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BIOPROCESS OPTIMIZATION FOR PECTINASE PRODUCTION IN A SUBMERGED CULTIVATION SYSTEM USING VARIOUS FILAMENTOUS FUNGI.

Ghulam Sughra Mangrio[1], Sippi Pirah[2], Nadia Mangrio[3], Farooque Ali Bughio[4], Asma Asghar[5], Bharat kumar[1], Altaf Ahmed Simair*[6]

[1]Department of Biotechnology, Sindh Agricultural University, Tandojam 70060, Sindh
[2]College of Chemistry, Chemical Engineering and Biotechnology, Donghua University, Shanghai, 201620, China.
[3]Sugarcane Research Institute, Agriculture Research Centre, Tandojam, 70060, Sindh
4Institute of Plant Sciences, University of Sindh, Jamshoro, Pakistan
[5]Institute of Molecular Biology and Biotechnology, Bahauddin Zakariya University, Multan, 60800, Pakistan
[6]Department of Botany, Government College University, Hyderabad, 71000, Sindh
*Corresponding author: altafsimair@gcuh.edu.pk

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ABSTRACT

The pectinase enzymes attack in several ways; the pectin is widely used in processing fruit juices, extracting vegetable oil, processing alcoholic drinks, and various uses in food industries. The present study was carried out to utilize an agro-industrial by-product (date syrup) as a carbon source for the growth of different fungi and the production of the pectinase enzyme. Four types of fungi (*Penicillium lilacinum*, *Mucor geophillus*, *Aspergillus niger* and *Aspergillus fumigatus*) were used in this study. The maximum production rate of pectinase was observed by *A. niger* when grown on a mineral medium incorporated with 2.5% and 5% date syrup compared to other fungi used. The highest rate of pectinase, 5.68 units/ml, was produced after 72 hours when *A. niger* was grown on 5% date syrup.

Key words: Biosynthesis, Pectinase, Agro industrial waste, Filamentous fungi, Date syrup *Aspergillus fumigatus*, *Aspergillus niger*, *Mucor geophillus*, submerged fermentation.

INTRODUCTION

Pectinases are industrially essential enzymes in the processing of agricultural products. They are used as extraction, clarification, and maceration aids for fruits and vegetables by breaking down pectin (complex heteropolysaccharide) (Haile & Ayele, 2022; Kashyap *et al*., 2001). The microorganisms used in fermentation foods and beverages and pectinase enzyme include (e.g. *Aspergillus fumigatus*, *Aspergillus niger*, *Aspergillus oryzae*, *Aspergillus sojae*, *Mucor geophillus*, *Penicillium lilacinum) (Haile & Ayele, 2022; Xiang et al., 2019).* Pectin is primarily found in higher plants' middle lamella and primary cell walls consisting of α–1–4 glycosidically linked galacturonic acid residues and α–1–2 linked rhamnose (Seymour & Knox, 2002). Due to the complex structure of pectin, its modification or complete breakdown requires many different enzymes. Pectolytic enzyme complex contains depolymerizing and demethylating enzymes. Depolymerizing enzymes are polygalacturonase which clears α–1–4–glycosidic bonds between two galacturonic acid residues, while pectin lyase catalyzes a β–elimination reaction between two methylated residues (Stutzenberger, 1992; Sughra *et al*., 2013). Desterifying enzyme Pectin- esterase catalyzes the demethylation of methylated pectin, producing methanol and pectin (Mata-Gómez *et al*., 2015; Stutzenberger, 1992). Commercial enzyme preparations used in food processing are almost exclusively derived from *Aspergillus* sp. and are traditionally a mixture of polygalacturonases, pectate lyase, and pectin esterase (El Enshasy *et al*., 2018; Sin *et al*., 2002). Pectin degrading enzymes have been extensively used to improve the stability of fruit and vegetable nectar and clarify fruit juices and wines (Bailey & Pessa, 1990; Fogarty & Kelly, 2012; Lang & Dörnenburg, 2000; Priest, 1985; Sin *et al*., 2002). Currently, they are widely used in industry for setting natural fibers and extracting oils from vegetables and citrus peels (Ros *et al*., 1993; Sreekantiah *et al*., 1971; Suri *et al*., 2022). The enzyme preparations used in the food industry are of fungal origin because fungi are potent producers of pectic enzyme and the optimal pH of many fruit juices ranges from pH 3–5.5 (Baracat *et al*., 1989; Siddiqui *et al*., 2012). Such preparation are not suited for the production of vegetables purées or

other preparations in which pH values are close to neutral (Fonseca & Said, 1995). Furthermore due to the relatively low temperature stability of the fungal enzyme preparation maceration needs to be carried out at temperature not exceeding 45°C, necessitating the incorporation of a pasteurization step to limit the growth of mesophilic microorganisms (Hyde *et al*., 2019; Ueda *et al*., 1982).

