetidus* NCIM 510 responded to the medium containing pectin without any additional sugars for pectinase production. Production cost of industrial enzymes is much influenced by the growth and production medium. Costs for pectic substrates, the chief carbon source, can be reduced by using fruit and vegetable waste as pectin sources for PGUase production. Earlier reports indicated the use of various agro-industrial wastes[20, 21, 22], such as, sugar cane bagasse, lemon peel and apple pomace, as substrates for PGUase production. Therefore, the present study concentrated on this aspect and pectin rich substrates like orange peel, jack fruit peel, apple pomace, pine apple peel, mango peel, banana peel and tomato pulp showed good response for PGUase production.

Nitrogen sources have a profound effect on the production of PGUase in culture medium. Nitrogen sources are very much required for enzyme biosynthesis as they supply amino acids and various cellular proteins. In the present study, urea, yeast extract, casein hydrolysate and potassium nitrate were found to be more significant variables for PGUase production (Table 4). Similar findings were reported for production of PGUase from *Mucor circinelloides* ITCC 6025[23]. Further, maximum PGUase activity was obtained when NH_4Cl was used as nitrogen source[24] and it was reported that nitrogen limitation had adverse effect on the PGUase production[25].

Mineral nutrients when tested for PGUase production by *A. awamori* MTCC 9166 revealed that among the 11 variables screened, NaCl, KH_2PO_4, $CaCl_2$ and K_2HPO_4 were more significant in affecting PGUase production (Table 5). This response could be due to the fact that NaCl and $CaCl_2$ showed good ion balancing nutrients, while phosphate sources like KH_2PO_4 and K_2HPO_4 provide the much necessary phosphates.

Thus, the present study was useful in providing a number cheaper and locally available carbon (pectin), nitrogen sources and mineral nutrients for PGUase production. Good response from cheaper pectin sources like fruit peels is significant as low cost production media can be designed. The statistical design allowed to efficiently screen n-1 variables in just n number of experiments saving both time and chemicals, which is also a very important aspect in designing production medium.

Acknowledgement

The authors (PNP & KA) are grateful to the management of BVB Vivekananda College for its encouragement to carry out the present work.

References

1 Ismail A-M S, Utilization of orange peels for the production of multienzyme complexes by some fungal strains, *Process Biochem*, 31(1996) 645-650.
2 Whitaker J R, Pectic substances, pectic enzymes and haze formation in fruit juices, *Enzyme Microb Technol*, 6 (1984) 341-349.
3 Whitaker J R, Microbial pectinolytic enzymes, in *Microbial enzymes and biotechnology* 2nd edn, edited by W M Fogarty & C T Kelly (Elsevier Science Ltd., London) 1990.
4 Alkorta I, Garbisu C, Llama M J & Serra J L, Industrial applications of pectic enzymes: A review, *Process Biochem*, 33 (1998) 21-28.
5 Jayani R S, Saxena S & Gupta R, Microbial pectinolytic enzymes: A review, *Procces Biochem*, 40 (2005) 2931-2944.
6 Sathyanarayana N G, & Panda T, Purification and biochemical properties of microbial pectinases—A review, *Process Biochem*, 38 (2003) 987-96.

7 Pedrolli D B, Gomes E, Monti R & Carmona E C, Studies on productivity and charecterisation of polygalacturonase from *Aspergillus giganteus* submerged culture using citrus pectin and orange waste, *Appl Biochem Biotechnol*, 144 (2008) 191-200.

8 Naidu G S N & Panda T, Production of pectolytic enzymes—A review, *Bioprocess Eng*, 19 (1998) 355-361.

9 Panda T & Naidu G S N, Rotating simplex method of optimization of physical parameters for higher production of extracellular pectinases in bioreactor, *Bioprocess Eng*, 23 (2000) 47-49.

10 Anuradha K, Naga Padma P, Venkateshwar S & Gopal Reddy, Fungal isolates from natural pectic substrates for polygalaturonase and multienzyme production, *Indian J Microbiol*, 50 (2010) 339-344.

11 Favela-Torres E, Volke-Sepulveda T & Vaniegra-Gonzalez G, Production of hydrolytic depolymerising pectinases, *Food Technol Biotechnol*, 44 (2006) 221-227.

12 Galiotou-Panayotou M, Rodris P & Kapantai M, Enhanced polygalacturonase production by *Aspergillus niger* NRRL-364 grown on supplemented citrus pectin, *Lett Appl Microbiol*, 17 (1993) 145-148.

13 Kawano C Y, Chellegatti M A, Said S & Fonseca M J, Comparitive study of intracellular and extracellular pectinases produced by *Penicillium frequentans*, *Biotechnol Appl Biochem*, 29 (1999) 133-140.

14 Blandino A, Dravillas K, Cantero D, Pandiella S S & Webb C, Utilisation of whole wheat flour for the production of extra cellular pectinases by some fungal strains, *Process Biochem*, 37 (2001) 497-503.

15 Plackett R L & Burman J P, The design of optimun multifactorial experiments, Biometrika, 33 (1946) 305.

16 Collmer A, Reid J L, & Mount M S, Assay procedures for pectic enzymes, *Methods Enzymol*, 161 (1988) 329-335.

17 Nelson N, A photometric adaptation of the Somogyi method for the determination of glucose, *J Biol Chem*, 153 (1944) 375-380.

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

19 Nair S R, Rakshit S K & Panda T, Effect of carbon sources on the synthesis of pectinase by Aspergilli, *Bioprocess Eng*, 13 (1995) 37-40.

20 Solis-Pereira S, Favela-Torres E, Viniegra-Gonzalez G & Gutiérrez-Rojas M, Effects of different carbon sources on the synthesis of pectinase by *Aspergillus niger* in submerged and solid state fermentations, *Appl Microbiol Biotechnology*, 39 (1993) 36-41.

21 Solis-Pereira S, Favela-Torres E, Gutiérrez-Rojas M, Roussos S, Saucedo-Castaneda G *et al*, Production of pectinases by *Aspergillus niger* in solid state fermentation at high initial glucose concentrations, *World J Microbiol Biotechnol*, 12 (1996) 257-260.

22 Abbasi H, Shafighzadeh H & Rahimi, A continuous production of polygalacturonases (PGUases) by *Aspergillus awamori* using wheat flour in surface culture fermentation, *Iran J Biotechnol*, 9 (2011) 50-55.

23 Thakur A, Pahwa R, Singh S & Gupta R, Production purification, and characterization of polygalacturonase from *Mucor circinelloides* ITCC 6025, *Enzyme Res*, (2010) 170549. [doi:10.4061/2010/170549]

24 Kashyap D R, Soni K S & Tewari R, Enhanced production of pectinase by *Bacillus* sp. DT7 using solid-state fermentation, *Bioresour Technol*, 88 (2003) 251-254.

25 Pattat N H C, Condemine G, Nasser W & Reverchon S, Regulation of pectinolysis in *Erwinia chrysanthemi*, *Annu Rev Microbiol*, 50 (1996) 213-257.