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BIOCONVERSION OF ECO-WASTE TO PECTINASE ENZYME BY FUNGAL ISOLATES

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ABSTRACT:

This study investigated production of pectinase enzyme and its application in sewage water treatment. Fungal isolates namely *A. oryzae, A. niger, A. fumigatus, A. flavus* were screened for pectinase production by using liquid state fermentation. Of the 4 selected species *A. oryzae* showed high and effective pectinase production. The substrate used for producing A. oryzae was kiwi fruit waste. High pectinase production was observed at czapek-dox agar medium, pH 7.0 with fermentation period 3 days. The carbon source used for pectinase production in A. oryzae was lemon peel and nitrogen equivalent used for pectinase production in *A. oryzae* was sodium nitrate. The aim of this study is utilization of agro-wastes for cost effective production and to provide ecofriendly method for pectinase production in large scale.

KEYWORDS:

PECTINASE, AGRO WASTES, LIQUID STATE FERMENTATION, KIWI FRUIT WASTE.

INTRODUCTION

Enzymes are biological catalyst produced abundantly by saprophytic fungi. They are widely applied in commercial purpose for their production efficiency. Pectinase is a heterogeneous enzyme present widely in middle lamella of primary cell wall in plants. They present as major component of cell as calcium pectate and magnesium pectate. Pectinase are involved in phytopathogenic process caused by microorganisms. Pectinase are group of enzyme contains polygalacturonase, pectin lyase, pectate lyase and pectin esterase. They breakdown glycosidic bond of long chain galacturonic acid residues of pectic substances(Gregorio et al., 2002).Microorganisms like filamentous fungi are favorable for pectinase production through fermentation. Fungi can produce both intracellular as well as extracellular enzymes. All fungi are heterotrophic and rely on carbon compounds synthesized by other living organisms. A fungus produces variety of enzymes that are useful in breaking down of complex polysaccharide to simple sugars. Small molecules like mono disaccharides, fatty acids and amino acids can easily pass through but for breaking down large products like pectin, it secretes some enzymes (Chowdhury et al., 2017). It is reported that microbial pectinase plays important role in global and food industries with total account of about 25%. This enzyme is also known as exo or endo enzymes based on their action pattern. Pectin can be extracted from fruit using acid such as hydrochloric acid or hydro nitric acid or chelating agents such as EDTA, ammonium oxalate or sodium hexametaphosphate. Pectinolytic enzymes are classified according to their way of attack on the galacturonase part of the pectin molecule. Pectins are heteropolysaccharides that consist of α 1-4 linked D-galacturonic acid residue and it has rhamnose on the main chain followed by arabinose, galactose, and xylose on the side chain (Kapoor et al., 2000) They can be distinguished between pectin de-polymers, which split the glycosidic linkage between galacturonase (methyl ester) residues. Endo PG'S randomly attack the 1,4 alpha glycosidic linkage of the polysaccharide chain producing a number of galacturonic acid oligomers, which exo PG'S specifically hydrolyses at the reducing end of polygalacturonase acid

Pectinase enzyme has great application in fruit juice clarification, paper making, textile industry, treatment of waste water and plays important role in maturity by catalyzing the extension of cell wall and softening of tissues. During ripening process pectinase enzyme break pectin, fruit becomes soft due to separation of cell to cell by breaking of middle lamella. The undesirable problem of spoilage, decay of processed food can be solved by treatment of efficient pectinase which can degrade homogalacturonan and rhamnogalacturonic acid of pectin to convert it into sugar and other useful compound. Pectinase is extensively used in food processing industry, scouring of cotton, degumming of plant fiber, bleaching of paper, tea and coffee fermentation (Thangaratham & Manimegalai 2014). Pectinase also play a vital role in industrial biotechnology in manufacturing process of enzymes, creation of biomaterials and alternate energy resources. It is one of the best alternatives for inorganic

chemicals. Microbial pectinase used widely due to high productivity rate and cost productivity. The most commonly used genera among them include *Aspergillus*, *Rhizopus*, *Trichoderma*, *Penicillium* and *Fusarium* (Ahmed *et al*., 2013). Production of enzyme with low-cost production technique is still a challenging area. A significant cost reduction can be attained with help of high yielding strains, optimal fermentation conditions and cheap raw material for growing microorganisms. Fruits like lemon, orange (Sibel *et al*., 2017), mosambi, pomegranate, kiwi, papaya, sapota, grape (Martin *et al*., 2004) are important constituents in pectinase production. Carbon source like orange peel, mosambi peel, lemon peel and rice bran have been used. Enzyme production conditions were optimized according to selective factors such as media, pH, time, temperature, and incubation period, concentration of carbon and nitrogen source. These factors should be optimized to get high quality and high yield of enzyme.

In view of current importance of fungi in enzyme industry this work is done to isolate pectinase from fungi. In this work agricultural waste and soil samples are used as the major substrate. To my awareness no work has been reported on pectinase enzyme production from kiwi fruit waste. Utilization of agricultural wastes save large amount of production cost and also creates non-polluting environment; these two aspects is the pillar of this research. Hence the present investigation was made in an attempt to deliver a basic understanding about the pectinase enzyme, factors involving production and its applications in current scenario which is applicable to the day-to-day needs of all the living organisms by an ecofriendly approach.