Homogenous polygalacturonase preparations are preferred for separating whole cells in manufacturing baby food, vitamins, color, and aroma (Chesson and Codner, 1978). Novel applications for producing polygalacturonases as functional food components can be investigated. Oligogalacturonides are sugars that possess properties that are beneficial to the health of consumers and are termed prebiotics (Silley, 1986). Prebiotics combines the effects of endogenous intestinal bacterial flora and the new probiotic organisms in a minimally processed plant food. It represents a perspective for one of the future trends of food biotechnology in the plant product market (Lang & Dörnenburg, 2000). As the beneficial functional properties of oligo galactosidase become more widely understood, purified polygalacturonase preparation will be necessary for their largescale production in good yields.

New enzymes for use in commercial applications with desirable biochemical and physic-chemical characteristics and a low cost of production have been the focus of much research. The application of agro-industrial waste as a carbon source in the enzyme production process reduces production costs and helps solve problems with their disposal (Crittenden & Playne, 1996). In this study, different concentration (2.5% and 5%) of Date syrups (by-product Date palm industry) was used as a carbon source for the growth of fungi and production of pectinase through the submerged fermentation process.

MATERIALS AND METHODS

Microorganisms: *Mucor geophilous* and *Penicillium lilacinum* were obtained from the Research Laboratory Department of Chemistry, Shah Abdul Latif University Khairpur, whereas *Aspergillus niger* and *Aspergillus fumigatus* were isolated and identified in this laboratory. The stock cultures of *A. niger, A. fumigatus, M. geophilous, and P. lilacinum were maintained* on agar slants containing (g/L) glucose 20, peptone 10, agar 20, and distilled water. The ingredients were thoroughly mixed and kept in culture tubes sterilized in an autoclave at 15 psi for 20 minutes. The sterilized slants were inoculated with *A. niger, A. fumigatus, M. geophillus,* and *P. lilacinum* and incubated at 37° C for five days to obtain luxuriant growth.

Inoculums: A spore suspension was prepared by adding sterilized water to the stock culture to get $50x10^6$ spores/ml

Mineral medium: The mineral medium for the growth of fungi and production of enzyme was used as reported by (Bhatti *et al*., 2004) with slight modification, which contains the following reagents per liter of KH_2PO_4 1.0 g, $FeSO_47H_2O$ 6.32 mg, $MgSO_4.7H_2O$ 0.25 g, $ZnSO_4.7H_2O$ 1.1mg, $MnCl_2.2H_2O$ 3.54 mg, $CaCl_2$ $2H_2O$ 46.7 mg, and NH_4NO_3 2.4 g. The pH of the medium was adjusted at 6.5

Fermentation medium: 50ml of mineral medium supplemented with 2.5 and 5% molasses as carbon source was taken in 250 ml flasks. The pH of the medium was adjusted to 6.5. The media were autoclaved within a foil-capped Erlenmeyer flask for 15 min at 121°C, 15 psi. The flasks were inoculated with 1.0 ml of inoculums containing $50x10^6$ spores/ml and incubated at 31± 2ºC in a shaking incubator at 90rpm. The culture broth was filtered from mycelium after 24 hours of incubation through Whatman No.1 filter paper. The recovered mycelium was dried at 80ºC and weighed.

2.4 Determination of Reducing Sugar: Reducing sugar content of broth was determined according to (Miller, 1959) Dinitrosalicylic acid (DNS) method.

Determination of Total sugar: Total Sugar content of broth was determined according to the method reported by (Montogomery, 1961).

Determination of Protein: The protein content in the culture broth was determined by the method (Kashyap *et al*., 1980) using bovine serum albumin as a standard.

Determination of pectinase Activity: The enzyme activity was determined by the DNS method (Miller, 1959). 1 ml of culture broth was added to 1 ml of 1% soluble citrus pectin solution (Sodium citrate buffer pH 5) and incubated for 15 minutes. The reaction was stopped by adding 2.0 ml of DNS (3, 5-dinitrosalicylic acid) reagent. The reaction mixture was heated in a boiling water bath for 5 minutes. After cooling, the absorbance of the reaction color was read at 540 nm.

One unit of pectinase activity is defined as one micromole of reducing sugar released per milliliter under the assay condition.

RESULTS AND DISCUSSION

In this study, four fungal species (*Aspergillus niger*, *Aspergillus fumigatus*, *Penicillium lilacinum,* and *Mucor geophillus*) were selected and grown in media containing molasses for the production of pectinase. The pectinase production capacity by *P. lilacinum* was observed when grown in a culture medium supplemented with 2.5% and 5% date syrup as a carbon source. The maximum production of pectinase [5.16 U/ml] was obtained at 72 hours and then decreased with the increased time. The concentration of total sugars, reducing sugars, and protein was decreased with the increase in fermentation time. Whereas pH values of culture medium initially declined and then increased throughout fermentation. The capacity of a

microorganism to produce extracellular enzymes is greatly influenced with different factors like carbon and nitrogen sources, temperature and pH (El-Refai *et al*., 1984).

