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Effects of Carbon and Process Parameters on the Production of Pectinase by *Aspergillus spp.* Isolated from Rotten Citrus Fruits

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ABSTRACT

The enzyme pectinases are key enzymes responsible for the breakdown of long and complex molecules of pectin and depolymerizing them. The enzyme has diverse applications in the juice processing, maceration of tea leaves, retting fiber, pulp and paper industries, textile industries, processing of cotton fabric and in various biotechnological approaches. It is well known that a wide range of microbial species are good source of pectinase including bacteria viz. *Clostridium sp.* and *Bacillus spp.*, yeast and large number of filamentous fungi viz. *Aspergillus niger, Aspergillus flavus, Fusarium oxysporum, and Botrytis cinerea*. The study was aimed to isolate some potential pectinase producer fungi and to check the effect of carbon and process parameters on the production of pectinase. In the present study, four *Aspergillus spp.* isolates recovered from rotten citrus fruit samples and have been designated as AS1, AS2, AS3 and AS4 respectively. All the 4 isolates were screened for pectinolytic activity and found positive with a zone of hydrolysis ranged from 2mm-4mm. AS1 shown maximum pectinolytic activity and was selected for pectinase production by submerged method in molasses and orange peel powder supplemented with 1% NaCl at pH 5.5, temperature 30^{o}C, with an incubation period of 72 hours has produced 242 IUmg^{-1} and 238.9 IUmg^{-1} protein respectively. From above study it can be concluded that molasses and orange peel powder supplemented with 1% NaCl at pH 5.5, temperature 30^{o}C, and incubation period of 72 hrs produced maximum pectinase by the submerged method.

Keywords: Aspergillus, Enzyme, Enzymatic activity, Pectinase, Pectinolytic activity

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INTRODUCTION

Pectinases are the enzymes which are accountable for the degradation of the long and complex molecules of pectin. The enzymes that hydrolyze pectin substances are known as pectic enzymes or pectinolytic enzymes. The three main types of pectinases are Pectinesterases, Depolymerizing enzymes and Protopectinase. A large amount of pectinases produced from microbes and account for 25 % of the global food enzyme [1].

Pectins are a family of complex polysaccharides that contain backbone of α-1, 4- linked D-galacturonic acid residues. Pectins are the heterogeneous polysaccharides which are not only

found in primary cell wall but also in the middle lamella between plants cells, where it helps to bind cells together. Three pectic polysaccharides (homogalacturonan, rhamnogalacturonan-I and substituted galacturonans) have been isolated from primary cell walls [2]. Cell walls of most plants viz. apple, pomace and citrus peel are the main sources of commercial pectin [3].

A large number of microbial strains have been studied for the production of pectinase. The main sources for the pectinolytic complex enzymes are yeast, bacteria such as *Clostridium spp.* and *Bacillus sp.* and large number of filamentous fungi such as *Aspergillus flavus, Fusarium oxysporum, and Botrytis cinerea* of which the most relevant ones are *Aspergillus spp. Aspergillus niger* is a haploid filamentous fungi which is used to produce various compounds such as citric acid, amylase, cellulase and pectinase and are considered GRAS (generally regarded as safe) by United States of food and drug administration. *Aspergillus spp.* are mesophilic fungi most commonly found in decaying environment, dust, and paint. This filamentous fungus exhibits a high tolerance to freezing temperature.

Pectinases are the enzymes which have extensive applications in juice and textile industries, maceration of tea leaves, retting of fibre, pulp and paper industry, processing of cotton fabric and in various biotechnological applications [4-9]. The utilization of renewable residues has focused worldwide attention because of their major components such as lignin, starch, cellulose, xylan and pectin [10]. Several microbes are capable of using these substances as carbon and energy sources by producing an immense range of enzymes [11]. The importance of the pectinase enzymes has gained momentum and lot of research is going on for efficient and economical productions of pectinase enzymes using cheaper substrates of agro- residue origin. Pectinases are produced during the natural ripening process of some fruit and they help to soften the cell walls in combination with cellulose. A large number of microbial strains have been studied for the production of pectinase. However, need for a newly isolated pectinolytic microbe and use of agro –residue for the production of pectinase to minimize the production cost along with waste management is prime focus of the study. The study makes a new plateform for the production of pectin along with the efficient management of agro- residue and make the producrion cost more cheaper.

