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Research Article

OPTIMIZATION OF CULTURAL CONDITIONS FOR PECTINASE PRODUCED BY FRUIT SPOILAGE FUNGI

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ABSTRACT

The purpose of this investigation was to isolate and identify some fruit spoilage fungi (FSF) and screening for cell wall degrading enzyme pectinase. Pectinases are a group of hydrolytic enzymes that play an important role in food processing industry and alcoholic beverage industry. 7 FSF cultures were isolated from apple, banana, kiwi, lemon, mango and orange and identified based on morphological and microscopic characteristics. Out of 7 fungal isolates 5 were identified as belonging to *Aspergillus* sp. and one each belonging to *Fusarium* sp. and *Trichoderma* sp. Identified fugal isolates were then screened for pectinase production. 4 isolates belonging to *Aspergillus* sp. showed pectinolytic effect as they produced zone of hydrolysis due to degradation of pectin by the pectinase. Further these isolates were subjected to pectinase production and pectinase activity was estimated by dinitrosalicylic acid method. Pectinase activity showed that FSF7 (isolated from orange) produced maximum (156.80U/ml) and FSF5 (isolated from lemon) minimum (102.54U/ml) enzyme after incubation of 120hours. Process parameters such as pH, temperature, incubation time, carbon and nitrogen source were optimized for FSF7 to maximize the pectinase production. Temperatue 35°C, pH 7, incubation time of 96hours, sucrose as carbon source and urea as nitrogen source were found optimum for pectinase production. Pectinase production was increased from 156.80U/ml to 358.22U/ml with optimization of culture conditions. In conclusion it can be said that *Aspergillus* sp. are widely distributed among the spoilage fruit fungi and secreted plant cell wall degrading enzyme pectinase.

Key words: *Aspergillus* sp., Pectinase, Incubation time, Carbon source, Urea.

INTRODUCTION

Once the fruit or vegetable has no connection to the living plant it cannot repair its cells or defend its tissues against natural deterioration. While fruits deteriorate naturally, predatory microorganisms like fungi can speed this process, but fresh fruits have an external toughness that functions as a barrier for entry of most plant pathogenic microbes. There are many reasons of fruit destruction but main cause of fruit spoilage is invasion by microorganisms such as mould, yeast and bacteria. Fruits contain high levels of sugars and nutrients and their low pH make fruits particularly desirable to fungal decay[1]. During postharvest handling, mechanical damage causes the release of enzymes present in the cell tissues. These enzymes break down the cellular material. Pectinases are the first enzymes to be secreted by fungal pathogens when they attack plant cell walls, weaken the plant cell wall and expose other polymers to degradation by hemicellulases and cellulases[2]. Various chemical reactions by the enzymes results in loss of flavour, nutrient, colour and texture. If temperature is not controlled during postharvest handling this may leads to the deterioration at an accelerated rate because enzymes are mainly composed of proteins, they are sensitive to heat. Pectinases are one of the most widely distributed enzymes in bacteria, fungi and plants. Fungi are considered to be prospective enzyme producing sources. Pectinases breaks down pectin and are of significant importance in the

current biotechnological era with their all embracing applications in fruit juice extraction and its clarification, scouring of cotton, degumming of plant fibers, waste water treatment, vegetable oil extraction, tea and coffee fermentations, bleaching of paper, in poultry feed additives, in alcoholic beverages and food industries[3]. Addition of vinegar, salts or sugars, removal of water and dehydration are used to prevent the growth of microbes as well as contamination but fruit spoilage cannot be prevented absolutely[4]. In spite of strategies to prevent fruit spoilage a portion of fruits are spoiled, may be because of postharvest processing, mechanical handling, invasion by microorganisms etc. but spoiled fruits and vegetable products are further used in many processes and industries. Moreover utilization of spoiled fruits also minimizes pollution[5]. Enzyme production by microorganisms has been reported to be highly influenced by many factors such as temperature, pH, incubation time and carbon sources[6,7]. Factors like carbon, nitrogen sources and their concentrations have always been of great interest to researchers in the industry for the low-cost media design. It is also known that 30-40% of the production cost of industrial enzymes is estimated to be the cost of growth medium. Therefore, it is of great significance to optimize the conditions for cost-efficient enzyme production[7]. Keeping in mind the applications of pectinases and utilization of spoiled fruits, present investigation was planned to isolate and identify some fruit spoilage fungi and screening for plant cell wall degrading enzyme pectinase.

