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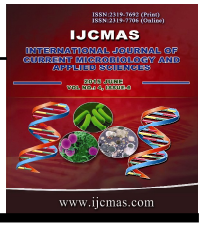

Original Research Article

Use of Plackett-Burman Design for rapid Screening of Diverse Raw Pectin Sources for Cold-Active Polygalacturonase and Amylase Production by *Geotrichum* sp

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A B S T R A C T

Cold–active polygalacturonases and amylases play significant role in extraction and clarification of fruit juices at industrial level. An optimized production medium with low cost substrates would be very useful for commercial production of these enzymes. The present study was done to screen low cost pectin and starch substrates for cold active enzymes production. The different pectin and starch sources screened were fruit and vegetables peels like citrus, pineapple, apple, banana, mango, guava, carrot, beetroot, bottle gourd, ridge gourd and potato. For efficient screening of the best sources a statistical design like Plackett-Burman was used as in this design n variables can be studied in just n-1 experiments only. A twelve experimental design Plackett-Burman was used as the best sources can be shortlisted in consideration with their interactive effects. The pectinolytic and amylolytic yeast isolate was identified as *Geotrichum sps* and was used for the present study. Cold-active pectinase and amylase enzyme activity was assayed by dinitrosalicylic acid (DNS) method. The flasks were incubated at room temperature (20-25 °C) and cold active enzyme activity was tested at 5°C. The best pectin sources selected for efficient cold-active pectinase production were banana and bottle guard and for amylase mango and guava peels. Thus, these studies indicate that low cost raw sources can be used for optimization of production media for commercial production of cold-active polygalacturonase and amylase producer which could have better application in fruit juice clarification studies.

Keywords

Amylase, Cold-active enzyme, *Geotrichum sps*, Poly galacturonase, Plackett-Burman, Pectin

Introduction

Pectic substances form a major component of the middle lamella of the primary cell walls of plant tissues and are degraded by special enzymes called pectinases. These are group of enzymes that are both depolymerizing and saponifying enzymes that catalyze the hydrolytic cleavage of the α-(1-4)-glycosidic bonds in polygalacturonic acid the constituent of pectin to form D-galacturonic acid (Jayani *et al.*, 2005; A.S. Ismail, 1996). Pectinases have diverse applications in food industry for clarification

of fruit juices, wines (Alkorta *et al.,* 1998), coffee and tea fermentations (J.R. Whittaker, 1984). Cold-active enzymes from psychrophiles are important for fruit juice clarification as cold stored juices can be clarified with at low temperatures and this offers potential economic benefits as substantial energy can be saved. The production of pectinolytic enzymes has been widely reported in bacteria and filamentous fungi (Pedroli *et al.,* 2008*)* but these enzymes are produced as a mixture of enzymes (pectinases). Yeasts from spoilt fruits have been identified to be a good source for pectinases especially polygalacturonases (K. Divya and P. Naga Padma, 2014; Parish and Higgins, 1989). Yeast pectinases are preferable as they are mostly single enzymes and not a mixture like those of bacteria and also for the fact that yeast is a GRAS organism (P. Naga Padma *et al.,* 2011). Cold-active enzymes specially polygalacturonases are attractive for usage in fruit juice industry as colder conditions hamper spoilage and favor milder conditions that avoid changes in organoleptic and nutritional properties (T. Nakagawa *et al.,* 2005 a; Gainvors *et al.,* 1994). The aim of the present study was to optimize the production medium using raw sources of pectin and to screen the efficient sources that could yield both highest cold-active pectinase and amylase activity. The sources were screened using a statistical design like Plackett-Burman (Plackett-Burman, 1946). With current stress on fruit production and processing in an agro based economy like India the need for such cold active enzymes is highly demanding as it is important to keep temperatures low during extraction and clarification to retain the organoleptic properties and hence the present study focused on it.

Materials and Methods

Inoculum preparation: The isolate under study was isolated from cold stored spoilt fruit (K. Divya and P. Naga Padma, 2014), and maintained as a glycerol stock. The culture was revived from glycerol stock using yeast extract peptone dextrose (YEPD) broth. An inoculum was prepared using the same broth by inoculating 10^6 cells /ml, incubated for 24 hours. The inoculum size used for the study was 5% and the inoculum broth contained 10^6 cells /ml.