Fig-01-05 shows the results of Pectinase production when A. *fumigatus* (*A. niger* + *A. fumigatus*), *A. niger*, *M. geophillus* and *P. lilacinum* grown on a culture medium supplemented with 2.5% date sugar as a carbon source. The date syrup is a liquid by-product of date palm industry containing 75% carbohydrates w/w small amount of fats and proteins along with other micro and macro elements (Al-Farsi *et al*., 2007; Al-Hooti *et al*., 2002). The maximum production of Pectinase 3.82 U/ml by *A. fumigatus* was achieved at 72 hours and then decreased with the increase of the time period (Fig-11).

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Fig-02 shows the results of pectinase production by, *A. niger*, mixed culture of *A. niger* + *A. fumigatus* and *M. geophillus* as 3.47 U/ml, 4.67 U/ml and 3. 9 U/ ml, respectively at 72 hours. Fig-05 shows the results of Pectinase production when *P. lilacinum* was grown on a culture medium supplemented with date sugar at 2.5 % at 30 ± 2 ºC and pH was maintained as 6.5. The highest production 4.32 U /ml were recorded at 72 hours' fermentation period. The decrease in pectinase production after 72 hours may be due to change in pH during the incubation period (Sampriya *et al*., 2012) or may be due to denaturation of enzyme inhibition or interaction with other components of medium (Soares *et al*., 1999). The low level of production could be also due to depletion of nutrients in the medium (Palaniyappan *et al*., 2009)

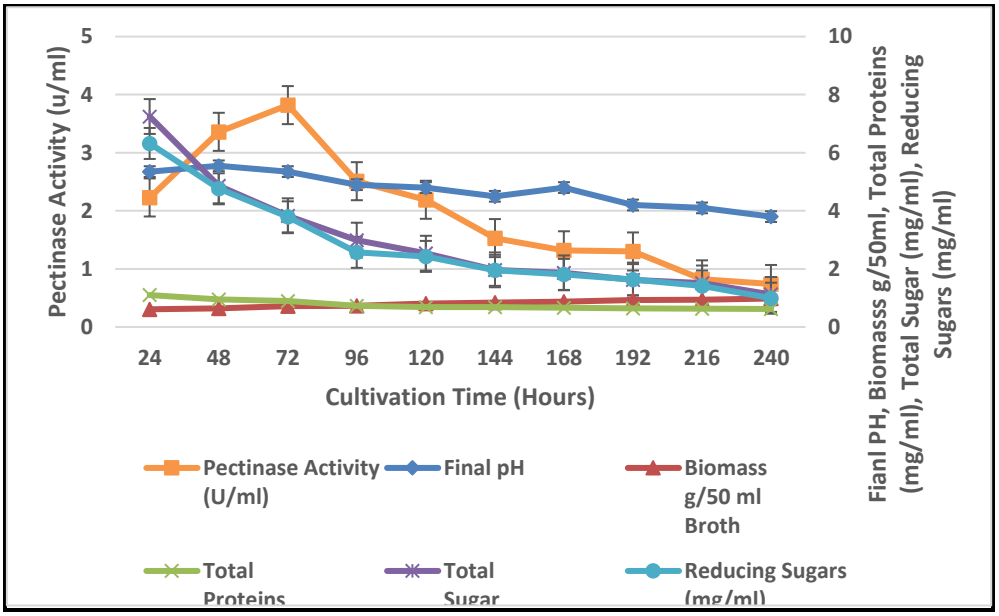

Figure -01: *A. fumigatus* was grown on mineral medium supplemented with 2.5% date syrup at 30 ± 2 ºC and pH was adjusted to 6.5.

Fig-06-10 show the results of pectinase production when filamentous fungi like *A. fumigatus*, *A. niger* + *A. fumigatus*, *A. niger*, *M. geophillus* and *P. lilacinum* were grown on a culture medium supplemented with 5% date syrup as a carbon source. *A. fumigatus* and a mixed culture of *A. niger* + *A. fumigatus*, produced pectinase 4.87U/ml and 4.21 U/ml respectively, and production decreased with the passage of time. Fig-08 shows the results of highest (5.68 U/ml) of [pectinase production by *A. niger* when grown on mineral medium supplemented with 5 % date syrup as carbon source. The increase of time period decreased the rate of enzyme production and concentration of sugar and proteins. Fig-09 and 10 show the results of Pectinase production by *M. geophillus* and *P. lilacinum*. The highest Pectinase production was recorded as 4.51U/ml and 5.16 U/ml respectively, while with the passage of time period decreased Pectinase production, concentration of total sugar, reducing sugar and total protein. The pH of the medium also changed gradually from acidic to alkaline except in *A. niger*.