MATERIALS AND METHODS

Isolation of Fruit Spoilage Fungi: Fruit spoilage fungi (FSF) were isolated from apple, banana, kiwi, lemon, mango and orange fruits. Fruits were incubated at 28°C for 5 days, after incubation, surfaces of fruits were washed off with sterile distilled water and serial dilutions of washed off water were made (10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}) for each fruit. 0.1ml aliquot from each dilution were spread on potato dextrose agar (PDA) plates and incubated in an inverted position at 28°C for 7 days. After 7 days, colonies were picked from each plate and sub-cultured on freshly prepared autoclaved PDA media, again incubated for 7 days and growth was observed after 7 days. Isolated fungi were maintained at 4°C for further processing.

Identification of Isolates: Fungal isolates obtained after sub-culturing were identified based on morphological and microscopic features of its sporulated structures. Morphology of FSF was observed on the basis of colour, size, surface and rate of growth of colonies by following the standard methods[8]. Microscopically the isolates were studied by staining with lacto phenol cotton blue using a compound microscope[9].

Screening of Fungal Isolates for Pectinase Production: Screening of FSF for pectinase production was done on pectinase screening agar medium. A well was created in centre of petri plate containing solidified pectinase agar medium, fungal spore suspension was poured into the well and incubated at 37°C for 24 hours. After 24 hours, plates were flooded with iodine for 15 minutes and observed for zone of hydrolysis around the wells[10].

Production of Pectinase Enzyme: Cell wall degrading enzyme pectinase from the isolated FSF was produced using their spoilage fruits as culture media in agitation phase. FSF were inoculated under aseptic conditions in Erlenmeyer flask containing 5% respective fruit peels and incubated at 28°C in incubator shaker at 150 rpm for 120 hours. Same procedure was carried in presence of potato dextrose broth as a control instead of fruit peels. The cell free broth was recovered by filtration using Whattman filter paper. Cell free broth was subjected to dialysis against 20mM Tris-Hcl buffer, pH 7.2 overnight. The dialyzed was centrifuged at 10000 rpm for 10 minutes and supernatant was used as crude extract[11].

Enzyme Assay: Pectinase activity was determined by estimating the liberated reducing end products and galacturonic acid as standard[12]. Enzyme activity was based on the amount of D-galacturonic acid released in crude extract. 1ml of crude extract was added to 3ml sodium acetate buffer (2M, pH-5) containing 1% pectin. Assays were carried out at 37°C for one hour. Amount of D-galacturonic acid was determined by dinitrosalicylic acid (DNSA) method. 0.5ml dinitrosalicylic acid reagent was added to each tube and tubes were heated in boiling water bath for 10minutes. After cooling to room temperature absorbance was taken at 560nm. Unit of enzyme activity was defined as the amount of enzyme that releases 1µmol of galacturonic acid per minute according to standard curve[11].

Parameters Controlling the Pectinase Production: Medium optimization for maximum production of the enzyme is an important step and involves a number of physico-chemical parameters such as the incubation period, pH, temperature and

carbon source & nitrogen source. Optimization was performed for the most potent FSF isolate screened as best producer of pectinase. Temperature range of 25°C-50°C and pH range of 5-9 was screened for optimization of temperature and pH parameters. Incubation time of 24 hours, 48 hours, 72 hours, 96 hours, 120 hours and 144 hours were used to study the optimal incubation time for pectinase production. Effect of various carbon compounds (fructose, glucose, lactose, sucrose and starch) and nitrogen compounds (peptone, ammonium nitrate, ammonium sulphate, urea and yeast extract) on pectinase production was also examined. Enzyme activity was determined by DNSA method.

RESULTS AND DISCUSSION

In this study, attempt was made to isolate pectinase producing fungi responsible for fruit spoilage. FSF exploit the fruit using extracellular lytic enzymes that degrade the cell wall of fruit to release water and other constituents to use as nutrients for their growth. FSF cultures were isolated from fruits and identified for the screening of cell wall degrading enzyme pectinase.

Isolation of Fruit Spoilage Fungi (FSF): Seven filamentous FSF were isolated from apple, banana, kiwi, lemon, mango and orange and sub cultured on PDA media to obtain pure colonies (Fig. 1).

