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Optimization of pectinase enzyme production using sour oranges peel *(Citrus aurantium* L) as substrate

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Abstract: The citrus fruit processing industries produce a large amount of waste material, which poses considerable disposal problems and ultimately leads to pollution. In the processing of citrus fruits, a large proportion of the produce goes waste in the form of peel, pulp and seeds. Dried citrus peel is rich in carbohydrates, proteins and pectin; pectin acts as the inducer for production of pectinolytic enzymes by microbial systems. The sour oranges peel was used for the production of crude pectinase using *Aspergillus niger* during submerged fermentation. The optimum conditions such as; optimization of substrate concentration (4%), optimization of inoculum size (9%), optimization of temperature (30ºC), optimization of time/duration (120 Hrs) and optimization of pH 5 were evaluated.

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INTRODUCTION

Citrus fruits constitute an important group of fruit crops produced all over the world. Pakistan is also a major producer of citrus fruits. Citrus fruits are utilized mostly for table purposes, but a significant portion is processed into various products, such as squashes, cordials, single strength juices, juice concentrates, marmalades, pickles, etc. The family of citrus fruits consists of Oranges, Kinnow, Khatta, Lime, Lemon (Galgal), Grapefruit, Malta, Mausami, Sweet orange etc. These all are known to contain appreciable amounts of pectin[1].

Fruit processing industries produce a large amount of waste material in the form of peel, pulp, seeds, etc. Some fresh orange peel is, however, used in shredded form in the preparation of orange-marmalade. This waste material presents considerable disposal problems and ultimately leads to pollution. Dried citrus peel is rich in carbohydrates, proteins and pectin; the fat content, however, is low. Various microbial transformations have been proposed for the utilization of food processing waste for producing valuable products like biogas, ethanol, citric acid, chemicals, various enzymes, volatile flavoring compounds, fatty acids and microbial biomass [6-11].

Citrus peel contains an appreciable amount of pectin and thus can be used as a substrate for the production of pectinolytic enzymes by microorganisms. Pectin acts as the inducer for the production of pectinolytic enzymes by microbial systems[12]. The advantage of using microorganisms for the production of enzymes is that these are not influenced by climatic and seasonal factors, and can be subjected to genetic and environmental manipulations to increase the yield[13].

The increasing energy demands have focused worldwide attention on the utilization of renewable resources, particularly agricultural and forest residues, the major components of which are cellulose, starch, lignin and pectin. These materials have attracted considerable attention as an alternative feedstock and energy source, since they are available abundantly. Several microbes are capable of using these substances as carbon and energy sources by producing a vast array of enzymes in different environmental niches[14].

Pectinases are a group of enzymes that catalyze the breakdown of pectin-containing substances and can be produced either by submerged[15] or solid-state fermentation procedures[16]. Submerged fermentation (SmF) systems have been extensively used for the production of high-priced materials and for the study of biochemical and physiological aspects of the synthesis of microbial metabolites[17]. Increasing applications have been found for pectinases in the food industry. Their use is essential for the clarification of fruit juices and they are an important aid in the extraction of juice and colored materials necessary for the preparation of fruit nectars and vegetable purees. Crude enzyme preparations have considerable commercial importance in the process of extraction and clarification of fruit juices[18,19].

The objective of the present work was to compare the overall physiological behavior of mould pectinase production in submerged fermentations. More specifically, we have evaluated the effect of the addition of substrate concentration on the activities of pectinase produced by SmF. Keeping in view the importance of enzyme pectinases in the food processing industry and the problems associated with the disposal of food processing industry waste, the present study was undertaken with the objectives

of utilizing citrus peels for the production of pectinase by the fungus *Aspergillus niger*.

MATERIALS AND METHODS

Procurement and preparation of substrate

Sour oranges peels (Citrus aurantium L) were procured from the fruit juice vendors of the local market of Lahore. The peels were sun-dried and ground to powder using an electrical grinder.

Physiochemical analysis of dehydrated sour oranges peel

Chemical analysis i.e. moisture, ash, fat, crude fiber, sugars (total, reducing, non reducing), lignin and vitamin C was performed in dehydrated sour oranges peel using standard methods 925.09, 923.03, 920.85, 920.86, 920.183, 973.18 and 967.21 respectively of AOAC[20]. Triplicate determinations were performed for all parameters and standard deviation was calculated[21].

Maintenance and growth of microorganism and inoculum preparation

The culture of *Aspergillus niger* was procured from the Food Biotechnology Center, PCSIR Laboratories Complex, Ferozpur Road Lahore. Spores of *Aspergillus niger* were grown and maintained on Potato Dextrose Agar (PDA) and inoculum were prepared as the methods described[22].

Enzyme extraction

The fermentation media was agitated at 250 rpm on rotary shaker for 20 minutes to extract the liquid from fungal mycelia and from the semisolid substrate. The extract was filtered through Whatman filter paper No. 1. This clear extract was used for enzyme assay.