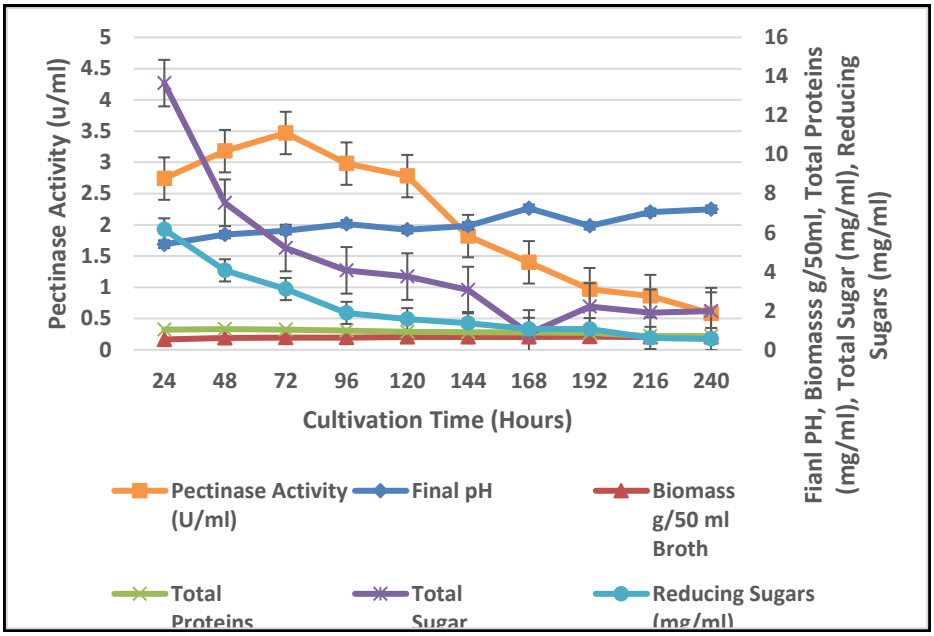

Figure- 02: A mixed culture of A. *niger* + *A. fumigatus* was grown on mineral medium supplemented with 2.5% date syrup at 30 ± 2 ºC and pH was adjusted to 6.5.

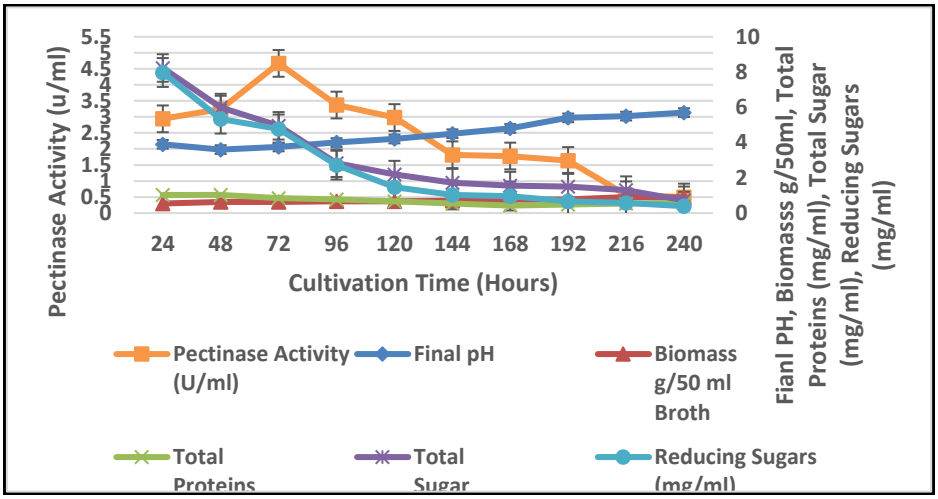

Figure-03: *A. niger* was grown on mineral medium supplemented with 2.5% date syrup at 30 ± 2 ºC and pH was adjusted to 6.5.