MATERIALS AND METHODS

Material used: All the chemicals, media, reagents used in the project were of analytical grade. The media used were procured from Hi media (India) and were used as per the manufacturer's directions. The glassware used during the project was of borosilicate glass.

Collection of samples: Peels of spoiled citrus fruits and vegetables were collected in a polythene bag from the dumping area of the canteen of Shaheed Udham Singh Group of Institutions, Tangori, Mohali (India).

METHODOLOGY

Isolation of *Aspergillus niger*: Isolation media of composition, g/L (Pectin, 10; sucrose, 10; tryptone, 3; yeast extract, 2; KCl, 0.5; $MgSO_4.7H_2O$, 0.5; $MnSO_4.5H_2O$, 0.01; $(NH_4)_2SO_4$, 2.0 and 20.0 agar) supplemented with mineral salt solution of composition g/100 mL

($CuSO_4.5H_2O$, 0.04; $FeSO_4$, 0.08; Na_2MoO_4, 0.08; $ZnSO_4$, 0.8; $Na_2B_4O_7$, 0.004, $MnSO_4$, 0.008) was used (Banu *et al*. 2010). Ampicillin (0.1%) was added to the above medium to restrict the bacterial growth. pH value was adjusted to 5.5 before autoclaving at 121°C for 15 min. Inoculated plates were incubated at 30°C for 5 to 7 days. The cultures were further purified by sub-culturing on PDA plates [12].

Characterization of fungal isolates: Characterization of fungal isolates was done with the help of Lactophenol cotton blue staining method [13]. Stained slides were examined under light microscope.

Screening of fungal isolates for pectinolytic activity: The isolates were assayed for pectinolytic activity using modified Czapek-Dox Agar, with citrus pectin as the sole carbon source by a modified plate method [11]. Potassium iodide- Iodine solution (5.0 g KI and 1.0 g I in 330 ml of distilled water) was used to determine the clear zone formed around the colonies.

Pectinase production with different agro-residue wastes: The fungal isolates were used to produce pectinase enzyme using the different agro -residue including orange peels and molasses as the sole carbon sources. The modified Czapek-Dox media contained 10g/L of the agro wastes as sole carbon source was used as the fermentation media. 100 ml of the sterile medium having pH (5, 5.5 and 6) was inoculated with spores of isolated fungus. Fermentation was carried out in 250 ml Erlenmeyer flask containing 100 ml of growth medium and incubated at different temperatures (25°C, 30°C, 35°C and 40°C) with agitations using shaking incubator. The medium was also optimized with different salt concentration. Cultures were harvested at intervals of 24 hour by centrifugation at 4000 g for 10 minutes. The supernatants were used as the crude enzyme which was further used to evaluate pectinase activity.

Enzymatic assay: The pectinase activity was determined by measuring the amount of reducing substances released from citrus pectin. For the enzymatic assay, the reaction mixture containing equal amounts of 1% citrus pectin prepared in sodium acetate buffer (0.05M; pH 5.5) and the crude enzyme solution was incubated at 50°C in water bath for 30 minutes. The reaction was stopped with 1.0 ml dinitrosalicylic acid solution (1% 3, 5-dinitrosalicylic acid (DNSA), 30% sodium potassium tartrate, and 0.4 M NaOH) after which the mixture was boiled for 10 minutes [14]. The colour was read at 540 nm using the spectrophotometer. The amount of reducing sugar released was measured using pectin as standard (Figure 1).