Enzyme assay

Enzyme assay was based on the determination of reducing sugars produced as a result of enzymatic hydrolysis of pectin by dinitrosalicylic acid reagent (DNS) method [23]. For this, to 0.2 ml of 1% pectin solution, 2.0 ml of sodium citrate buffer of pH 5.0 and 1.0 ml of enzyme extract was added. The reaction mixture was incubated at 35°C±1°C for 25 in After 25 min, 1.0 ml of this reaction mixture was withdrawn and added to test-tubes containing 0.5 ml of 1M sodium carbonate solution. To each test-tube, 3.0 ml of DNS reagent was added and the test tubes were shaken to mix the contents. The test tubes were heated to boiling on the boiling water-bath for 10-15 min.

Instrumentation

The test tubes which contain DNS reagent, Crude enzyme and substrate along with buffer were cooled and 20 ml of distilled water was added to the contents of each tube and the absorbance was measured at 570 nm using Spectronic 20 (Shimadzu). The enzyme and substrate blanks were run parallel. The standard curve was prepared for reducing sugars with glucose. One enzyme unit of Pectinase is the number of µM of reducing sugars measured in terms of glucose, produced as a result of the action of 1.0 ml of enzyme extract in 1 minute at 35±1°C.

RESULTS AND DISCUSSION

The study was undertaken to evaluate the chemical characteristics of the peels (sour oranges) and on the basis of its nutrient contents the pectinase enzyme was prepared by utilizing these peels. In chemical composition i.e. moisture, ash, crude fat, crude fiber, sugars (total, reducing, non-reducing), pectin and lignin were reported as percent (%), while the vitamin C as mg/100g (Table 1). The mean and standard deviation were also determined.

It was observed that the sour oranges peels contain moisture content (12.5±0.5%), ash (3.5±0.1%), fat (1.7±0.2%), crude fiber (17.2±0.2%), total sugars (14±1.3%), reducing sugars (10±1.0%), non reducing sugars (3.8±0.4%), pectin (7.0±0.3%), lignin (5.4±0.3%) and ascorbic acid (84±2.5mg/ 100g). Different conditions for optimization of substrate for the production of pectinase enzyme were also evaluated (Tables 2-6).

Table 1: Chemical composition of sour oranges peel

No	Parameters	Sour orange peels
1	Moisture (%)	12.5*±0.5**
2	Ash (%)	3.5±0.1
3	Fat (%)	1.7±0.2
4	Crude fiber (%)	17.2±0.2
5	Total sugar (%)	14±1.3
6	Reducing sugar (%)	10±1.0
7	Non reducing Sugar (%)	3.8±0.4
8	Pectin (%)	7.0±0.3
9	Lignin (%)	5.4±0.3
10	Vitamin C (mg/100g)	84±2.5

*Average of triplicate determination
**Standard deviation values

Table 2: Effect of substrate concentration on enzyme activities

No.	Substrate concentration	Enzyme activity (µM/ml/min)
1	1%	92.80
2	2%	97.94
3	3%	103.66
4	4%	110.63

Optimization of substrate concentration for the production of pectinase

The results of substrate concentration on pectinase production were shown in Table 2. Its were found that the maximum production were obtained at

4% substrate concentrations, followed by 3%, 2% and 1% substrate concentration. The study carried out using *A. giganteus* produced PG activity in medium concentration of orange waste, with peak production at 3% (w/v)[24]. Our results were in agreement to the previous findings[25].

Table 3: Effect of Inoculum size on Enzyme activities.

No.	Inoculums size	Enzyme activity (µM/ml/min)
1	3%	67.09
2	6%	75.66
3	9%	101.38
4	12%	79.66

Table 4: Effect of temperature on enzyme activity.

No.	Temperature	Enzyme activity (µM/ml/min)
1	20 °C	92.23
2	30 °C	104.80
3	35 °C	99.09
4	40 °C	95.091

Table 5: Effect of duration on enzyme activity.

No.	Duration	Enzyme activity (µM/ml/min)
1	24 hrs	61.38
2	48 hrs	92.23
3	72 hrs	98.52
4	96 hrs	87.6

Table 6: Effect of pH on enzyme activity.

No.	pH	Enzyme activity (µM/ml/min)
1	4.0	78.52
2	4.5	89.94
3	5	93.37
4	5.6	75.091

Optimization of inoculum size for the production of pectinase

The effect of inoculum size for the production of pectinase was shown in Table 3. 3%, 6%, 9% and 12% inoculum size were used for the production of pectinase production. It was found in this study that 9 % were optimum inoculum size for the production of pectinase. The pectinase production was increase when size of inoculum increase, but it reached maximum at 9 % and decreased at 12%. Our results were in agreement of the previous findings[26] in which 10% inoculum for the production of polyglacturonase was used.

Optimization of temperature for the production of pectinase

Thermal stability and activity of pectinases are of great significance in biotechnological process. It has been indicated in one study that the optimum incubation temperature was 25 ºC for the production of PME [27], but in our study we found that 30ºC was the optimum incubation temperature. It has been reported that strain of *Thermoascus aurianticus*, *Aspergillus aculeatus* and *Fusarium oxysporum* have produced PGases with optimal activities at a temperatures from 60 to 65º C, respectively[28-30].