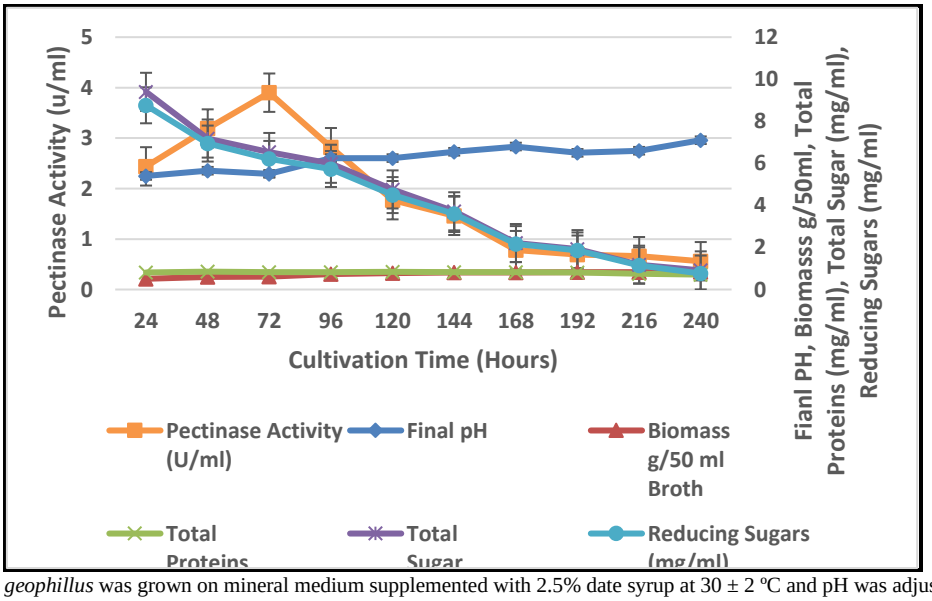

Fig-04: *M. geophillus* was grown on mineral medium supplemented with 2.5% date syrup at 30 ± 2 ºC and pH was adjusted to 6.5

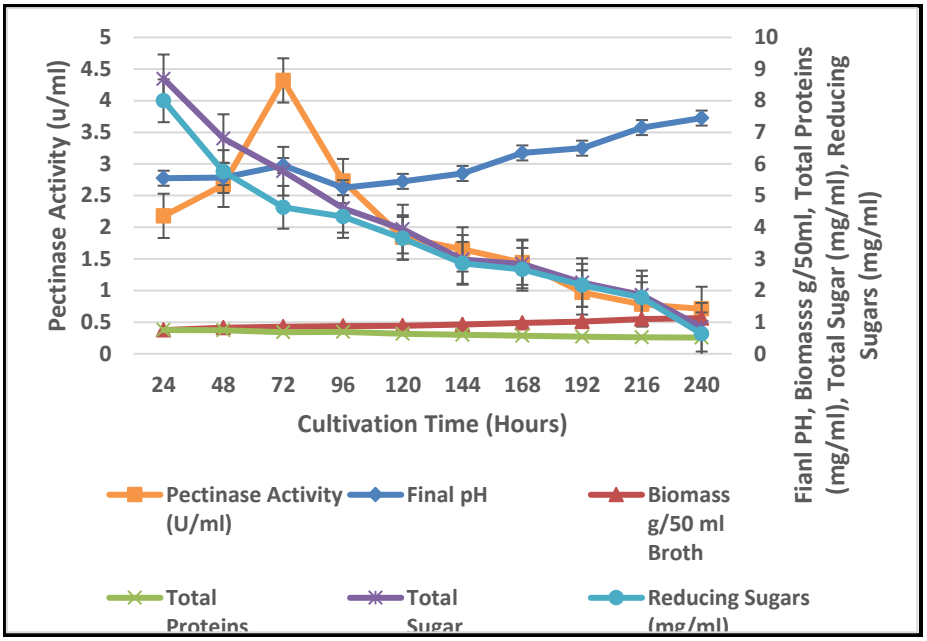

Fig-05: *P. lilacinum* was grown on mineral medium supplemented with 2.5% date syrup at 30 ± 2 ºC and pH was adjusted to 6.5.