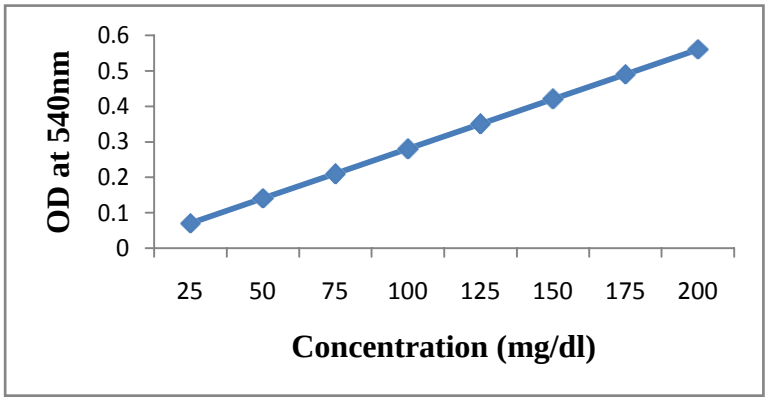

Figure 1: Standard curve of pectin

RESULTS

Isolation of *Aspergillus spp.:* A total four fungal isolates of *Aspergillus spp.* were recovered from various sources by growing these on basal medium containing pectin as a sole carbon source and designated as AS1, AS2, AS3 and AS4.

Cultural and morphological characterization: All the four isolates were then characterized in terms of cultural and morphological properties. Cultural characteristics were observed on potato dextrose agar. It showed white colored colonies on potato dextrose agar plate, but with the passage of time it matured and gradually turned into greenish –blue and then to black (Figure 2). After the observation of cultural characteristics, the colonies were observed for morphological characteristics under microscope after lactophenol-cotton blue staining [15]. Single-celled spores (conidia) have been observed in chains developing at the end of the sterigmata arising from the terminal bulb of the conidiophores (Figure 3).

Figure 2: Cultural appearance of isolates

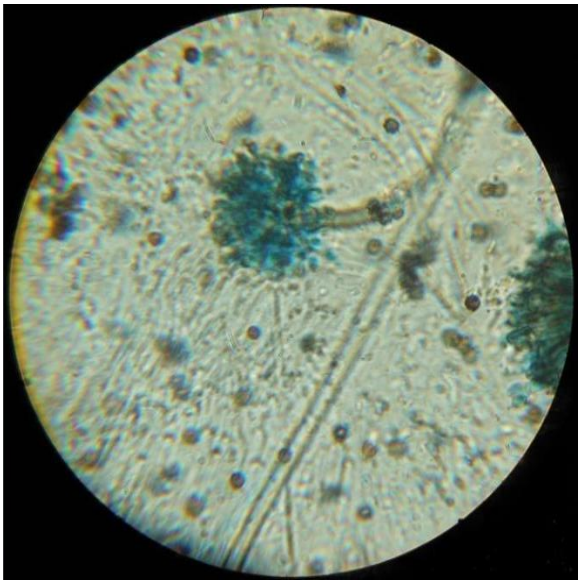

Figure 3: Microscopic observation of isolates

Screening of *Aspergillus spp.* for pectinolytic activity: All the isolates have been screened for pectinolytic activity on Czapek-Dox Agar plates. Clear zones have been observed in all the plates. These zones of clearance around the fungal growth showed the evidence of pectinolytic activity of the isolates. Figure 4 showed the relative pectinolytic activity of the fungal isolates as indicated by their relative clearance zones. The diameter of zone of clearance was shown in table 1.

Table 1: Diameter of zone of hydrolysis of different isolates

ISOLATE NUMBER	ZONE OF HYDROLYSIS
AS1	4 mm
AS2	3 mm
AS3	2 mm
AS4	2 mm

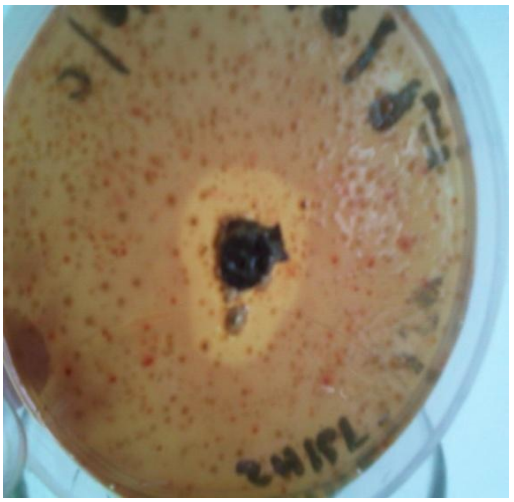

Figure 4: Clear zone from AS1

Enzymatic activity: The enzymatic activity was calculated with the help of DNSA method. Standard curve of pectin was prepared to calculate the enzymatic activity.