Identification of Fungal Isolates: Fungal isolates were identified on the basis of morphological and microscopic characteristics (Table 1). Based on morphological and microscopic features the fungal isolates were probably identified as belonging to *Aspergillus sp., Trichoderma sp. and Fusarium sp.* (Table 2).

From the morphological and microscopic characterization of fruit spoilage isolates it can be inferred that out of 7 isolates, 5 were probably identified as belonging to *Aspergillus* sp. and one each to *Trichoderma* sp. and *Fusarium* sp. *Aspergillus* sp. was isolated from apple, kiwi, lemon, mango and orange. Both *Trichodema* sp. and *Fusarium* sp. (probable identity) were isolated from banana only. The frequency of occurrence shows that *Aspergillus* sp. had the highest frequency occuring in 5 out of 6 fruits, revealed that *Aspergillus* sp. is highly pathogenic (Table 2). The results of the present study was found to be similar with earlier study[13] where *Aspergillus* sp. was spoiled 5 out of 12 fruits thus found most common pathogenic fungi responsible for the deterioration of the fruits

Screening of FSF for Pectinase Activity: 7 isolated fungal cultures were screened for pectinolytic activity. Out of 7 isolates, 4 isolates (FSF1, 4, 5 & 7) belonging to *Aspergillus* sp. showed zone of hydrolysis means they had pectinolytic effect but zones were absent in *Trichoderme* sp. and *Fusarium* sp. FSF 7 isolated from orange showed largest zone of hydrolysis (25mm) followed by FSF 4 from kiwi (23mm), FSF 1 from apple (20mm) and FSF 5 from lemon (16mm) indicating that FSF 7 produced highest amount of pectinase followed by FSF 4, FSF 1 and FSF 5 (Table 3 & Fig. 2).

Pectinase Production and Enzyme Assay: All the fruit spoilage fungal isolates were screened for quantitative estimation for pectinase production using selective enrichment approach to draw comparative account. This method was based on the enzymatic hydrolysis of pectin, the galacturonic acid was determined spectrophotometrically. FSF 7 was found to produce maximum pectinase (156.80U/ml) followed by FSF 4 (142.72 U/ml), FSF 1 (124.84 U/ml) and FSF 5 (102.54 U/ml). FSF 7 was found to be best producer of cell wall degrading enzyme pectinase out of all the tested FSF isolates so it was further used for optimization of process parameters (Table 3).

Parameters Controlling the Pectinase Production: FSF 7 giving the maximum zone of inhibition and pectinase production (Table 3) was subjected to different pH, temperatures, incubation time, carbon and nitrogen source for pectinase production. Effect of pH on pectinase production was studied by conducting the experiments at pH l 5, 6, 7, 8 and 9 by keeping the temperature at 28°C. As the pH was increased from 5 to 7, pectinase activity was also increased but decreased with further increase in pH value. Total pectinase activity was more at pH 7 throughout the incubation when compared with other pH range (Fig. 3).

Optimum temperature for pectinase production was determined by performing the experiment at temperatures 25°C, 30°C, 35°C, 40°C and 45°C by keeping all other conditions constant for incubation period of 144 hours. As the temperature was increased from 25°C to 35°C the enzyme production was increased and maximum pectinase activity (186.32U/ml) was observed at 35°C after incubation of 96 hours. Further increase in temperature decreased the enzyme activity and the decrease in enzyme activity at higher temperatures may be due to denaturation of enzyme (Fig. 4).

Pectinase activity was found to increase with incubation time and was maximum (358.22U/ml) after incubation of 96 hours and later on started depleting when the incubation time was increased. The decrease in the activity can be due to the depletion of nutrients in the medium (Fig. 5).

Maximum pectinase activity was observed with sucrose as carbon source and minimum with starch (Fig. 6). Among the used nitrogen sources, urea was found best for optimum production of pectinase followed by ammonium nitrate, ammonium sulphate, peptone and yeast extract after incubation of 96 hours (Fig. 7). Urea is a cost effective nitrogen source so pectinase can be produced in large quantity economically by using urea as nitrogen source.

