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Polygalacturonase Production by *Aspergillus niger* MTCC 3323 Utilizing Kinnow Peel Waste As A Substrate

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ABSTRACT

The present study was carried on exo-PGase production from Kinnow waste using A.niger MTCC 3323 which produced good amount of exo-polygalacturonase activity under various process conditions suc as pH, temperature organic and inorganic sources of nitrogen and incubation time.And Maximum PGase activity was observed at pH 5.0 and at 30^0 C and Maximum PGase activity with peptone as organic source and ammonium chloride as inorganic source of nitrogen and submerged fermentation was more suitable for exo-PGase activity than solid state fermentation.

Key words: *Polygalacturonase; Pectinase; Solid state fermentation; Submerged fermentation; Kinnow peels.*

INTRODUCTION

Pectic substance is a generic name used for the compounds that are acted upon by pectinolytic enzymes. These are negatively charged acidic glycosidic macromoleculese and have high moleculer weight .They form the major components of the middle lamella and primary plant cell wall. Pectinase is a general term for enzymes such as pectolyase pectozyme and polygalacturonase commonly reffered to as pectic enzymes which break down pectin of plants. One of the most studied and widely used commercial pectinases is polygalacturonase. It is useful because pectin is the jelly – like matrix which helps to cement plant cells together and in which other cell wall components, such as cellulose fibrils are embedded. Therefore pectinase enzymes are commonly used in processes involving the degradation of plant materials, such as speeding up the exrtraction of fruit juice from fruit, incliding apples and sapota. Pectinases have also been used in wine production since the 1960s. The function of Pectinase in brewing is twofold, first it helps breakdown the plant (typically fruit) material and so helps the extraction of flavours from the mash. Secondly the presence of pectin in finished wine causes a haze or slight cloudiness, Pectinase is used to break this down and so clear the wine Alkorta *et al.*[2], Lang and Dornen-burg[5]. Microorganisms are used for the production of pectinases because the microbes can be easily cultivated under controlled conditions.

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In the last fifteen years, *Erwinia, Bacillus, Saccharomyces, Kluyeromyces, Aspergillus, Penicillium, Fusarium and Rhizopus* have been the genera most frequently used for the production of pectinases *et al.*[1], Rangarajan *et al.*[10]. However strains of *Aspergillus, Penicillium* and *Erwinia* mainly used for the enzyme production studies because there are exhibiting higher pectinase activities.

Citrus fruits are native to south eastern Asia and are among the oldest fruit crops to be domesticated by humans. Brazil is the world's largest producer of citrus fruits (14.4 million tons) during the season and exports frozen fruit juice concentrates of > 1 million tons per year followed by United states other important producing countrie include china, India, Spain, Morocco, Italy etc Giese *et al.*[12],. Citrus fruits have well documented nutritional and health benefits as well as industrial uses Davies *et al.*[4],. Edible citrus is generally divided into sweet oranges, sour oranges, mandarians, grapefruit, pummelos, lemons, limes, citrons and kinnows. Kinnow is a variety of citrus fruit cultivated extensively in India. It is a hybrid of two citrus cultivars "King" (*Citrus mobilis*) × "willow leaf" (*Citrus deliciosa*), first developed by H.B. frost at the Citrus Research Centre of University of California, Riverside USA.IN India this variety was introduced by J.C. Bakshi in 1954 at Punjab Agricultural University, Regional fruit Research station Abohar.

Dried kinnow peels are rich in carbohydrates particularly (fructose, sucrose, glucose), proteins and pectins as well as insoulable cellulose and hence can be used as fermentation feedstock for the production of fermentation products and hence can be utilized in obtaining valuable products like peel oil such as d-limonene, pomace powder, dietry fibres and predominantly various enzymes like pectinase. Processing and utilization of Kinnow into various end products eventually leads to generation of waste in the form of peels and pomace (approximately 50% of the fruit weight) which offers potential applications in biotechnology. The solid material arising from processing of Kinnows for juice consists of peel, seed, and pulp (collectively reffered as Kinnow bagasse), and primarily constitute a waste product, which leads to serious environmental issues, like increased BOD, accumulation of waste which may under go decomposition and lead to foul smell and nausence hence polluting the area and all these problems need to be tackled or corrected for healthy and fresh environment. Using Kinnow waste as a substrate, fermentation has been extensively employed for the production of pectinases Pereira *et al.*[9], patil and dayanand[7, 8].

Pectinases have wide applications in food industries like (fruit juice extraction, coffee and tea fermentation, oil extraction, improvement of chromocity and stability of red wines), textiles, paper and pulp industries and in waste water treatment Patil *et al.*[7, 8]. They also have been used in simultaneous saccharification of solid state fermentation processes of citrus peel wastes into bioethanol wikkins *et al.*[11]. Due to the potential and wide applications of pectinases, it is necessary to study on several aspects related to pectinase production. The idea of using cheaper and easily available raw material for the pectinase production is an important parameter for cost effective production.

So keeping in view the above application of pectinases the following work was carried out with the objective of developing low cost and eco friendly approach of pectinase production from easily available raw material i.e Kinnow.