MATERIALS AND METHODSSAMPLE COLLECTION

Fruit wastes like Kiwi, mosambi, orange, lemon, pomegranate, sapota and papaya which are pectin abundant source. They were collected from local fruit shops and its juice was extracted, filtered and sorted in test tubes each of 25ml.

Orange waste was collected from nearby fruit shop. They were mixed with soil in a pot. Water was sprinkled in low quantity. After 4 weeks, the soil was well degraded with fruit sample, it is then used as an inoculums.

ISOLATION AND IDENTIFICATION OF PECTINOLYTIC FUNGI

Serial dilution of inoculums sample was done. 1ml of inoculums sample with 9 ml of distilled water was added in test tube. This dilution process was done for 3 times. At the concentration of. It 10^3 was inoculated in media plate containing Czapek dox agar medium. The solid medium contained (1000ml): 2.0 g Sodium nitrate, 1.0 g Dipotassium phosphate, 0.50 g Magnesium sulfate, 0.50 g Potassium chloride, 0.01 g Ferrous sulfate, 15.0 g Pectin, 15.0 g Agar was added to the medium. The pH value was adjusted to 7.3 before autoclaving at 121^0C for 30 minutes. Inoculated plates were at 30^0C with help of hot air incubator for 5 days. Pure cultures were obtained by repeated sub culturing on Czapek-dox agar plates. The cultured microorganisms are identified by slide culture technique. PDA medium was prepared and sterilized in autoclave. The solidified media was made into sliced squares using sterile knives and scalpel and placed on glass slide. A microorganism was inoculated on it and culture at 30^0C for 3-5 days. After the growth of organism, it was flooded with lacto phenol cotton blue and observed under binocular microscope was used and the photographs were taken fungal isolates were identified with the help of microbiology department in PSG college of arts and science, Coimbatore and with the help of the book described by (Kenneth *et al*., 1965; Domsch *et al*.,1980).

SCREENING OF FUNGAL ISOLATES FOR PECTINOLYTIC ACTIVITY

The isolates were cultivated on modified Czapek-dox agar with 1.5% pectin as substrate with 2% fungal suspension was inoculated for 5 days at 30^0C and screened for pectinolytic activity. It was detected by flooding culture plates with freshly prepared Potassium iodide- iodine solution (Hankin *et al*., 1971). This solution produces characteristic red to brown color. After 20 minutes translucent halo region appears where pectin is degraded. It forms zone around the organism. Based on the zone formation, pectinase producing isolates were identified.

PREPERATION OF INOCULUMS

Czapek-dox agar medium was taken in Erlenmeyer flask of 250ml capacity was inoculated with fungal spores from mother culture plates. Then it was incubated at 30^0C for 5 days under stationary condition. Then 9% sodium chloride solution was added and shaked to obtain the fungal spores. It was stored in small aliquots at 4^0C for further use.

OPTIMIZATION STUDIES

The operating variables for fermentation were optimized which include media (Czapek- dox agar and sabouraud agar media) pH range 4.5, 5, 5.5, 6.5, 7, 7.5 (Martin *et al*., 2004), (Ahmed *et al*., 2015; Nithinkumar *et al*., 2010; Uroosa *et al*., 2018), temperature (30^0C) (Roheena *et al*.,2018; George *et al*., 2017), carbon source (lemon peel, orange peel, mosambi peel and rice bran), nitrogen source (sodium nitrate-0.1% ,0.2%, 0.3%, Pepetone-0.5%, 1%, 1.5%) and incubation period (3^{rd} day and 5^{th} day) for effective pectinase production. All the experiments were carried out in 100ml conical flask. After inoculation all the flasks were kept in a mechanical shaker at 200 rpm for 3-5 days using duplicates. The optimized culture conditions were used for in-vitro enzyme production.

LIQUID STATE FERMENTATION

Fermentation of fungal isolates was carried out in 100 ml of Czapek-dox agar and Sabouraud agar broth were taken in 250ml conical flask. From the observations obtained through the optimization studies, various parameters were adjusted for each organism. For adjusting media to alkaline nature (pH 7.0>), 5g of sodium hydroxide pellets were dissolved in 100ml of distilled water (freshly prepared) and it was used. For adjusting the media to acidic nature (pH 7.0<), 355 hydrochloric acids (35ml concentrated

Hydrochloric acid with 65ml of distilled water) was used. Carbon source and nitrogen source equivalents were added and kept in autoclave for sterilization at 120^{0}C for 20 minutes, 2% of fungal spore suspension from each sample was taken and poured into the culture flask. The culture flask was kept in mechanical shaker at 150 rpm for 3-6 days.

CRUDE ENZYME PRODUCTION

The fermentation flask was maintained at 30^{0}C for 5 days. Later the liquid was filtered through what man No. 1 filter paper and their clear extract from fermentation (culture filtrate) and the fungal residues were thrown off. It is maintained in glass aliquots and was named as crude enzyme. It was used for further enzyme assay. It was used for further enzyme assay. The crude enzyme was stored at 4^{0}C for further enzyme assay.