Effect of carbon source: The production medium was optimized with two different carbon sources i.e. orange peel powder and molasses. The highest pectinase activity of 242 IU mg^{-1} protein was showed with molasses as sole carbon source. The maximum activity with orange peel powder was 238.9 IU mg^{-1} protein. It has been concluded from the graph (Figure 5) that the molasses is the better sole carbon source than orange peel powder.

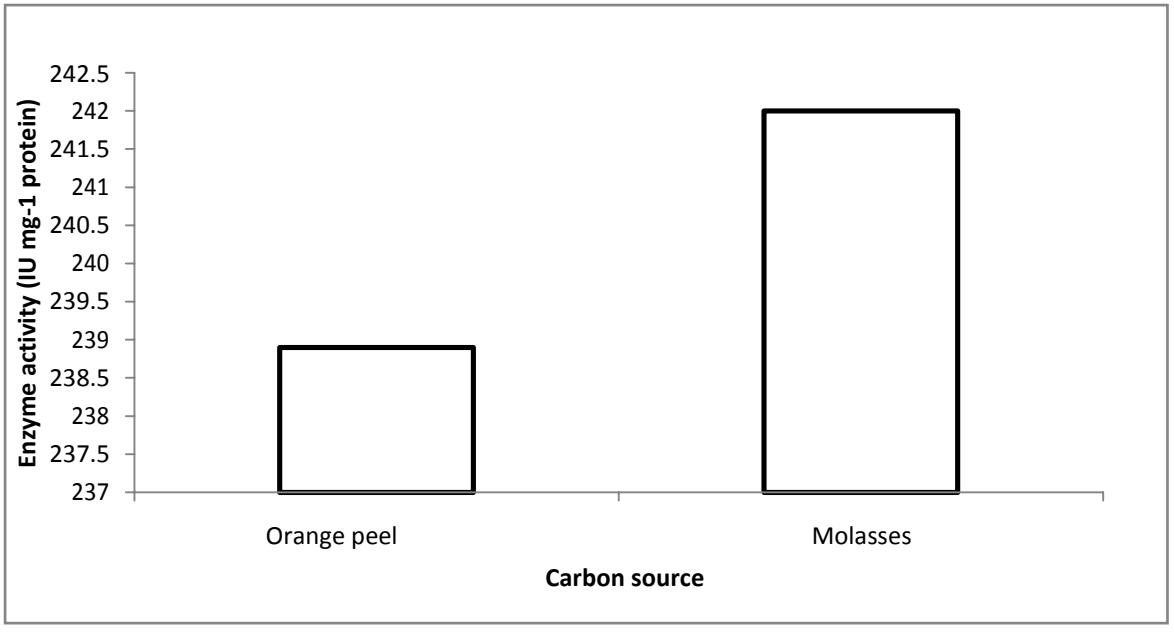

Figure 5: Pectinase activity of *Aspergillus spp*. fermented on orange peel and molasses

Effect of pH: The production medium was optimized with different pH viz. 5, 5.5 and 6 along with above mentioned agro-residue as carbon sources. It has been found that the pectinase activity was maximum at pH 5.5. The minimum pectinase activity was observed at pH 6. It has been concluded from the graph (Figure 6) that enzymatic activity decreases with increase in pH after 5.5.