Pretreatment of raw pectin peels: Peels of different fruits and vegetables like citrus, pineapple, apple, banana, mango, guava, carrot, beetroot, bottle gourd, ridge gourd and potato were collected and dried in a hot air oven at 60°C to remove the total water content. The dried peels were then made into fine powders using a blender and preserved. The percentage pectin present in the peels was determined by carbazole method (Ranganna S, 1979).

Polygalacturonase/Amylase production: Submerged fermentation studies for enzyme production were carried out at flask level in 250ml Erlenmeyer flasks containing 50ml modified YEPD broth to which different dried raw pectin peels were added according to the Plackett-Burman 12 experimental design.

The flasks were inoculated with actively growing yeast culture with the inoculum size of 5% containing $1x10^6$ cells/ml and incubated at RT (20-25° C) and assayed for enzyme production at 5°C. Broth samples were collected from the incubated flasks every 24hrs and assayed for enzyme activity at 5°C. The peak enzyme production was considered for statistical analysis using Indostat software.

Polygalacturonase assay: One ml of culture broth was cold centrifuged at 4°C, 5000 rpm for 10 minutes. Supernatant was taken as enzyme source. The enzyme was

assayed by measuring the D-galacturonic acid released from polygalacturonic acid as substrate by dinitrosalicylic acid (DNS) method (Collmer *et al.*, 1988, Miller., 1959) at 5°C as assay temperature. One unit of enzyme activity is defined as the amount of enzyme required to produce 1 µM of galacturonic acid per minute at incubated temperature (5°C).

Amylase assay: The enzyme was assayed using 1% soluble starch of HI media make as substrate. The reducing sugars liberated on incubation of enzyme with substrate were determined by DNS method (Miller, 1959). One unit of amylase activity is defined as the amount of enzyme required to liberate 1 µ mole of reducing sugars (glucose equivalents) per minute.

Experimental design (Plackett-Burman design): Plackett-Burman designs are very efficient statistical screening designs used to screen n-1 variables in just n number of experiments with interactive effects of variables in consideration. Screening of various efficient pectin sources has been done using Plackett-Burman statistical design for 12 runs and 11 two-level factors. This design requires that the frequency of each level of a variable should be equal and that in each test the number of high and low variable should be equal. Then the effects of changing the other variables cancel out while determining the effect of a particular variable. The main effect was calculated as the difference between the average of measurements made at the high level setting (+1) and the average of measurements observed at low setting (-1) of each factor (K.Anuradha *et al* , 2014).

Results and Discussion

Eleven different cheaper pectin sources like citrus, pineapple, apple, banana, mango, guava, carrot, beetroot, bottle gourd, ridge gourd and potato were screened using a twelve Plackett-Burman statistical experimental design. The pectin content of the eleven sources was determined by carbazole method (Ranganna S, 1979) (Table 1). The cold active enzymes yield varied in different flasks the pectinase enzyme yield varied from 120-196 U/ml and amylase enzyme yield varied from 9-19 U/ml (Table 2). The peak enzyme production values were considered for statistical analysis using Indostat software. The analysis yielded regression coefficients and t-values. Based on the highest positive regression coefficients and highest t-values the best raw sources were selected. Sources with highest positive regression coefficients and their corresponding t-values for the peak production of cold active pectinase production as indicated in (Table 3) were banana and bottle gourd. Similarly for cold-active amylase production mango and guava peels were found to be efficient (Table 4).

Pectin present in fruits and vegetables encourages the growth of pectinolytic organisms as it is good carbon and energy source (K. Anuradha *et al.,* 2010) Presence of carbon source at required concentrations in the culture medium is very important for optimized production of enzymes. Taking this into consideration different raw pectin sources were screened using a statistical method like Plackett-Burman (Plackett-Burman, 1946). This design is reliable, fast, significant and time saving procedure as up to n-1 variables can be screened in just n number of experiments and so is also cost effective.

Yeasts have been reported to produce polygalacturonase (Blanco *et al.,* 1994; Birgisson *et al.,* 2003; P. Naga Padma *et al.,* 2011) among which *Geotrichum sps* is considered to be a good producer of cold-active pectinase and amylase (Natalia Lorena Rojas *et al.,* 2008).

Table.1 Pectin content in peels of different fruits and vegetables. (K. Anuradha et al., 2008)

Pectin Source	% Pectin
Citrus	14.8
Pineapple	9
Apple	1.8
Banana	7.0
Mango	13.0
Guava	3.89*
	*(Norma and Hannah, 2000)
Carrot	16.5
Beetroot	12
Bottle gourd	6
Ridge gourd	0.18
Potato	0.05

Table.2 Cold-active pectinase and amylase enzyme production at 24 hrs (Peak enzyme) by *Geotrichum* sps.