PARTIAL PURIFICATION OF ENZYME

The fermentation broth was filtered using what man No.1 filter paper and centrifuged at 10,000 rpm for 20 minutes at 4^{0}C to remove the unwanted particles of the organism. It was then mixed with three volumes of ice-cold acetone and allowed to stand for 15 minutes. The entire content was centrifuged at 4000 rpm at 4^{0}C for 20 minutes. Then the precipitate was dissolved in sodium acetate buffer (0.465 g sodium acetate, 50 ml Distilled water, pH-5.5) and stored at 4^{0}C for further use (Yarkanni *et al*., 2002).

DETERMINATION OF PROTEIN PECTINASE ACTIVITY

The protein content of the crude enzyme was estimated by the Folinciocalteau method (Lowry *et al*.,1951) using reagent blank.

CRUDE ENZYME USED FOR SEWAGE WATER TREATMENT

The obtained crude enzyme was treated with sewage water collected from sewage treatment plant, PSG college of Arts & Science, Coimbatore. Initial pH and TDS (Total Dissolved solvents) value of the water was noted. Different enzyme concentration (3%, 5%, 10%) were taken and added to sewage water. The reaction of enzyme was determined. It was then kept at mechanical shaker for 150rpm for 3 hours. Then the TDS value of the enzyme water mixture observed. The enzyme activity was calculated on comparison with initial and final values.

STATISTICAL ANALYSIS

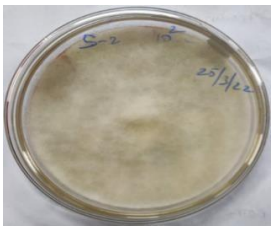
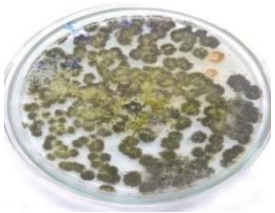
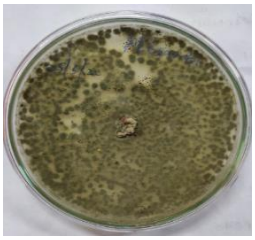
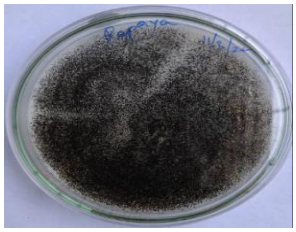
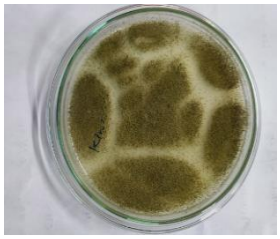
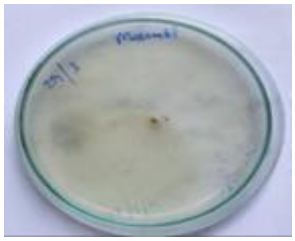
Results were given as the mean standard deviation of two independent determinations. All the statistical analyses were performed with two-way analysis of variants (ANOVA). Using Agrees stat V3.1. Difference was considered to be significant at $p<0$.

RESULTS AND DISSCUSION

Pectinolytic enzymes are of significant importance in the current biotechnological era with their embracing applications in fruit extraction and clarification, food, leather and textile industries. Pectinase are commonly used in many industrial sectors and the demand for more stable, highly active and specific enzymes are growing rapidly.

Serial dilutions were made for isolation of pectin rich fungal isolates it was known that 8 fungal species belonging to 4 different genera were isolated namely *Aspergillus oryzae, A. Niger, A. fumigatus, A. flavus, Fusarium oxysporum, F. solani, Rhizopus oryzae, Penicillium citrinum.* Out of 8 fungal species grown on screening medium, only 4 fungal isolates *A. oryzae, A. Niger, A. fumigatus, A. flavus* showed clear zones and found as effective pectinase enzyme producing organism. The fungal isolates were then purified by repeated sub culturing using Czapek-dox agar plates and stock culture was maintained.

PLATE 1: FUNGAL ISOLATES:

			
FUSARIUM SOLANI	***ASPERGILLUS FUMIGATUS***	***ASPERGILLUS FLAVUS***	***ASPERGILLUS NIGER***
			
PENICILLIUM CITRINUM	***ASPERGILLUS ORYZAE***	***RHIZOPUS ORYZAE***	***FUSARIUM OXYSPORUM***

OPTIMIZATION OF CULTURE MEDIA

It is essential to optimize the fermentation medium for effective pectinase production. Optimization studies on media, pH, carbon source, nitrogen source and incubation period were observed. From the optimization studies of media Czapek-dox agar (Anuradha *et al*., 2010) wasfound to be effective in pectinase production by *Aspergillus oryzae* and *A. Niger*. Sabouraud agar medium (Denis et al., 2005) was found to be effective in production by *A. fumigatus* and *A. flavus*. Czapek-dox agar medium showed maximum pectinase production in *Aspergillus* sp (Anuradha *et al*., 2010).