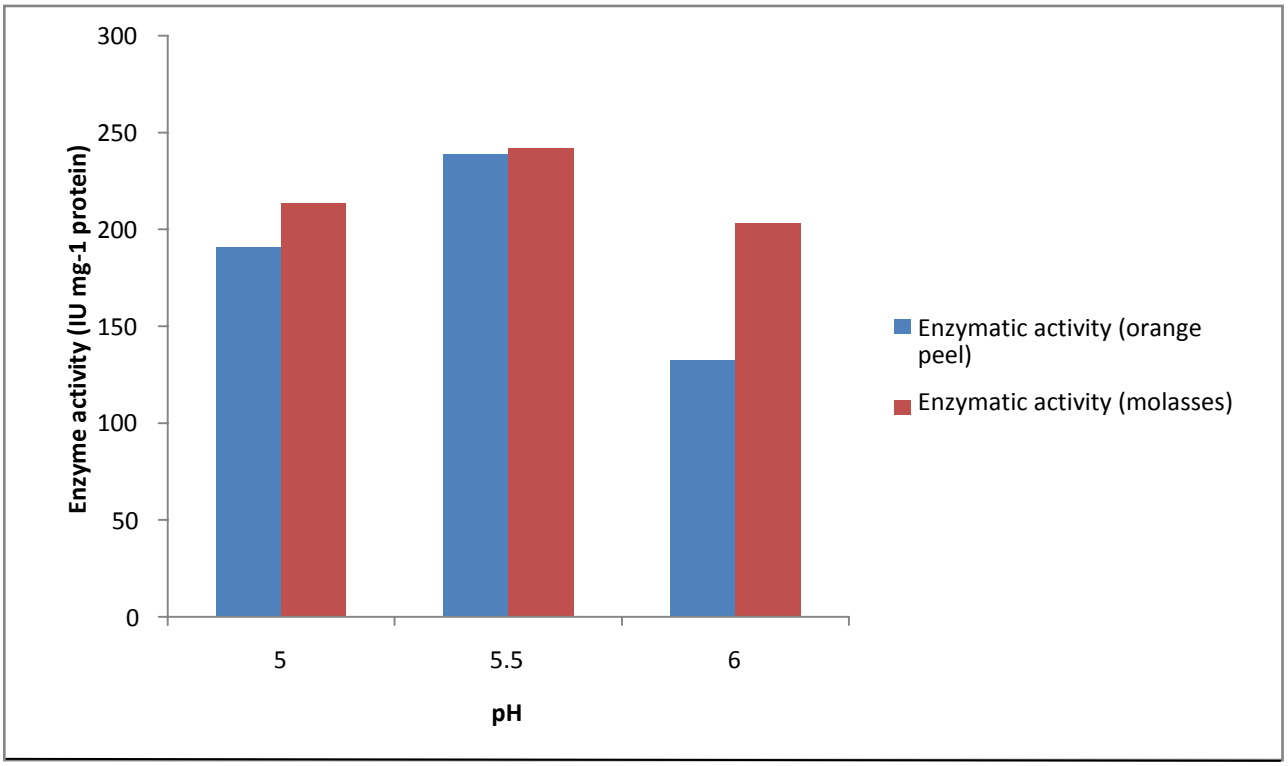

Figure 6: Effect of pH on the production of pectinase

Effect of incubation period: The production medium was observed for pectinase activity at time intervals of 24 hours. The maximum enzymatic activity has been observed after 72 hours. The least enzymatic activity was observed after the incubation period of 24 hours. It has been observed that the enzymatic activity was gradually increasing with incubation period up-to 72 hours and it suddenly start decreasing after 72 hours (Figure 7).