Temperature 35°C (186.32 U/ml), pH 7 (358.22 U/ml), incubation time of 96 hours (358.22 U/ml), sucrose as carbon source (165.40 U/ml) and urea as nitrogen source (302.10 U/ml) were found best for optimum production of pectinase by FSF 7. The present study indicates that *Aspergillus* sp. might be an efficient and economical source of pectinase enzyme.

Table1
Morphological and Microscopic Characterization of Fungal Isolates

Fungi	Colony Shape	Colony Colour	Texture	Microscopic Description
FSF1	Circular	Brownish- green	Rough	Round sporangia with brown smooth textured unbranched clustered sporangiospores and non-septate hyphae
FSF2	Circular	Dark green	Smooth	Round, smooth-rough conidia and hyaline, branched conidiophores
FSF3	Circular	Pink	Rough	Sickle shaped conidia pointed at the tip, conidiophores were short and branched
FSF4	Circular	Black	Rough	Smooth, mature conidia, vesicles were observed
FSF5	Circular	Brown	Rough	Long, smooth to rough conidia, irregular and radiated conidial heads
FSF6	Circular	Yellow- green	Rough	Radiated conidial heads, conidia were round, globose, smooth to rough walls appeared in chains with rough conidiophores
FSF7	Circular	Initially brown then turned black	Smooth	Conidia were round, globose, rough with radiated conidial heads, septate hyphae smooth long and hyaline conidiophores

Table 2
Probable Identity of Fungus Isolated from Various Fruits

Fruit	Fungi	Probable Identity
Apple	FSF1	*Aspergillus* sp.
Banana	FSF 2	*Trichoderma* sp.
Banana	FSF3	*Fusarium* sp.
Kiwi	FSF4	*Aspergillus* sp.
Lemon	FSF 5	*Aspergillus* sp.
Mango	FSF6	*Aspergillus* sp.
Orange	FSF7	*Aspergillus* sp.

Table 3
Zone of Hydrolysis and Pectinase Activity of Pectinolytic Fruit Spoilage Fungal Isolates

Fungi	Fruit	Zone of Hydrolysis (mm)	Pectinase Activity (U/ml)
FSF1- *Aspergillus* sp.	Apple	20	124.84
FSF2- *Trichoderma* sp.	Banana	No zone	-
FSF3- *Fusarium* sp.	Banana	No zone	-
FSF4- *Aspergillus* sp.	Kiwi	23	142.72
FSF5- *Aspergillus* sp.	Lemon	16	102.54
FSF6- *Aspergillus* sp.	Mango	No zone	-
FSF7- *Aspergillus* sp.	Orange	25	156.80