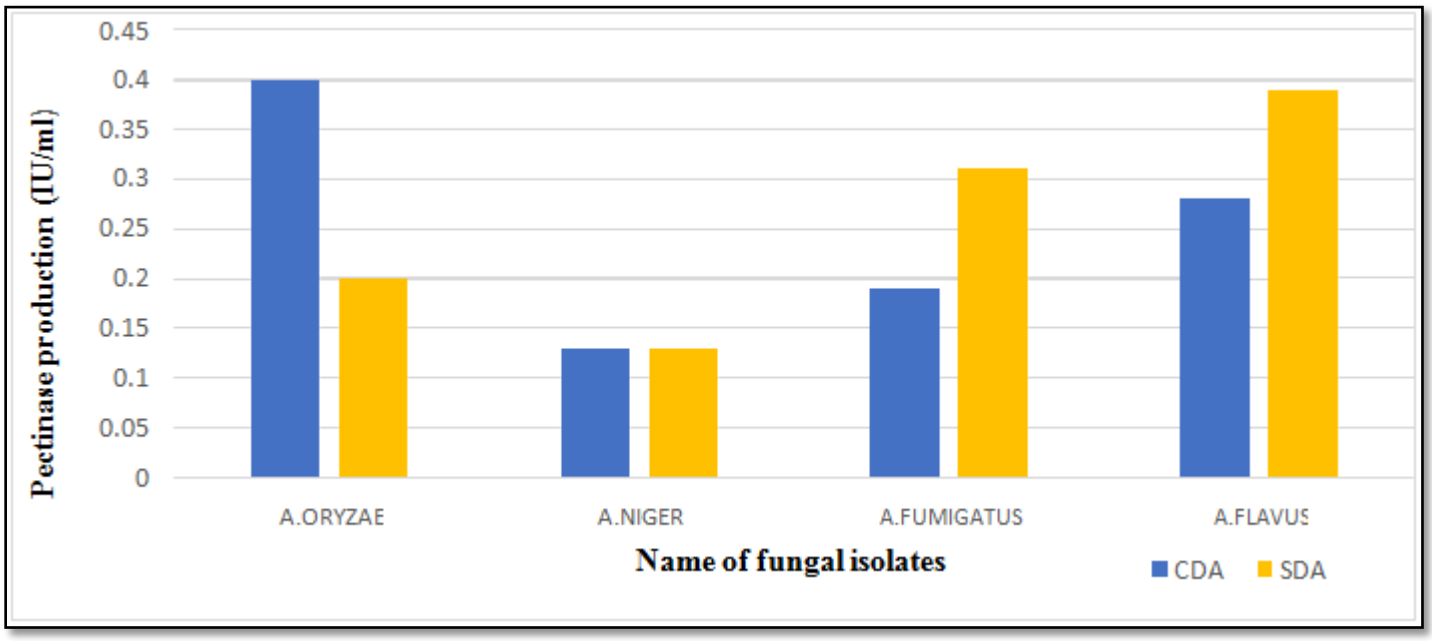


FIGURE 1. OPTIMIZATION OF CULTURE MEDIA FOR MAXIMUM ENZYME PRODUCTION.

OPTIMIZATION OF pH

Figure 2 depicts the effect of different pH on the production of pectinase by *Aspergillus* sp. The pH variations in Czapek-dox agar medium were carried out at the range of 6.5,7.0 and7.5 and the pH variations in sabouraud agar medium were carried out at the range of 4.5,5.0and5.5. The Maximum production of pectinase was obtained at pH7.0 in *Aspergillus oryzae* when compared to other fungal isolates. The pH of the medium also were found to be limiting the growth of culture or extent influence upon catalytic activity of enzyme (Johnson *et al.*, 2012)

