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Production and Optimization of Pectinase from *Bacillus* sp. MFW7 using Cassava Waste

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ABSTRACT

This study investigated pectinase production and optimization by bacterial strain MFW7 isolated from fruit market wastes. Initial screening process identified the best pectinolytic strain and it was characterized by 16S rDNA sequencing and designated as Bacillus sp. MFW7. Optimization of process parameters were carried out by altering the physico-chemical environment of the production medium. High pectinase production was observed at 72 h of incubation at 35°C with the initial pH of 6.5. Various nutrients were investigated in terms of their effect on the production of extracellular pectinase using Cassava waste as substrate. Lactose in combination with peptone supported maximum pectinase production. Purified pectinase showed a molecular weight of 37 kDa observed by means of SDS-PAGE.

Keywords: *Bacillus subtilis*, Pectinase, Cassava waste, Lactose, Peptone

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INTRODUCTION

There is an increasing energy demands worldwide towards the utilization of renewable resources, from agricultural and forest residues. The major components of these residues are cellulose, lignin and pectin [1]. These materials have paid more attention as an alternative feedstock and energy source, since they are abundantly available. Several microorganisms are capable of using these substances as carbon and energy sources by producing a vast array of enzymes in different environmental conditions [2].

Pectinases are the group of enzymes that catalyzes the degradation of pectic substances through de-polymerization and de-esterification reactions [3]. Pectinolytic enzymes are classified according to their mode of action on the galacturonan part of the pectin molecule. Pectinases have major applications in food industries for the production and clarification of wines and juices [4].

In the world market, pectinases accounts for about 10% of total enzyme production. Pectinolytic enzymes are produced by many organisms like bacteria, fungi, yeasts, insects, nematodes, protozoan and plants. Of that, microbial pectinases possess more advantages as it involves in the phytopathologic process, plant–microbe symbiosis and the decomposition of dead plant materials [3].
Many studies have been conducted on the production of pectinases from various microorganisms. But a few works have been published about cost-effective production of enzymes. The difficulties to obtain the appropriate substrate

might be the biggest problem to develop such studies. A suitable substrate should be cost-effective and to provide all necessary nutrients to the microorganism, if not, it would be necessary to supplement them externally [5].

There are different low-cost substrates like cassava waste which is used for cost-effective production of the pectinase enzyme [6]. In this paper we report the production of pectinases by a newly isolated strain of *Bacillus* sp. by submerged fermentation using cassava waste.

MATERIALS AND METHODS

Sample Collection and Screening for Pectinase Producers

Bacteria for the production of pectinase were isolated from soil sample collected from fruit market, Chennai. The sample was diluted and spreaded on nutrient agar plates and incubated at 37 °C for 48 h. The isolated colonies were picked up and streaked on nutrient agar plates to get pure culture. Screening of pectinase producing bacteria was carried out in pectinase screening plate [7] containing (g/l): yeast extract-1, ammonium sulfate-2, Na_2HPO_4-6, KH_2PO_4-3, citric pectin 5, Agar 20 (pH 4.0). After 24 h incubation, the colonies sowing clear zones upon flooding with 1% cetyltrimethyl ammonium bromide were confirmed as pectinase producers and the colony with maximum zone diameter was preceded for further studies.

Identification of the Bacterial Strain

The bacterial strain was identified by morphological characteristics as well as comparison of 16S rDNA gene [8]. Genomic DNA (gDNA) of the bacteria was isolated by CTAB method. Partial DNA fragment of the 16S rDNA was amplified by polymerase chain reaction (PCR) using gDNA as template. The primers (63f 5′-CAGGCCTAACACATGCAAGTC and 1387r 5′-GGGCGGWGTGTACAAGGC) used were designed from the conserved sequences of bacteria by Marchesi et al. [9]. The phylogeny and family of the bacterial strain was accessed using BLAST search.

Substrate

Cassava waste (g/100g dry residue); moisture: 11.2%, starch: 63.0%, crude fibre: 10.8%, crude protein: 0.9% and total ash: 1.2%), was collected from local Starch extraction industry in Chennai. The cassava waste was oven dried (80 °C for 24 h) [6] and the powder was stored at 4 °C till use.

Pectinase Production Medium

A basic liquid medium showing the following composition (g/l): Cassava waste 0.3, Sucrose 10, KNO_3 0.6, KH_2PO_4-1, $MgSO_4$ 0.25, $CaCl_2$ 0.1, $NaNO_3$ 2, K_2HPO_3 0.5, KCl 0.5 and yeast extract 1 [10] was used for the production of pectinase. Erlenmeyer flasks containing 100 ml of basal medium amended with 3% cassava waste were inoculated with one ml of overnight grown bacterial culture and incubated for 3 days at 37 °C under agitation (125 rpm). The culture medium was centrifuged and the supernatant was used as crude enzyme source.