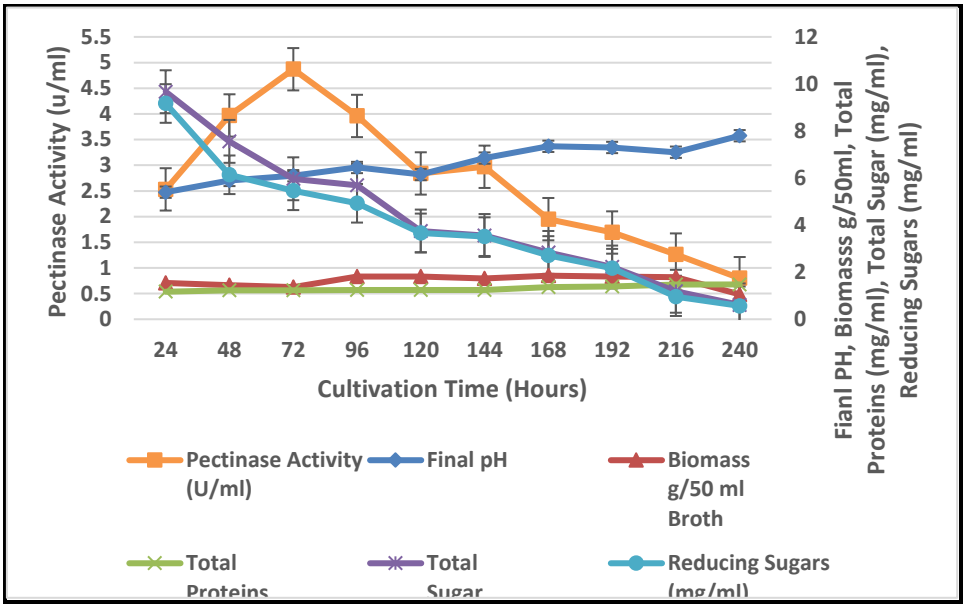

Fig-06: *A. fumigatus* was grown on mineral medium supplemented with 5% date syrup at 30 ± 2 ºC and pH was adjusted to 6.5.

Fig-11: shows the comparative results of pectinase synthesis by different filamentous fungi like *A. fumigatus*, (*A. niger* + *A. fumigatus)*, *A. niger*, *M. geophillus* and *P. lilacinum* when grown on medium supplemented (2.5% and 5%) of date sugar as carbon source. Results of pectinase production indicate that *A. niger* produces a higher level of pectinase when the culture medium containing 2.5% and 5% date syrup as a carbon source in comparison to other fungi .

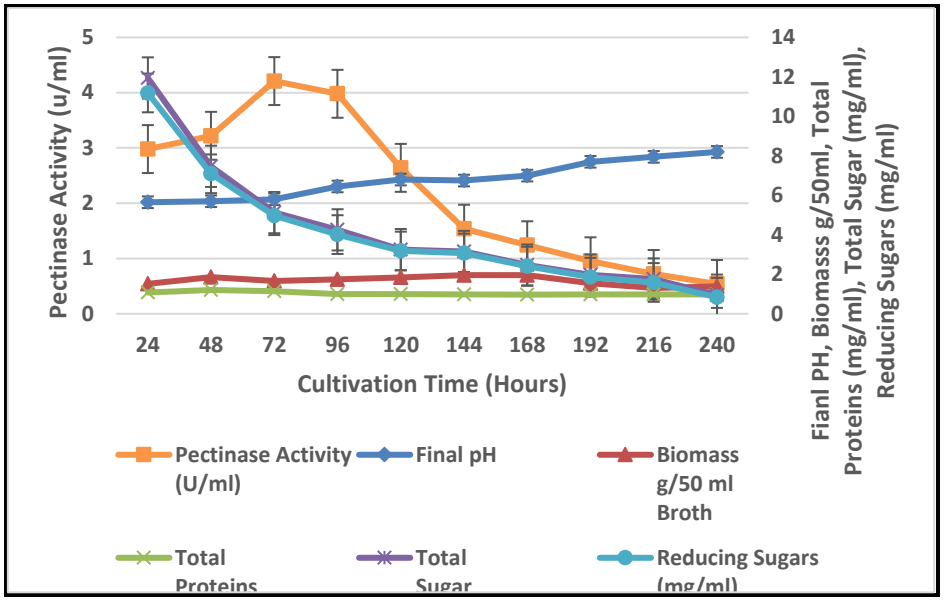

Fig-07: A mixed culture of A. *niger* + *A. fumigatus* was grown on mineral medium supplemented with 5% date syrup at 30 ± 2 ºC and pH was adjusted to 6.5.