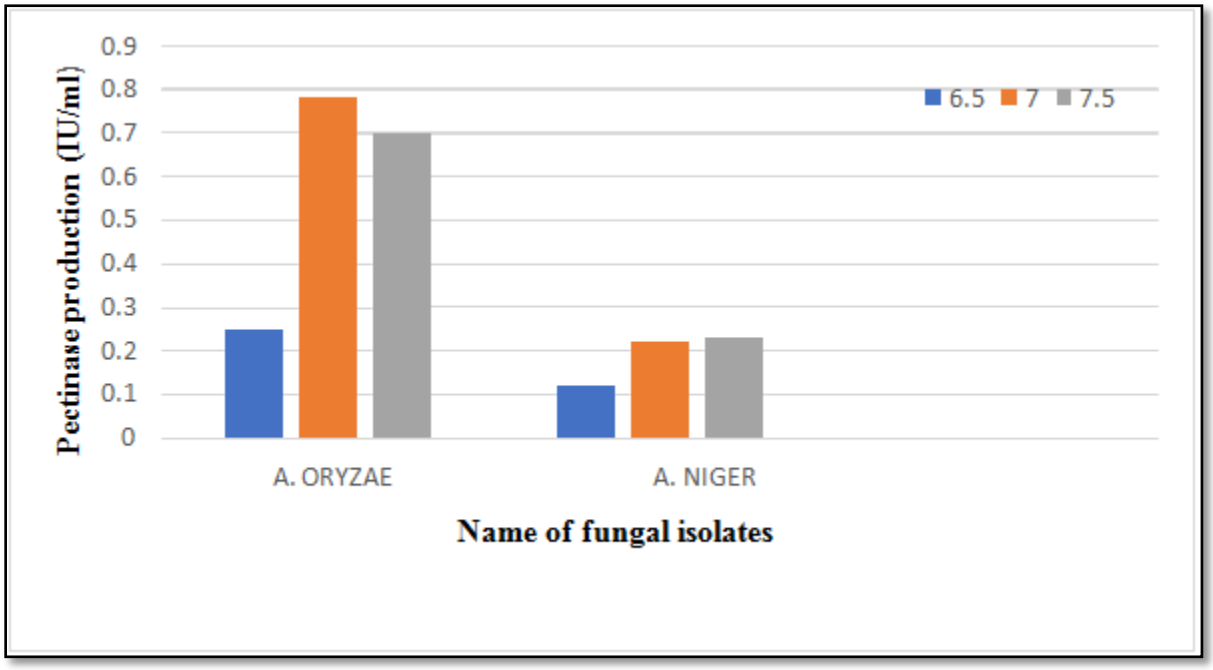


FIGURE 2 OPTIMIZATION OF PH IN CZAPEK-DOX AGAR MEDIA FOR EFFECTIVE PECTINASE PRODUCTION

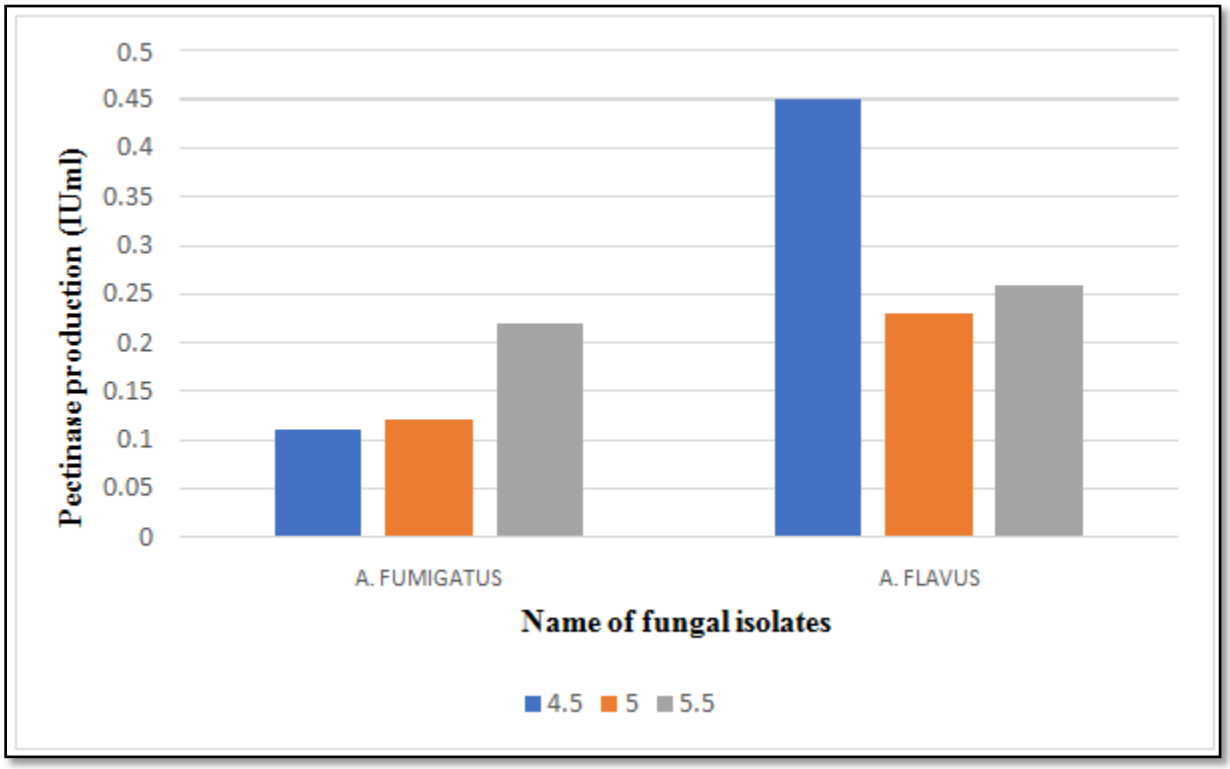


FIGURE 3 OPTIMIZATION OF PH IN SABOURAUD AGAR MEDIUM FOR EFFECTIVE PECTINASE PRODUCTION.

OPTIMIZATION OF CARBON SOURCE

Considering the effective pectinase production required by large industries, using agricultural wastes as carbon source will be eco-friendly and cost effective. Carbon source like Mosambi peel, orange peel, wheat bran (Dange & Harke 2018; Roheena *et al*., 2018), rice bran, citrus waste (Davanso *et al*., 2019) was used. An experiment was carried out to find the effect of various agro wastes as a carbon source namely lemon peel, orange peel, mosambi peel and rice bran in suitable media for enriched pectinase production. Lemon peel proved to be more effective in pectinase production in *Aspergillus oryzae* which is first in report for effective pectinase production, followed by *Aspergillus fumigatus*. Least production was observed in *Aspergillus flavus*.