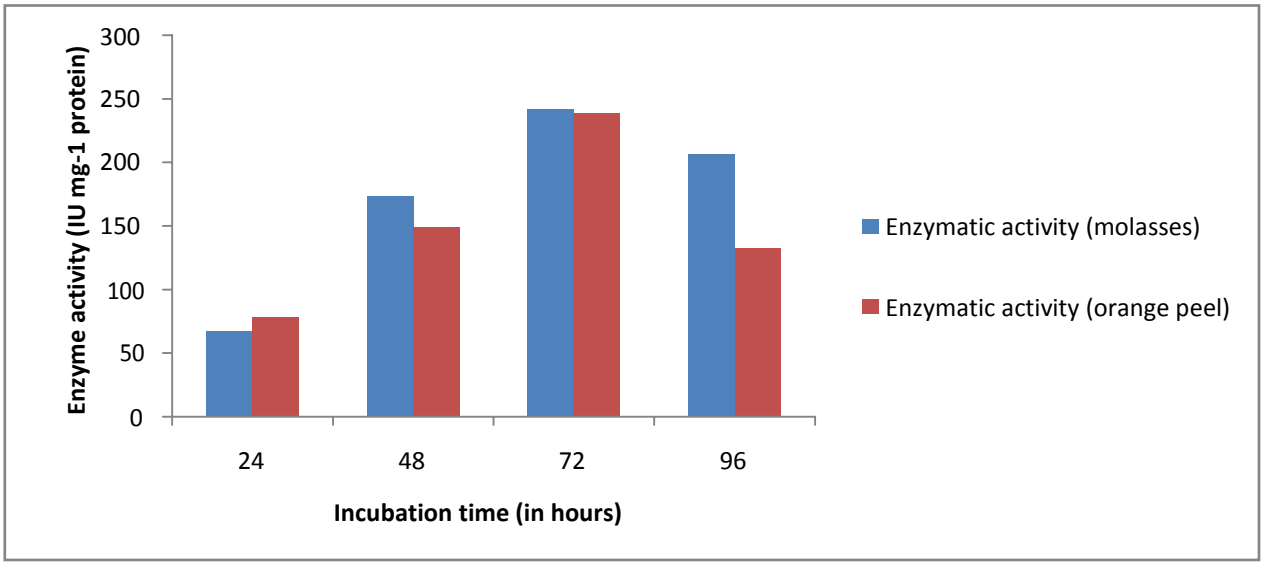

Figure 7: Effect of incubation time on the production of pectinase.

Effect of temperature: The growth medium was incubated at different temperatures viz. 25^{o}C, 30^{o}C, 35^{o}C and 40^{o}C. The maximum enzymatic activity was observed at 30^{o}C after that the enzymatic activity suddenly decreased at higher temperatures (Figure 8).