Quantitative Assay for Pectinolytic Activity, Biomass and Protein Concentration

The quantitative assay of pectinase was done by using pectin as substrate [11]. The reaction mixture containing 0.5 ml of the crude enzyme and 0.5 ml of pectin in 0.1M acetate buffer with pH 6.0 was incubated at 40 °C for 10 min. The reaction mixture was then added with 1 ml of DNS reagent and the mixture was boiled for 5 min at 90 °C. One milliliter of Rochelle's salt was added to stop the reaction. The absorbance was read at 595 nm. Standard glucose solution was used to generate standard graph. One unit of Pectinase activity was defined as the amount of enzyme which liberated 1µm glucose per min. Bacteria biomass was determined by measuring the absorbance at 600 nm [12]. The total soluble protein was determined by Bradford method [13] using BSA as standard.

Effect of Incubation Time, Temperature, pH and Substrate Concentration

The bacterial isolate was subjected to different culture conditions to derive the optimum conditions for pectinase production. Growth and pectinase production were estimated at regular time intervals (12, 24 36, 48, 72, 96, 120 and 144 h) and selected temperatures (25, 30, 35, 40, 45, 50 and 55 °C), substrate (cassava waste) concentrations (0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and, 3.5%) and pH (3.5, 4.5, 5.5, 6.5, 7.5, 8.5, 9.5). All the experiments were carried out in 500 ml Erlenmeyer flask containing 100 ml of basal medium.

Effect of Carbon and Nitrogen Sources

Carbon sources such as glucose, maltose, lactose, starch, xylose and sucrose, and nitrogen sources such as peptone, urea, yeast extract, KNO_3, NH_4Cl, and $NaNO_3$ were supplemented as individual components to the basal media to check their effect on pectinase production.

Extraction and Purification

The crude enzyme source was precipitated by 65% saturation of ammonium sulphate. The precipitate was dissolved in Na_2HPO_4−KH_2PO_4 buffer (pH 6.0) and separated by ion exchange chromatography [14]. The enzyme solution was applied to sepharose column (1.0 cm × 20 cm) pre-equilibrated with Na_2HPO_4−KH_2PO_4 buffer (pH 6.0). The flow rate was adjusted to 18 ml/h and the fractions with maximum enzyme activity was pooled and concentrated. Homogeneity of the fraction containing pectinase activity was checked by SDS-PAGE as described below.

SDS-Polyacrylamide Gel Electrophoresis

The pectinase purified from *Bacillus* sp. MFW7 was electrophoresed according to Laemmli [15]. Aliquots of 20 µl were loaded onto a 12% SDS polyacrylamide linear resolving gel overlaid with a 6% stacking gel. Gels were stained with Coomassie Brilliant blue R-250.

RESULTS AND DISCUSSION

Enzyme production is one of the broad areas of biotechnology which accounts for about 1.5 billion of the world market **[10]**. The enzymes of microbial origin were found to be more advantageous than others. In the present study production of pectinase using cassava waste was carried out and the production conditions were optimized.

The serially diluted soil samples were screened for pectinase producing bacteria. Out of 17 bacteria grown on screening medium, only 7 strains showed clear zones and found as pectinase producers. The strains were then purified using repeated subculture using NA plates and stock culture was maintained in NA slants.

Identity and phylogeny of the isolate was analysed using 16S rDNA analysis. The strain shows maximum similarity ratio towards *Bacillus* spp. using BLAST and hence the isolate was also confirmed as *Bacillus* sp. and submitted to Genbank (Genbank accession: JQ904291). Of the many microorganisms, *Bacillus* spp. are known to produce variety of extracellular enzymes and they have a wide range of industrial applications [16]. The bacterial colony with maximum zone diameter was selected as the best strain and preceded for further studies.

The best strain was studied in detail for the enzyme production with respect to development of low-cost and easy available medium ingredient. The culture was further screened for pectinase production by submerged fermentation using cassava waste as substrate. Studies on production and secretion of pectinase are important to develop enzyme systems which could be directly used for converting biomass into enzymes.

Of the various wastes accumulating in the environment, Cassava wastes were considered as the most predominant substrate due to its vast usage in starch processing industries in India [6]. The cassava wastes consists of nutrients which can acts as nutrient source as well as substrates for the production of various enzymes and hence, it was attempted to study on its role as substrate for pectinase production.

It is essential to optimize the fermentation medium for cost-effective production of pectinase. In submerged fermentation the pectinase production reached maximum at 96 h of incubation. Further increase in incubation period did not show any significant increase in pectinase production rather it was decreased. Thus, optimum time of pectinase synthesis was to be 96 h after inoculation (Fig. 1).