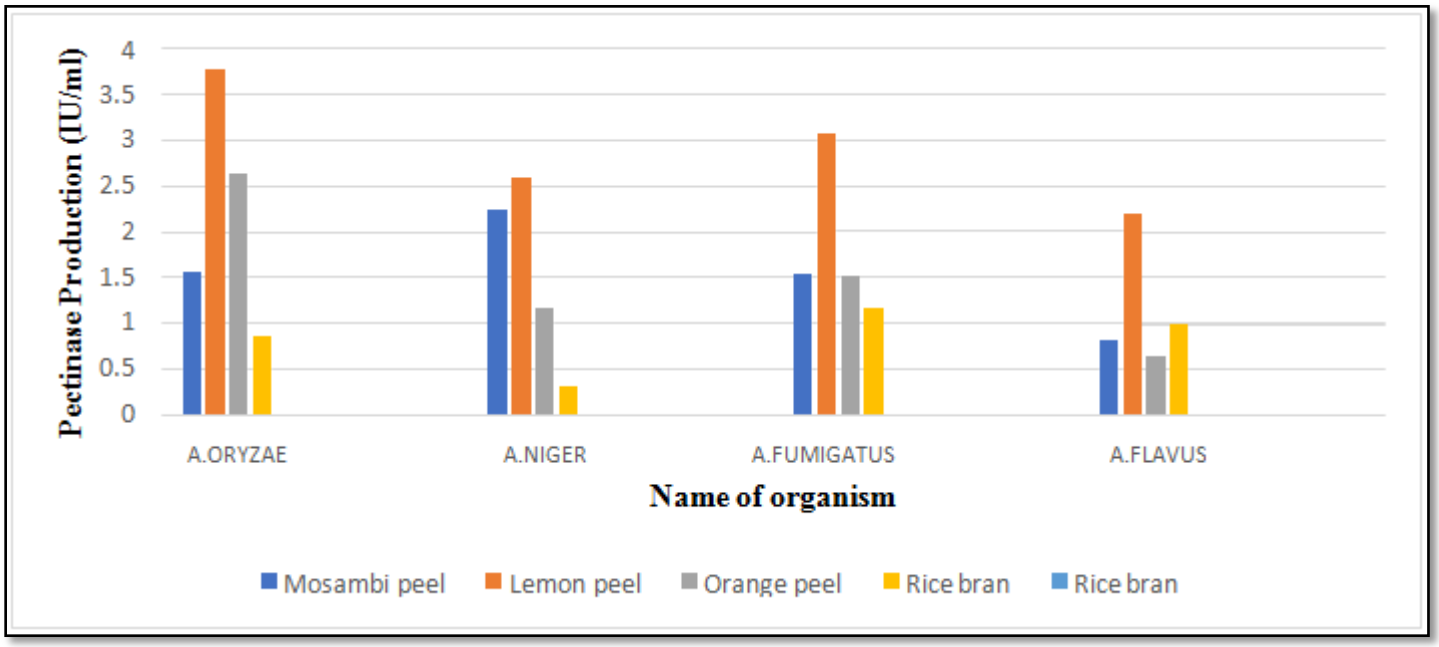


FIGURE 4 OPTIMIZATION OF CARBON SOURCE FOR EFFECTIVE PECTINASE PRODUCTION

OPTIMIZATION OF NITROGEN EQUIVALENTS

The effect of various nitrogen equivalents in pectinase production with different fungal isolates was studied. In selected media, nitrogen source was replaced with equivalent amounts of sodium nitrate at different concentrations (0.1%, 0.2%, 0.3%) in czapek-dox agar medium and peptone at different concentrations (1%, 2%, 3%) in sabouraud agar medium. It was determined that *Aspergillus oryzae* in sodium nitrate at0.1% concentration shows better results, followed by 2% concentration of peptone in *Aspergillus fumigatus*.