MATERIAL AND METHODS

Collection of Kinnow waste

Kinnow peels were collected from various fruit vendors of Mohali Punjab and were sorted manually. The peels were washed several times so that the dirt and dust was cleaned off, then the peels were sun dried and grinded in mechanical grinder and the powder was cleared with the help of sieve, with a mesh size of 5.6 and were used as a substrate for solid state fermentation and extraction.

Maintenance and Growth of Micro-organisms

The ampule containing *A. niger* MTCC 3323 was broken under aseptic conditions in Laminar air flow bench. Sufficient amount of inoculum was inoculated in the flask of 250 ml capacity containing containing 50 ml of potato dextrose broth and incubated at 30^o C for 4-5 days under stationary conditions for the development of fungal matt. After 5-6 days, the matt was used as inoculums to make the slants of potato dextrose agar. Slants were incubated at 30^0 C for 5-6 days for the growth of *A. niger* and were than stored in refrigerator at 4^0 C and sub-culturing was done periodically.

Preparation of inoculums

In flask of 250 ml capacity, 50 ml of potato dextrose broth was added and inoculated with matt of fungus which was previously maintained on PDA slants and incubated at 30^0 C for 5-6 days under stationary conditions for development of matt, Inoculum was prepared by suspending one loop full of inoculating needle of matt in 2ml of sterilizes distilled water, the inoculums strength was set at 0.3 OD at 550 nm, which to be used as inoculums.

Extraction process

The extract from kinnow peels was prepared as described by Yoeh *et al*.[12]. Abnout 100 grams of kinnow peel powder were placed in a thimble and 1000 ml distilled water was added in extraction flask. The assembly was placed over a heating mantle and the temperature was maintained at 100^0 C for 3 days. After 3 days of extraction the extract was collected and stored at 4^0 C. This extract was used as a substrate for submerged fermentation.

Analytical procedures

- After fermentation the culture sample was centrifuged at 10,000 rpm for 10 min and 5 ml sample was taken.
- In case of solid state fermentation extraction of enzyme was done in equal volume of distilled water.
- The supernatant was used for estimation of exo- polygalacturonase activity.

Estimation of Exo polygalacturonase activity

Exo polygalacturonase activity was analysed by incubating the culture supernatant for 1 hr at 50^0 C with 0.5% (w/v) polygalacturonic acid in 50 mM citrate buffer pH 4.8 (2.5g of polygalacturonic acid in 500ml of 50 mnM citrate buffer on hot plate was added slowly stirred until completely dissolved and stored at 4^0 C). Reducing sugars in the reaction mixture was determined by DNS acid method[6] using galacturonic acid as standard.

The enzyme activity was calculated from a standard curve (galacturonic acid 0.1-0.8mg/ml).

Estimation of Polygalacturonic acid by DNS method

Reagents used: Sodium potassium tartarate, DNS reagent (3,5 – dinitroslicylic acid), NaoH solution.

Procedure

- 1 ml of reaction mixture was taken
- 1 ml of DNS reagent was added and heat the mixture for 5 min at boiling water bath.
- Measure the absorbance at 540 nm and record the result.

RESULT AND DISCUSSION

Processing of Kinnow peel solids

Processing of Kinnow peel was done after drying and grinding of Kinnow peels. Kinnow peel powder was placed in the soxhlet extraction assembly. About 100 grams of Kinnow peel powder was taken and 1000 ml of distilled water was placed in thimble and the whole assembly was placed on heating mantle maintained at 100^0 C by Yoeh *et al*[12]. The extraction was carried out for 3 days and the extract was stored at 4^0 C. The extract obtained was further used as substrate for submerged fermentation. However, dried kinnow peel was used as such as a substrate for solid state fermentation.

Effect of pH on the production of polygalacturonase from *A. niger* MTCC 3323 by submerged fermentation (SmF) and solid state fermentation (SSF) utilizing Kinnow peel waste as substrate

The effect of various pH (3-6) of the fermentation medium on the production of

PGase by *Aspergillus niger* MTCC 3323 was studied. It is shown in the (table 1.1) that maximum PGase activity from *A. niger* MTCC 3323 (3.21 % 2.79) was obtained at pH 5 for both SmF and SSF However more PGase activity was in smF as compared to SSF.

Effect of temperature on the production of polygalacturonase from *A. niger* MTCC 3323 by submerged fermentation (SmF) and solid state fermentation (SSF)

The fermentation medium flasks (SSF & SmF) in the temperature range of 25-35^0 C using production medium with pH 5 containing 100 ml peel extract for SmF and 10 gm peel powder for SSF. In the SSF the initial moisture content was adjusted to 70%. The results given in (table 1.2) revealed that the maximum PGase activity that is (3.27 IU) and (2.9 IU) was obtained at 30^0 C for SmF and SSF respectively, Above 30^0 C temperature decrease in PGase activity was observed.