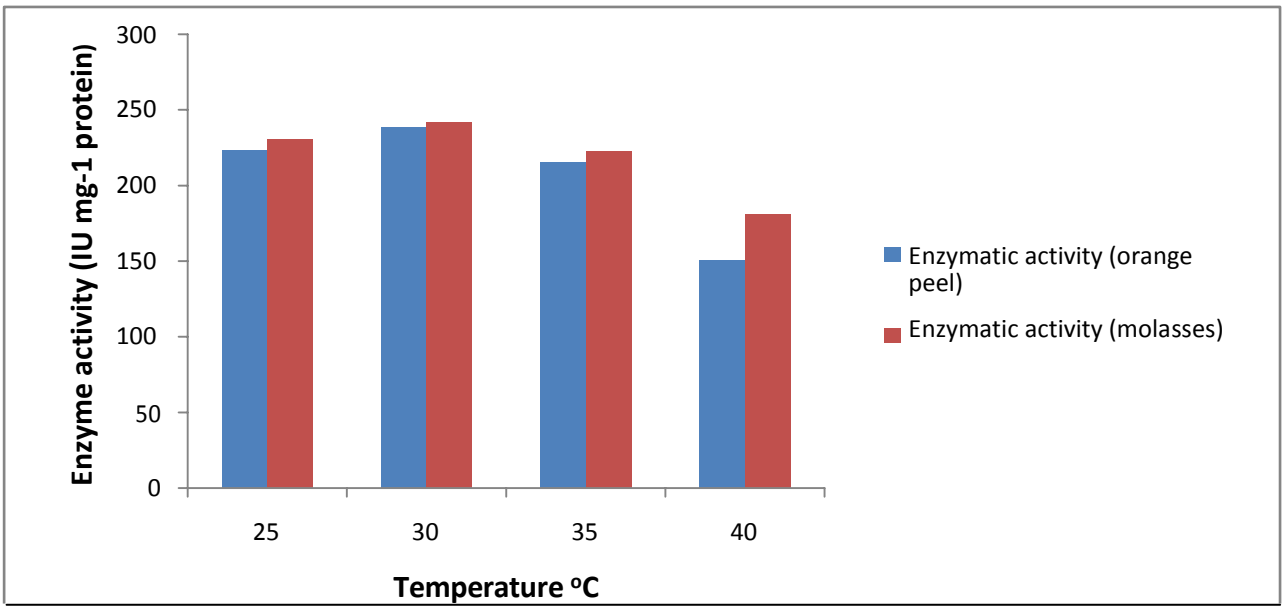

Figure 8: Effect of temperature on the production of pectinase

Effect of salt concentration: The growth medium was optimized with different NaCl concentrations of 0.5%, 1.0%, 1.5%, 2.0%, 2.5% and 3.0%. The enzymatic activity was observed maximum on salt concentration of 1% for both orange peel powder and molasses. It was observed that enzymatic activity was decreasing with the increase in salt concentration and there was no enzymatic activity after the concentration of 2.5% i.e. the enzymatic activity was zero in the salt concentration 3.0% (Figure 9).

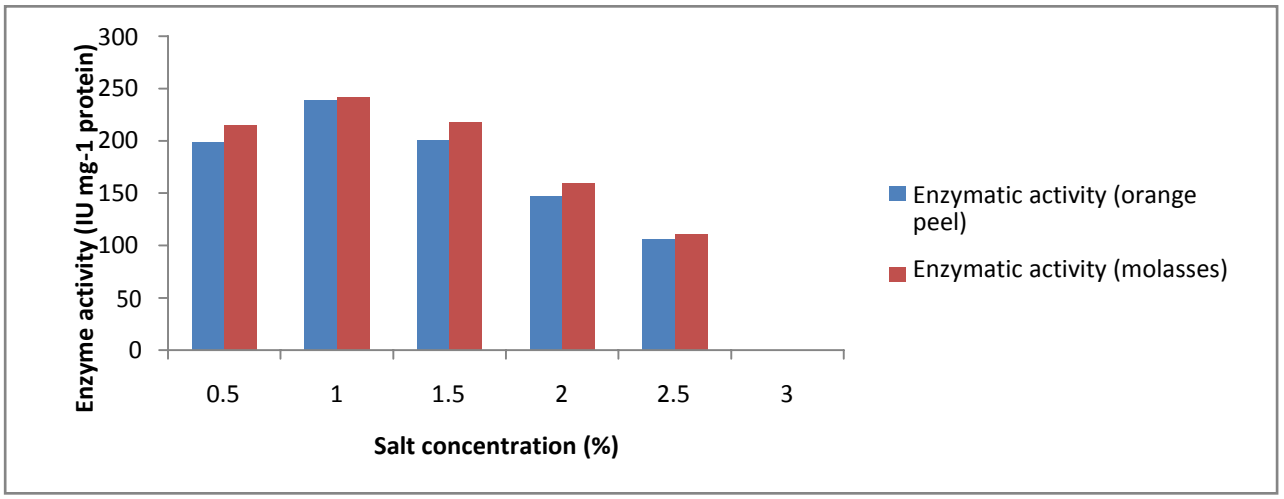

Figure 9: Effect of salt concentration on the production of pectinase

DISCUSSION

Pectinases or petinolytic enzymes hydrolyze pectic substances. Pectinases are one of the most widely distributed enzymes in bacteria, fungi and plants. It has been reported that microbial pectinases account for 25% of the global food enzymes sales. Almost all the commercial preparations of pectinases are produced from fungal sources. *Aspergillus niger* is the most commonly used fungal species for industrial production of pectinolytic enzymes [16].