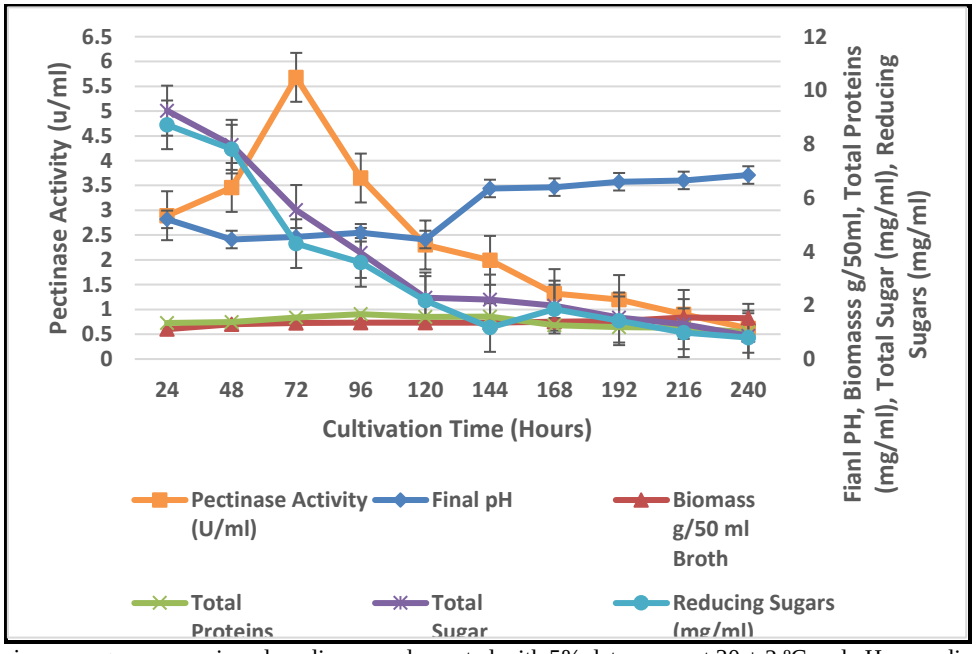

Fig-08: *A. niger* was grown on mineral medium supplemented with 5% date syrup at 30 ± 2 ºC and pH was adjusted to 6.5.

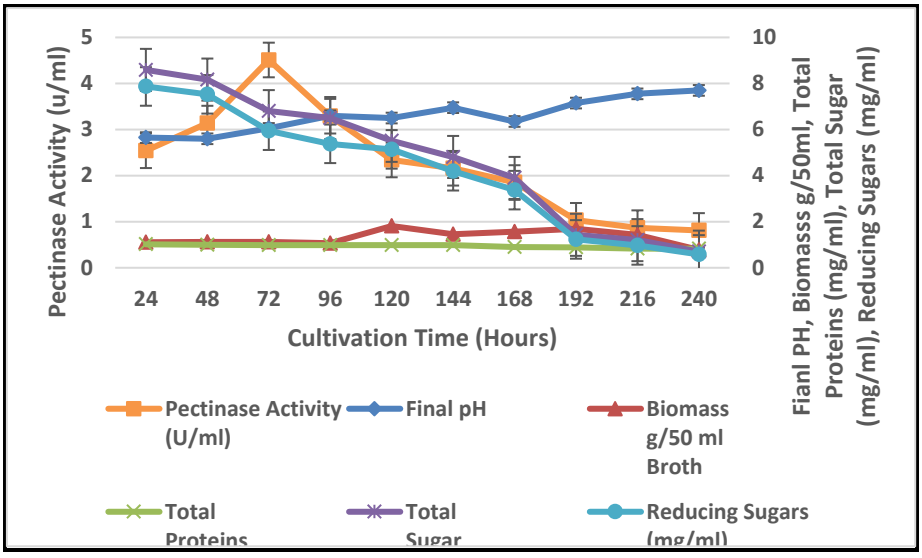

Fig-09: *M. geophillus* was grown on mineral medium supplemented with 5% date syrup at 30 ± 2 ºC and pH was adjusted to 6.5.

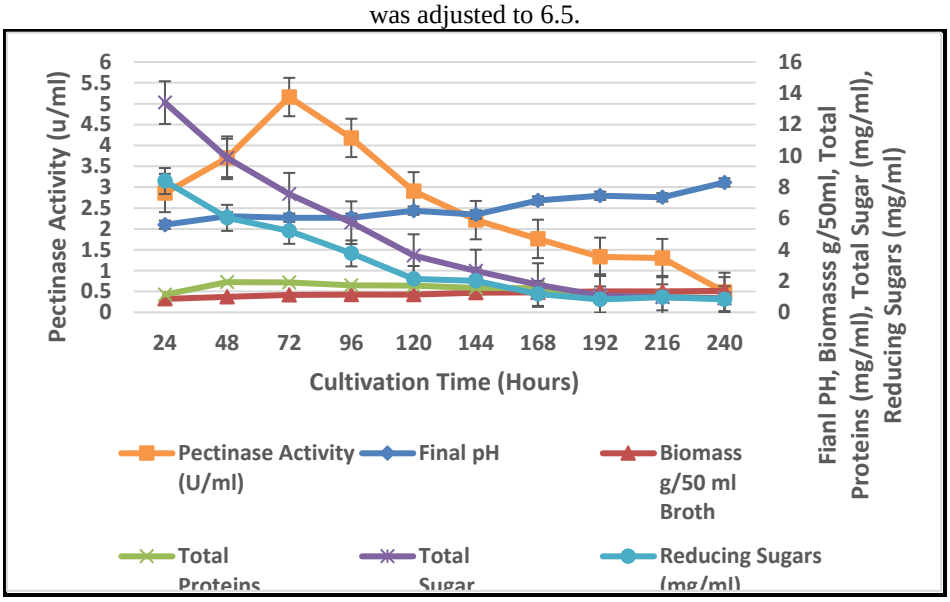

Fig-10: *P. lilacinum* was grown on mineral medium supplemented with 5% date syrup at 30 ± 2 ºC and pH was adjusted to 6.5