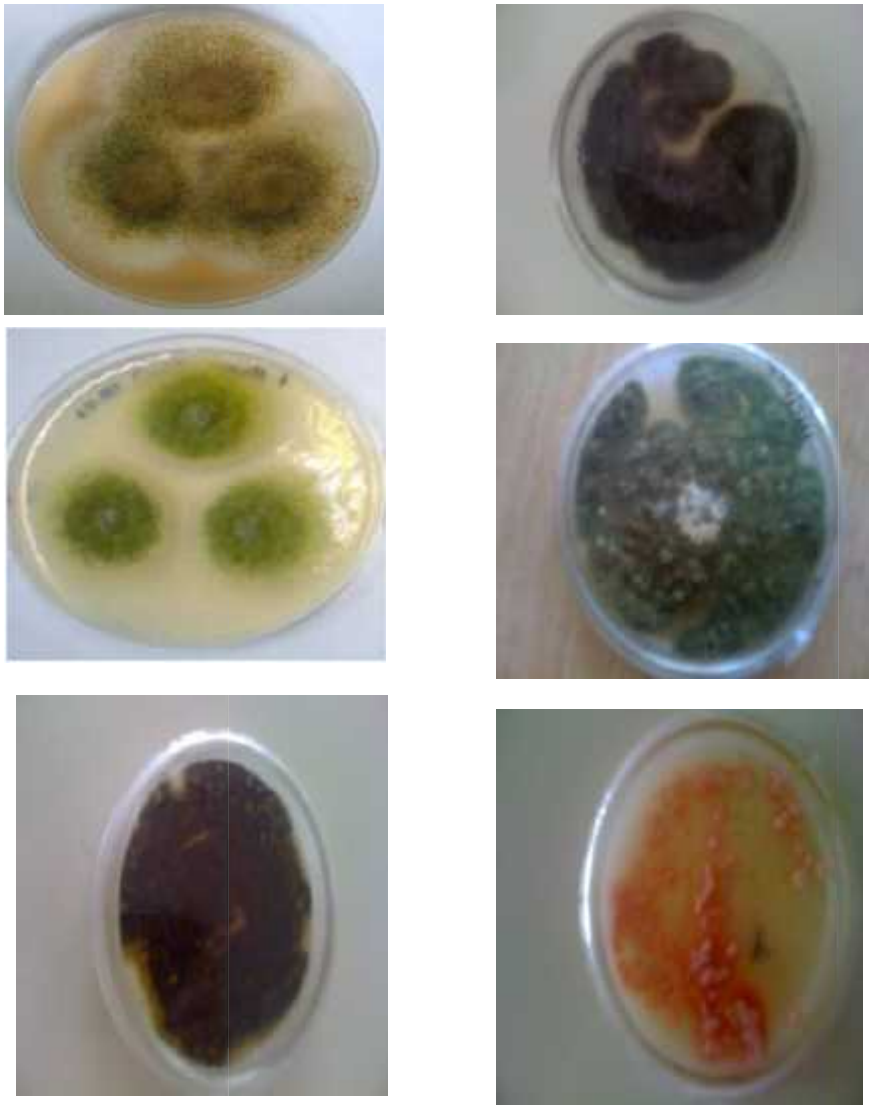

Fig.1
Fruit Spoilage Fungi on PDA Media

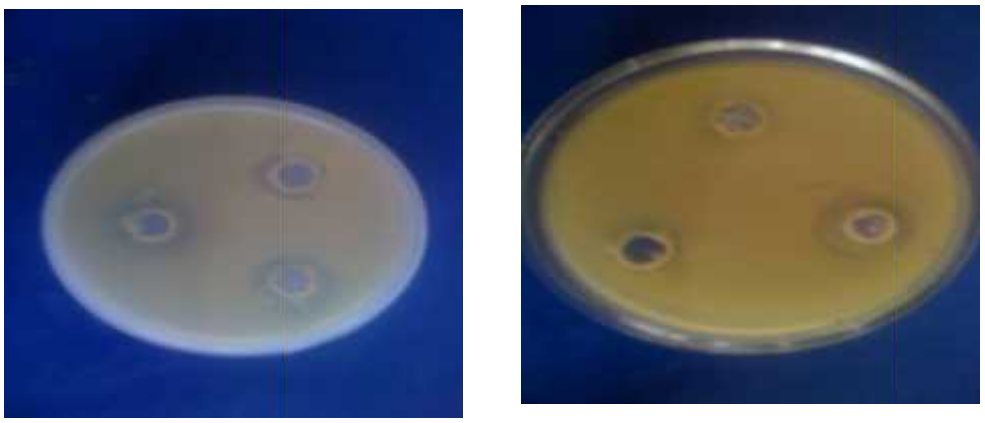

Fig. 2
Zone of Hydrolysis Produced by FSF

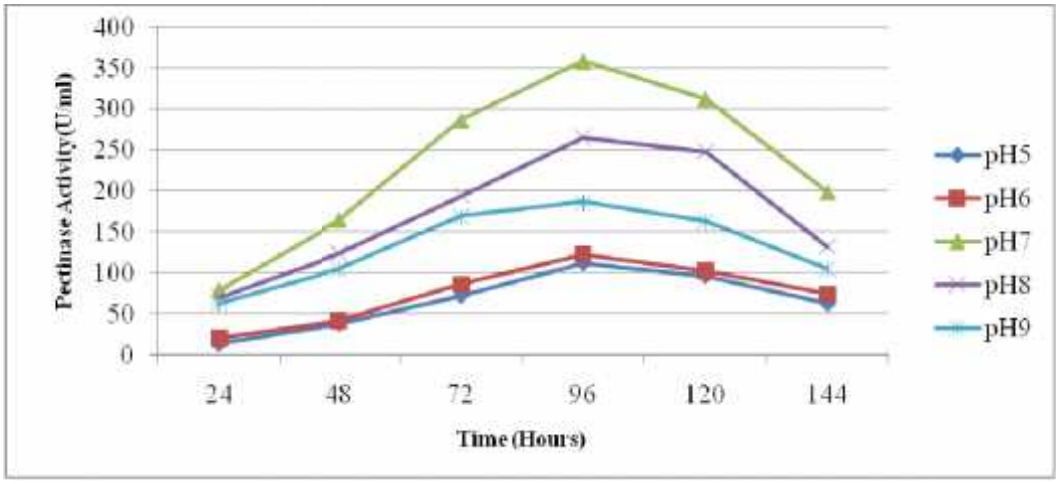

Fig. 3
Effect of Different pH on Pectinase Production

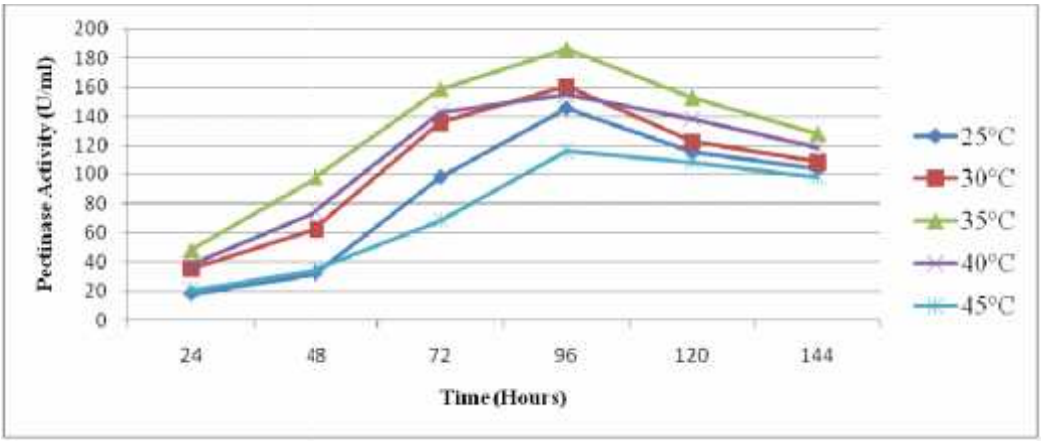

Fig. 4
Effect of Different Temperatures on Pectinase Production

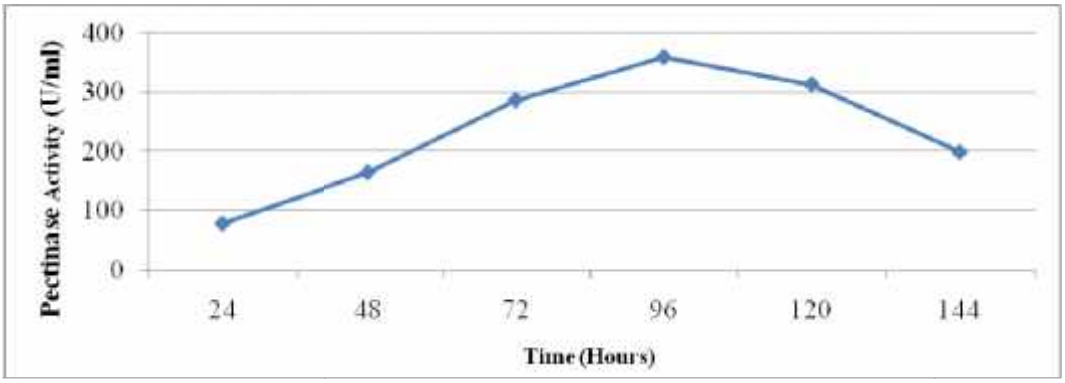

Fig. 5
Effect of Different Incubation Time on Pectinase Production

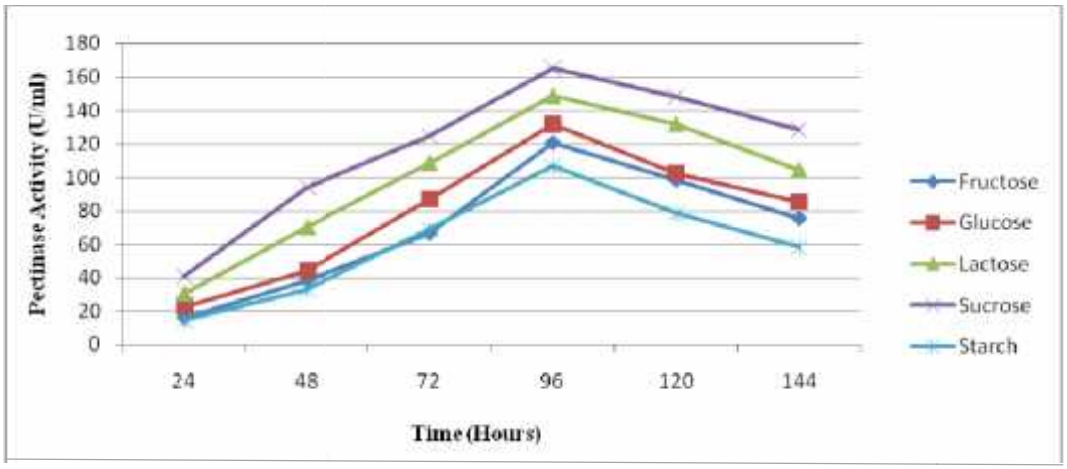

Fig. 6
Effect of Different Carbon Sources on Pectinase Production

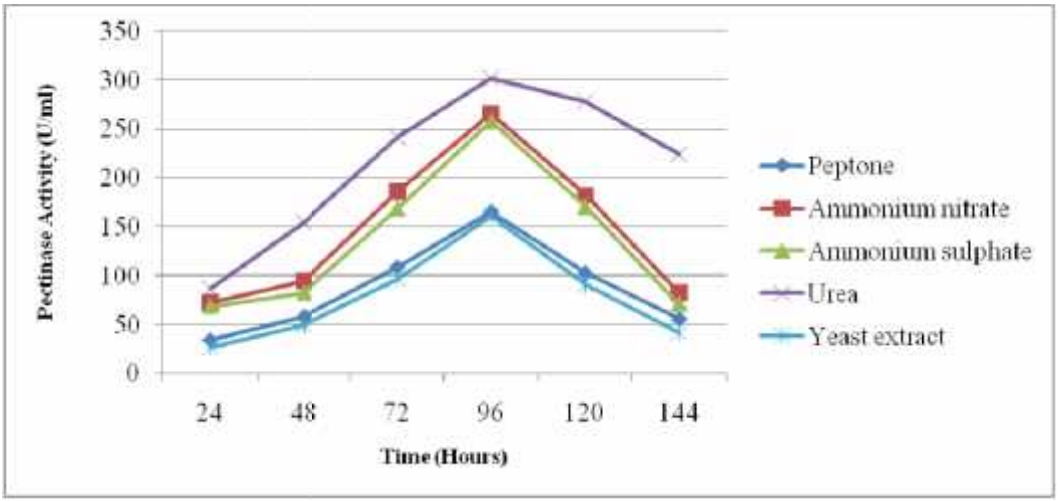

Fig 7
Effect of Different Nitrogen Sources on Pectinase Production