Effect of inorganic nitrogen on the production of PGase from the Kinnow waste by submerged fermentation under different incubation time by *A. niger* MTCC 3323

To study the effect of inorganic nitrogen sources on the production of PGase, ammonium sulphate and ammonium chloride at (2% w/v) concentrations were added to the fermentation medium separately and their effect at different incubation period (24-96hr) were studied. Data presented in (table 1.3) showed the effect of inorganic nitrogen sources during SmF and revealed that after 72 hrs of incubation maximum PGase production (3.94 IU) was obtained with 25% (w/v) ammonium chloride. However with ammonium sulphate 2 % (w/v) maximum PGase activity (3.52 IU) was obtained after 72 hrs. With both inorganic nitrogen sources, further increase in the incubation time decrease in the enzyme activity was observed.

Effect of inorganic nitrogen source on the production of PGase from the Kinnow waste by solid state fermentation under different incubation time by MTCC 3323

Effect of inorganic nitrogen 2% (w/v) was studied on production of PGase by *A. niger* MTCC 3323 during SSF. Fermentation was carried out at 30^0 C. It was observed that maximum PGase activity was upto 72 hrs followed by decrease in activity (table 1.4).On studying the effect of ammonium chloride concentration (1-4 5, w/v) with incubation time ,3 % (w/v) ammonium chloride gave highest activity after 72 hrs of fermentation.

Effect of organic sources on the production of PGase from Kinnow waste by submerged frementation under different incubation time

To study the effect of various organic sources on the production of PGase in SmF using different organic sources like peptone and yeast extract at (2% w/v) concentration was added to the fermentation medium separately and their effect on PGase production at different incubation period (24-96 hrs) were studied. Among peptone and yeast extract, peptone showed maximum (7.79 IU) PGase activity after 72 hrs of incubation period (table 1.5). There was a drastic increase in enzyme activity on addition of organic nitrogen sources with increase in incubation time from 48 to 72 hrs.

Effect of different organic nitrogen sources on the production of PGase from Kinnow peel waste by solid state fermentation by using *A. niger* MTCC 3323 under different incubation time

Data presented in (table 1.6) showed the effect of organic nitrogen sources on polygalacturonase production from *A. niger* MTCC 3323 during SSF utilizing Kinnow peel waste. Fermentation was carried out at temperature 30^0 C (initial pH 5).Similar to the submerged fermentation, peptone gave maximum PGase activity(4.39 IU) as compared to yeast extract (3.74 IU) after 72 hrs.

Table 1: Effect of pH on the production of polygalacturonase from *A. niger* MTCC 3323 by submerged fermentation (SmF) and solid state fermentation (SSF) utilizing Kinnow peel waste as substrate

pH	Polygalacturonase activity (IU)	
	SmF	SSF
3	0.98	2.71
4	2.63	2.72
5	**2.89**	**3.21**
6	2.13	2.21

Table 2: Effect of Temperature on the production of polygalacturonase from *A. niger* MTCC 3323 by submerged fermentation (SmF) and solid state fermentation (SSF) utilizing Kinnow peel waste as substrate

Temperature ^{0}C	Polygalacturonase activity (IU)	
	SmF	SSF
25	1.85	2.78
30	2.95	3.27
35	1.25	2.71

Table 3: Results of submerged fermentation using inorganic sources of nitrogen under different incubation time

Incubation Time (hr)	Polygalacturonase activity (IU)	
	Ammonium sulphate (2%)	Ammonium chloride (2%)
24	3.03	3.67
48	3.33	3.82
72	3.52	3.94
96	2.84	3.20

Table 4: Effect of inorganic sources on PGase activity in SSF by using *A.niger* MTCC 3323

Incubation Time (hr)	Polygalacturonase activity (IU)	
	Ammonium sulphate (2%)	Ammonium chloride (2%)
24	3.03	3.67
48	3.33	3.82
72	3.52	3.94
96	2.84	3.20

Table 5: Effect of organic sources on production of PGase from kinnow waste by submerged fermentation under different incubation time by using *A. niger* MTCC 3323

Incubation Time (hr)	Polygalacturonase activity (IU)	
	Yeast extract (2% w/v)	Peptone (2% w/v)
24	2.58	2.91
48	4.96	7.84
72	**5.95**	**7.97**
96	4.83	6.89

Table 6: Effect of organic sources on production of PGase from kinnow waste by solid state fermentation under different incubation time by using *A. niger* MTCC 3323

Incubation Time (hr)	Polygalacturonase activity (IU)	
	Yeast extract (2% w/v)	Peptone (2% w/v)
24	3.07	3.60
48	3.76	3.21
72	**3.74**	**4.39**
96	3.20	2.43

CONCLUSION

In current study on PGase production from Kinnow waste by using *A. niger* MTCC 3323 produced good amount of ex. Polygalacturonase activity under various process conditions such as pH, temperature ,organic inorganic sources of nitrogen and incubation time. Maximum PGase activity was observed at pH 5.0 and at 30^0 C. Maximum enzyme activity was with peptone as organic nitrogen source and ammonium chloride as inorganic nitrogen source in submerged fermentation as compared to solid state fermentation. The results derived from the current study revealed that Kinnow waste can be a promising raw material for production of several products. This study will act as a first line information to the researchers who are exploring the possibilities of converting waste to wealth, the concept which is currently evolving rapidly in the applied science branches from all possible

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