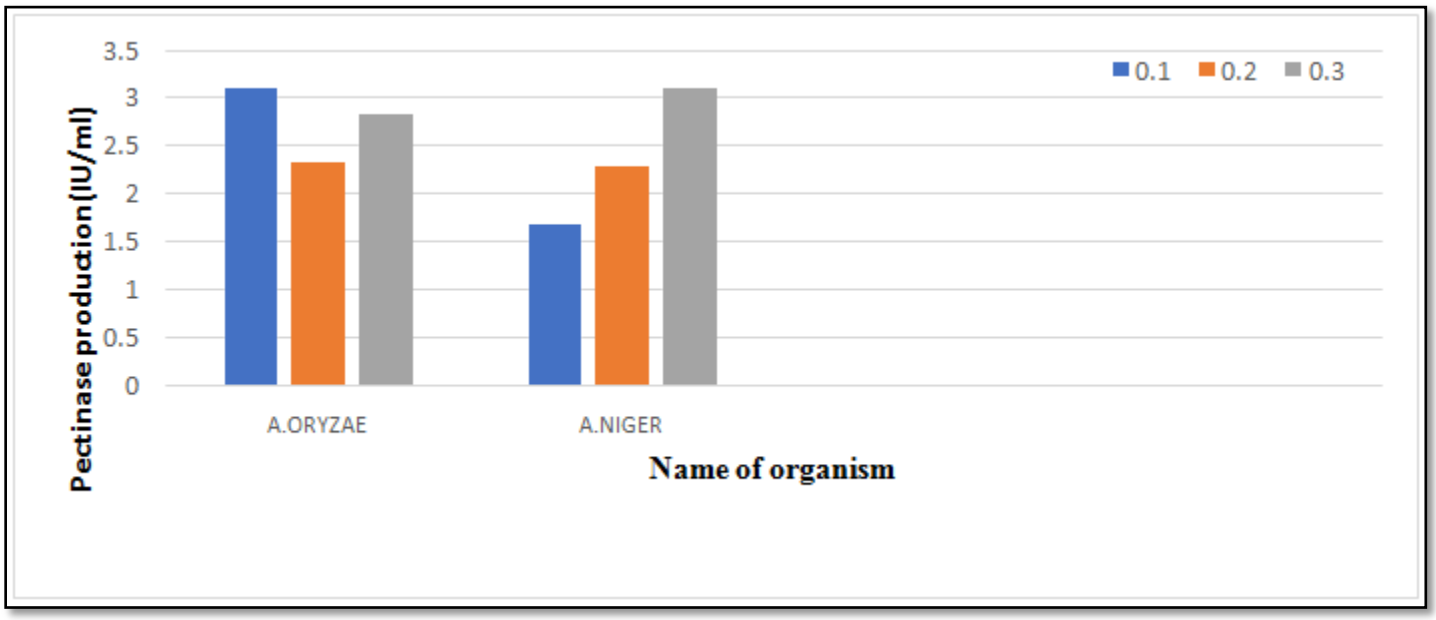


FIGURE 5 OPTIMIZATION OF NITROGEN EQUIVALENTS IN CZAPEK-DOX AGAR MEDIUM FOR EFFECTIVE PECTINASE PRODUCTION

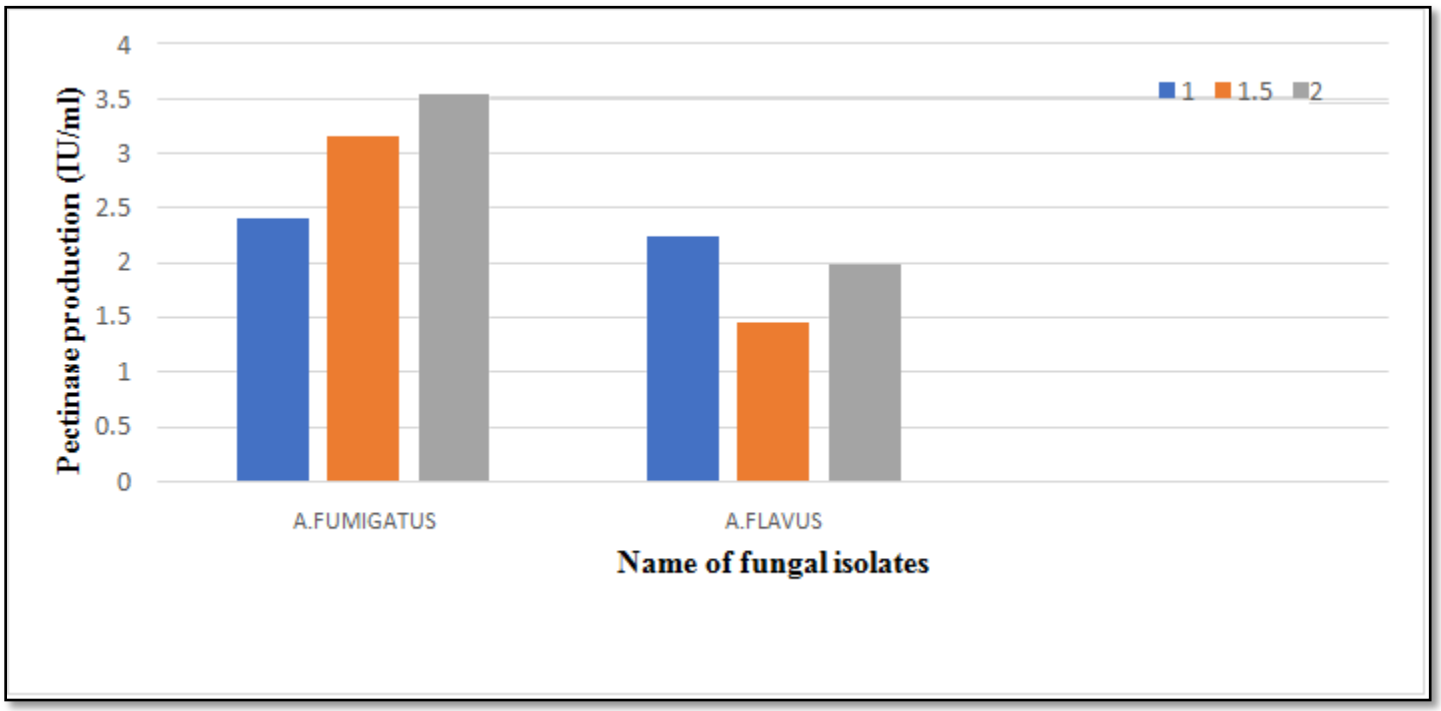


Figure 6 Optimization of nitrogen equivalents in sabouraud agar medium for effectivepectinase production.

OPTIMIZATION OF FERMENTATION PERIOD

Fermentation period is one of the major important factors affecting the process of pectinase production. A study was carried out for determining the fermentation period in 3rd and 5th day. It was known that *Aspergillus oryzae* showed maximum pectinase production in 3rd day and 5th day.