Ahmad *et al.*[14] found that fungal species responsible for the production of pectinase had maximum activity at 48 hours after which the activity gradually decreased. According to Khairnar *et al.*[10] maximum pectinase production was obtained in between 24 hours to 72 hours from *Aspergillus* sp. Results of Naderi *et al.*[15] demonstrated that the maximum exopectinase enzyme production was achieved on second day. Akinmusire[13] reported that *Aspergillus* sp. is the common organism associated with the spoilage of orange. Present results of maximum pectinase production by using sucrose as carbon source and urea as nitrogen source were found similar with the results of Ibrahim *et al.*[16] and Rajmane and Korekar[17] respectively. Pectinase production was enhanced by addition of nitrogen sources such as yeast extract, peptone and ammonium chloride but was inhibited by glycine, urea and ammonium sulphate[18].

CONCLUSION

Microbial pectinase can be considered as the most important enzyme for many industries. Cost effective technologies are needed for the production of enzymes and fungus is suitable for economical production of pectinases using spoiled fruits. Major parameters affecting the enzyme production were studied and optimal levels were identified. It is concluded from the findings that pectinase production was optimized by optimizing the process parameters and considerable amount of enzyme was produced by a newly isolated strain of *A. niger* FSF7 under laboratory conditions. This isolate can be identified by molecular methods to confirm its novelty and can be used for commercial production of pectinases with optimized process parameters.

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