In the present study four fungal isolates were recovered from rotten fruit peels and morphologically characterized and identified as *Aspergillus spp.* and designated AS1 to AS4. All the isolates were further subjected to check their pectinolytic activitiy shown by presence of zone of hydrolysis around the growth. All the isolates were found to be positive for pectinoytic activity indicates potential pectinase producer. Similarly, strain of *Aspergillus niger* was screend for pectinolytic activity. *Aspergillus niger* demonstrated a large zone of hydrolysis around the large colony on pectin agar medium [17]. The zone of clearance was seen, degradation was evidenced by a clear zone around fungal growth. Okafor *et al.* [12] cultivated the fungal isolates on modified Czapek-Dox agar plates for the screening of isolates for pectinolytic activity. He reported that *Aspergillus niger* and *Penicillium chrysogenum* gave the highest pectinolytic activity as shown by large clearance zones.

Further for the production of pectinase the production medium was optimized with two different agro-residue carbon sources viz. molasses and orange peel powder. The highest pectinase activity of 242 IU mg^{-1} protein was showed with molasses as sole carbon source. The activity with orange peel powder was 238.9 IU mg^{-1} protein. Okafor *et al*. [12] optimized the medium with different agro wastes like orange peel, sawdust, wheat bran, sugarcane pulps and pineapple peel and they concluded that the highest pectinase activity was showed at 48 hours with wheat bran as sole carbon source. The maximum pectinase activity obtained with wheat bran was 350.28 ± 2.82 IU mg^{-1} protein. Sugarcane pulp yielded the second best value of 282.13 $\pm$ 5.23 IU mg^{-1} protein at 72 hours. The least pectinase activity was obtained with orange peel powder. The maximum pectinase production by *Aspergillus niger* using different agro wastes was in the order: wheat bran> sugarcane pulp> pineapple peels> saw dust> orange peels.

After optimization of carbon source further different process parameter i. e. pH, temperature and incubation period and salt concentration were also optimized to maximize the production of pectinase. The production medium was set with different pH (5, 5.5 and 6) along with different agro- residue. It has been found that in both the medium pectinase activity was maximum at pH 5.5. Kutateladze *et al*. [18] cultivated the microscopic fungi at pH range from 2.0 to 10.0. They reported that for *Aspergillus niger* T 1-1 culture, the optimal pH for the pectinase production is 6.0. Mrudula *et al*. [19] optimized the medium with pH range of 3-8. They reported that for *Aspergillus niger* strain the pectinase activity was maximum at pH 5. They also observed that enzyme activity decreases when pH was increased to 6 and keep on decreasing as pH value increased up-to 8.

The effect of incubation period on pectinase production was also evaluated and found that the enzymatic activity was increases with the time observed maximum at 72 hours and again it was slowly decreases upto 96 hours. Mrudula *et al*. [19] reported that *Aspergillus niger* showed best enzymatic activity after 96 hours of incubation period. Okafor *et al.* [12] reported that *Aspergillus niger* showed the maximum pectinase activity after 72 hours of incubation time when orange peel was used as sole carbon source and after that the enzymatic activity keeps on decreasing. Temperature also effects the production of pectinase. As we know that molds are grown best at temperature range 25-30^{o}C. The AS1 had shown maximum enzymatic activity at temperature 30^{o}C and slowly decreases at higher temperature. In a similar study, Panda *et al*. [20] reported that *Aspergillus niger* and *Aspergillus flavus* showed maximum enzymatic activity at

30^{o}C. They also observed that pectinase activity decreased gradually at temperatures higher than 30^{o}C. The production media was also supplemented with various concentration of table salt to check the effect of enzymatic activity. From this study it was revealed that the 1% NaCl concentration is the best proportion to enhance the production of enzyme. Makky, [21] supplied the medium with different salt concentrations viz: 0, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 %. He grew the isolates of *Aspergillus flavus* on two natural pectic substrates (dead tree leaves and rice straw) in the presence and absence of salt. He reported that enzymatic activity was not affected till 1% concentration, after 1% the enzymatic activity started to decrease and no enzymatic activity was reported when the salt concentration was 3.0%.

CONCLUSION

The results of the investigations shows that the molasses and orange peel powder is a good carbon source with production medium pH 5.5, temperature 30^{O}C, supplemented with 1% NaCl concentration and incubation period of 72 hrs give maximum enzymatic activity (pectinase production) by submerged fermentation method using fungus *Aspergillus spp*.

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