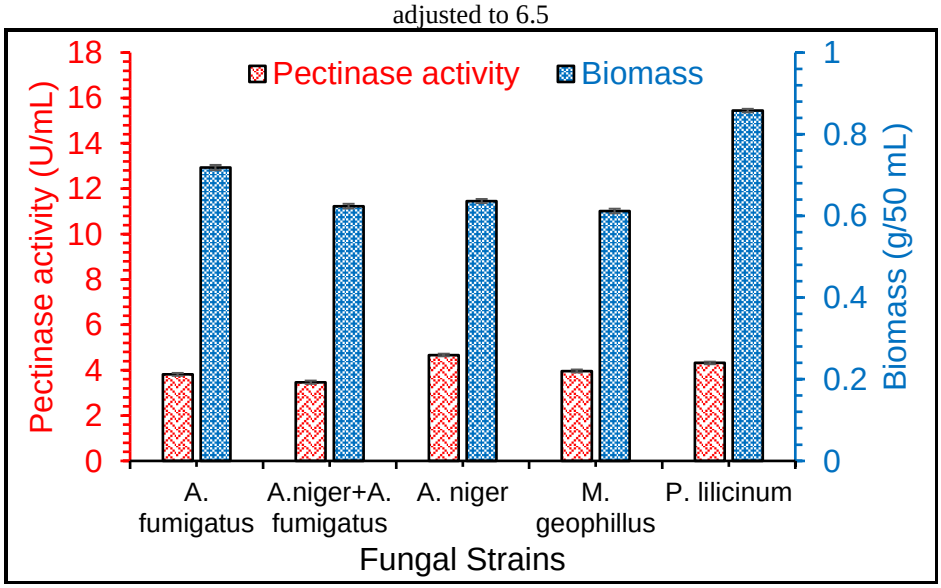

Fig-11: Effect on growth and pectinase production by different fungi when grown on mineral medium supplemented with 2.5% date syrup at 30 ± 2 ºC and pH was adjusted 6.5.

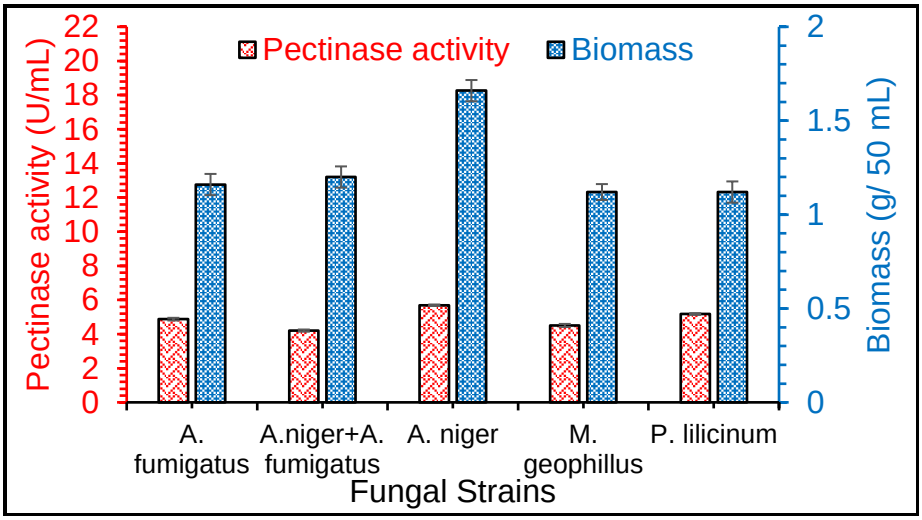

Fig-12: Effect on growth and pectinase production by different fungi when grown on mineral medium supplemented with 5.0% date syrup at 30 ± 2 ºC

CONCLUSION

It is concluded that highest production of pectinase (approximately 5.68 Units / ml) was recorded by *Aspergillus niger*, when it was grown on 5% date syrup at 72 hours in comparison to other species of fungi. On the basis of higher production of pectinase, it is suggested that the 5% date syrup is the best and cheapest source for the synthesis of pectinase and *Aspergillus niger* is the best producer of pectinase in comparison to other species of fungi.

In concern with the use of date palm which is an agro-industrial by product in the present study suggested high pectinase production by *Aspergillus niger* and *Penicillium lilacinum* with date syrup as sole carbon source. It is economic to use date syrup for the production of pectinase as compared to pure sugars. In Pakistan date syrup is a by-product of date palm industry and it can also be used to produce ethanol; however, it can be successfully used in enzyme production. Its careless dumping in nature can causes environmental pollution, hence it can be eco-friendly used as a good substrate for enzyme production.

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