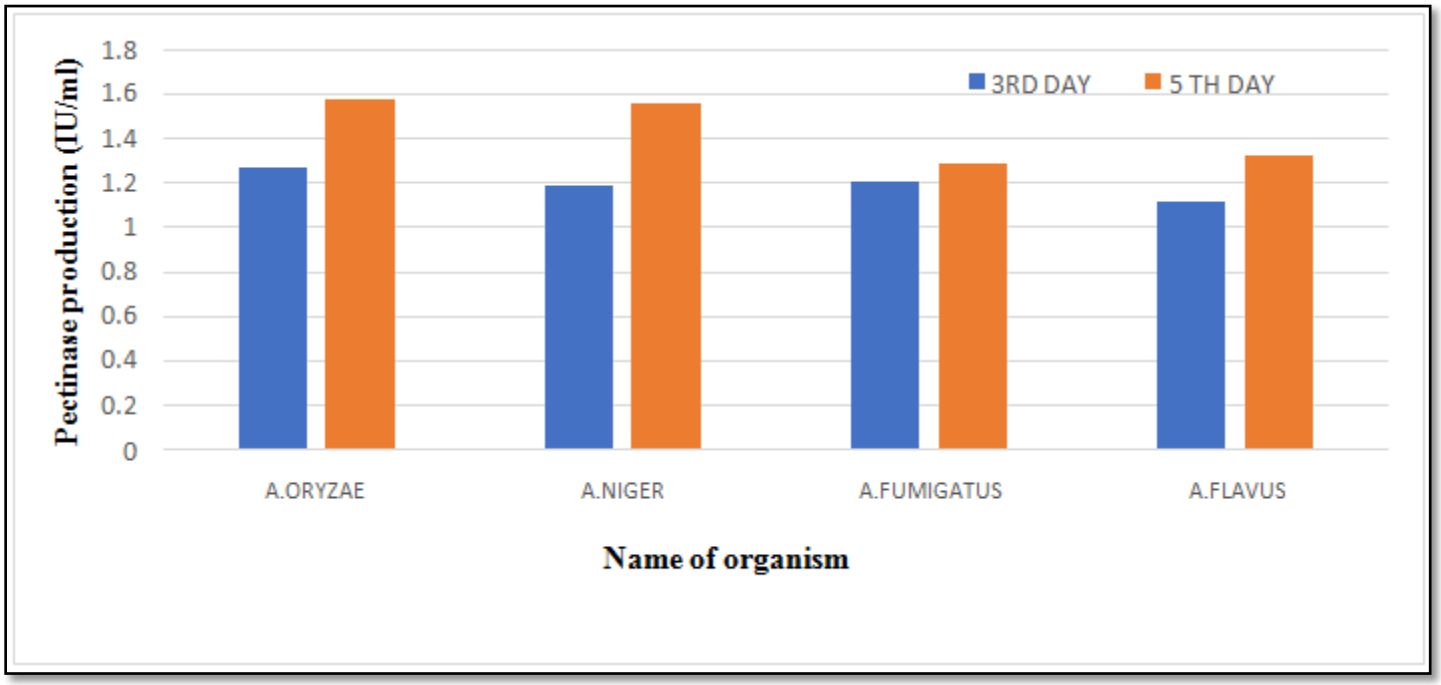


FIGURE 7 OPTIMIZATION OF FERMENTATION PERIOD FOR EFFECTIVE PECTINASE PRODUCTION.

PARTIAL PURIFICATION OF ENZYME AND ITS APPLICATION

For effective pectinase production, liquid state fermentation was used (15, 21). The optimized enzyme was partially purified following the method of yarkanni et.al. The partially purified enzyme was used in sewage water treatment. Various concentrations (3%, 5%, and 10%) were used to check the activity of enzyme against impurities in sewage water. pH and TDS were parameters used. The average pH of the treated water must be 7.5-9.0 after treating the partially purified enzyme it was found that sewage water treated with enzyme at 5% concentration showed pH 8.0 which indicates the enzyme acts on sewage water to clear the impurities. The average TDS of the treated water should be below 1780ppm. After treating the partially purified enzyme it was found that enzyme at 5% concentration shows 1758 ppm which indicates the enzyme acts on sewage water to clear the impurities.

ESTIMATION OF PROTEIN

Estimation of protein in crude enzyme was performed (Lowry *et al.*, 1951). The crude enzyme of Aspergillus oryzae was used for estimation. Standard values were obtained. The crude enzyme was taken in 0.1 and 0.2 concentrations for estimation. It was observed that higher the concentration of enzyme higher the protein content, which was directly proportional.

CONCLUSION

Pectinase was one of the first enzymes to be employed commercially in various sectors dating back to 1930. Microbial pectinase account for 25% of all food enzyme sales worldwide. As a result, pectinase are leading enzyme in commercial sector at present. Instead of using high yielding strains, the cost of production can be reduced by using ideal fermentation conditions and inexpensive raw materials as carbon source and substrate are proved. As a result, the production of pectinase from agro-wastes appears to be promising and requires extensive studies. In addition, effective sewage waste water treatment from kiwi fruit proves its capability in this domain. Further research into the molecular and nano aspects of pectinase, as well as the development of mutants of improved pectinase generating strains, can be done in the future.

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