The PME activity was recorded maximum at 25ºC, decrease in PME production was observed both at higher and lower temperature than 25ºC[27]. The production of PGase by *Aspergillus niger* using orange peel as inducer found that 30°C was the best temperature for the production of the said enzyme[31]. The effect of temperature on PG production were studied and it was found that in medium with initial pH 3.5 the highest PG production, expressed either as activity per unit volume or activity per milligram of protein, was obtained at 30 °C, with the mean values of 43.0 U/ml and 69.5 U/mg protein when the medium initial pH 6.0 and when the initial pH 3.5, the highest PG activity per unit volume 6.0I U/ml was produced at 30°C, while the maximum values of specific activity were obtained in the range 20-30 °C the mean values being 25.9 U/mg of protein[24].

Orange waste was used as carbon source with the incubated temperature 28°C produced 3.33U/ml of PG [24]. In our study it was found that 30°C was the optimum temperature for the production pectinase which is accordance to the studies showed a higher pectinase (24.1U/ml) activity under the growth condition temperature was 28°C [32] and pectin lyase was produced at 30°C[33].

Optimization of duration on for the production of pectinase

The production of pectinlytic enzyme profile of *streptomyces lydicus* under SmF for 4 days on rotary shaker at 175 rpm and 30°C was studied. Difference pectinolytic activities exhibited by the culture were PG and PL. PE production was detected in the culture supernatant. In this research work the time course study was conducted and found that PG titer after 96 h was the highest and the highest enzyme activity recorded was 0.98 U/ml. Pectin layese activity reached its peak after 48h incubation and then decreased. The highest enzyme titer recorded was 0.4 U/ml[26].

The results on production of PME in terms of incubation periods show that with the increase in the incubation period, the PME activity increase. The lowest PME activity was observed at 24 hrs of incubation, while the maximum was recorded at 96 hrs of incubation period and therefore, only negligible increase in PME production took place [27].

The optimized fermentation period indicated a wide range in SmF (40-120h) and SSF (90-120 h) [34, 35]. It has been showed that the production of pectinase in SmF, its activities decreased rapidly after 72 h of fermentation. Endo-p was not detected

at 96 h of culture and endo-p reached 0.85 U/ml at 120 h, but this was due to pH related that above pH 6.5, endo-p and exo-p produced by *A. niger* CH4 under the same culture conditions were unstable [34].

The effect of incubation time on PGase production, after intervals of 15, 24, 39, 48, 63, 72, 87, 96, 111 and 120 h have been reported[31]. This study showed that maximum enzyme production at 63-72 h after which, there was a sharp decrease in the enzyme activity, which may be because of the exhaustion of nutrients and or accumulation of metabolites in the fermentation medium. The time of maximum enzyme production is significant because it is considered to be the cycle time for reusability transfer in repeated bath fermentation.

The time-course of PG was studied which indicates that the peak production of PG seen after 3.5 h after growth[24]. The cultural condition times for the production of pectinase were 108 h[32]. Our present study indicate that 120 h was the best time/duration for the production of pectinase, but in another study it was found that 96 h were the optimum time for the production of pectin methyleserase (PME) by *A. niger* [27].

The study showed that pectinase production started with the 7th incubation day by *A. niger*, while *T. Viride* showed later production. For the former mould the maximum PGase production was recorded about the 14th incubation day while in *T. Viridia* it was detected later, at 25th incubation day, when the observed units (9.0I U/ml) were about seven times more than in *A.niger* (1.27U/ml)[25]. The times of incubation used for the pectinase production using different carbon sources (orange waste, apple pomace, citrus pectin) were 7 days[24] which were found less as compared to our study.

Optimization of pH for the production of pectinase

It has been reported that besides acidic PGase produced by fungi and yeast, some bacteria strains produced PGase with optimum activity at low pH values, pectinase produced by Streptomyces sp. QG-11-3 optimally activate at pH 3. Since this enzyme was stable in the pH range of 2 to 9, it could be used for reaction under acidic or alkaline conditions[36]. It was found that PGase production by *Aspergillus aculeatus* was optimally active at pH 3, however most of studies on acidic PGase production usually determine their activity at temperatures ranges a pH values from 4.0 to 4.5[29]. In this study it is found that the optimum pH condition for the production of pectinase were 5, but it was reported that the optimum pH value were 4.0 for the production of PME using apple pomace as substrate by *A. niger* [27].

It has been reported that optimum pH in both cases of fermentation SSF and SmF was the same (pH 4). This may be due to the fact that the optimum pH for the production of PME is more related to the optimum conditions required for the growth of specific microorganism employed to conduct the fermentation than other factors, so it may have remained in a particular range for the some microorganism, irrespective of the type of fermentation[27]. It was previously evaluated that the pH medium was adjusted before inoculation to values from 3.0 to 10.0. The fungal growth and PG production are detected over the whole pH range tested best results. The highest PG production was obtained in growth media initially adjusted to pH 3.5[24] and the crude pectinase production from lemon pulps, the medium pH was kept about 3.7[25]. Our results showed that the pH 5 of the medium were the best production for pectinase. The initial pH 4.5 of the cultural median was kept for the production of pectinase[34].

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