Run	a	b	c	d	e	f	g	h	i	j	k	Pectinase U/ml		Amylase U/ml	
												24 hrs SET I	24 hrs SET II	24 hrs SET I	24 hrs SET II
1	+	+	+	+	+	+	+	+	+	+	+	120.1	124.1	15.4	17.4
2	-	+	-	+	+	+	-	-	-	+	-	150.5	157.1	17.2	19.4
3	-	-	+	-	+	+	+	-	-	-	+	151.0	152.4	15.4	17.0
4	+	-	-	+	-	+	+	+	-	-	-	192.1	194.8	15.0	16.0
5	-	+	-	-	+	-	+	+	+	-	-	150.0	157.1	15.9	15.0
6	-	-	+	-	-	+	-	+	+	+	-	151.8	157.1	12.5	13.4
7	-	-	-	+	-	-	+	-	+	+	+	173.7	175.9	12.5	14.0
8	+	-	-	-	+	-	-	+	-	+	+	151.9	153.9	15.0	17.4
9	+	+	-	-	-	+	-	-	+	-	+	151.9	153.9	10.0	12.4
10	+	+	+	-	-	-	+	-	-	+	-	131.3	133.5	12.4	13.0
11	-	+	+	+	-	-	-	+	-	-	+	150.2	152.4	12.4	15.2
12	+	-	+	+	+	-	-	-	+	-	-	196.2	194.8	9.0	10.0

Table.3 The regression coefficient and t-values for 11 raw pectin sources for cold active pectinase production by *Geotrichum* sps.

S.No	Ingredients	Cold-active Pectinase Production at 24 hrs	
		Reg. Coeff	t-value
1.	Intercept	157.4042	459.4078
2.	Citrus	0.8042	2.3471
3.	Pineapple	-13.0625	-38.1249
4.	Apple	-6.1625	-17.9862
5.	Banana	7.7542	22.6317
6.	Mango	-2.4792	-7.2358
7.	Guava	-2.6708	-7.7952
8.	Carrot	-2.7375	-7.9898
9.	Beet root	-2.7792	-8.1114
10.	Bottle gourd	1.4792	4.3172
11.	Ridge gourd	-8.9958	-26.2557
12.	Potato	-6.4542	-18.8375

Table.4 The regression coefficient and t-values for 11 raw pectin sources for cold active amylase production by *Geotrichum* sps

S.No	Ingredients	Cold-active Amylase Production at 24 hrs	
		Reg. Coeff	t-value
1.	Intercept	14.2875	96.9410
2.	Citrus	-0.7042	-4.7778
3.	Pineapple	0.3542	2.4030
4.	Apple	-0.6958	-4.7212
5.	Banana	0.1708	1.1591
6.	Mango	1.0542	7.1525
7.	Guava	0.8042	5.4563
8.	Carrot	0.6292	4.2689
9.	Beet root	0.7625	5.1736
10.	Bottle gourd	-1.1625	-7.8876
11.	Ridge gourd	0.6792	4.0682
12.	Potato	0.2208	1.4984

Different raw pectin sources may contain pectin and starch in different concentrations which effects the production of both cold-active pectinase and amylase and the present screening strategy is useful as it screens nutrients considering their interactive affects also. The present study was useful in screening the most significant and cheaper pectin and starch sources for optimizing the production medium for cold-active enzymes production. These enzymes are mostly inducible and hence need a good inducing substrate for commercial production. Use of cold active enzymes for fruit juice clarification is not only cost effective at industrial scale (L. Ceci and L. Lozano, 1998; R. Margesin and F. Schinner, 1994; T. Nakagawa *et al.*, 2005 b) but also commercially significant as it cuts down energy inputs as directly cold stored juices can be clarified. This also gives scope for labour intensive fruit processing industries in a fruit producing and populated country like India.

Diverse cheaper raw pectin and starch sources were efficiently screened using Plackett-Burman 12 experimental design. The best sources screened for cold-active pectinase production were banana and bottle gourd, for amylase mango and guava peels. Cold-active pectinase production was good and there was an increase in the cold-active enzyme yield when the cheaper raw pectin sources were used. Thus the statistical design was significant in choosing the best sources for media optimization.

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