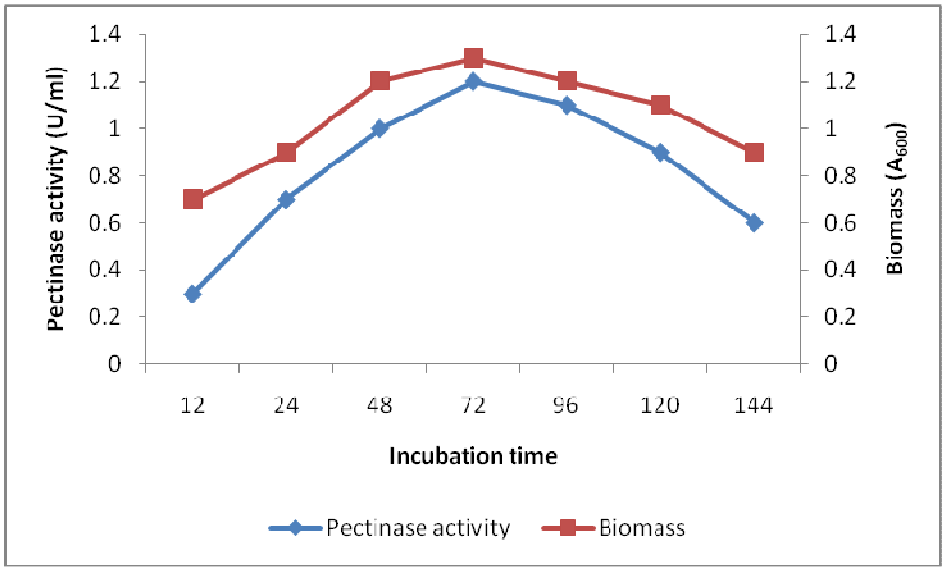

Fig. 1: Growth and enzyme activity profile of *Bacillus* sp. MFW7 With respective to different incubation time

The pectinase production by *Bacillus* sp. MFW7 was found maximum at 35 °C. Further increase in the temperature results in the decrease of pectinolytic activity. However, the temperature of the fermentation medium was found to be optimum at 35 °C (Fig. 2). When temperature is altered below or above the optimum the activity is decreased or becomes denatured. The maximum production of pectinase enzyme was obtained at 37 °C by the *Bacillus subtilis* isolated by Arpita et al. [17].

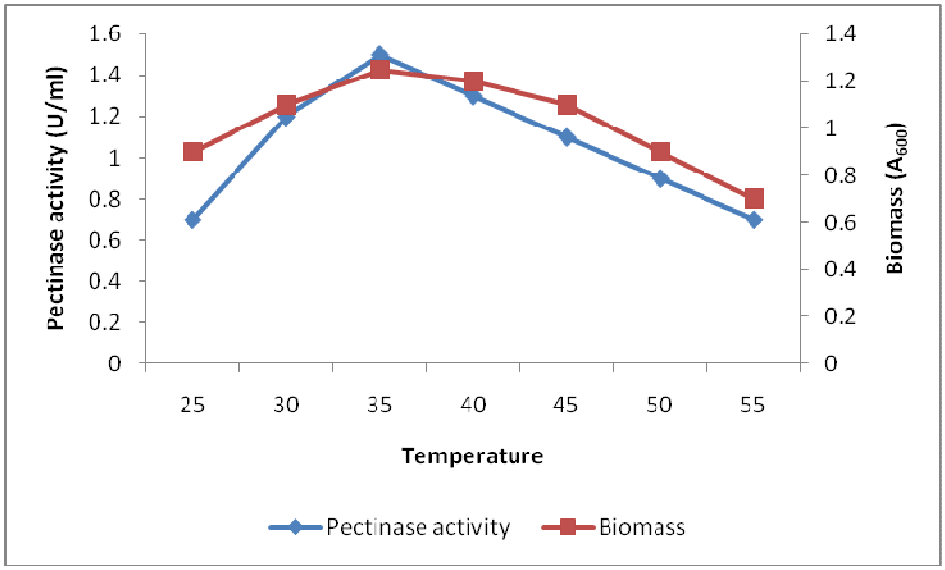

Fig. 2: Growth and enzyme activity profile of *Bacillus* sp. MFW7 With respective to different temperature

Fig. 3 depicts the effect of different pHs on the production of pectinase by *Bacillus* sp. MFW7 The maximum production of pectinase was obtained at pH 6.5 and hence, considered as optimum. Increase in pH further decreased the production of enzyme. Orange peel as substrate at the concentration of 1% with the pH of 7.0 was found to be optimum for pectinase production by *Bacillus* sp. [17].

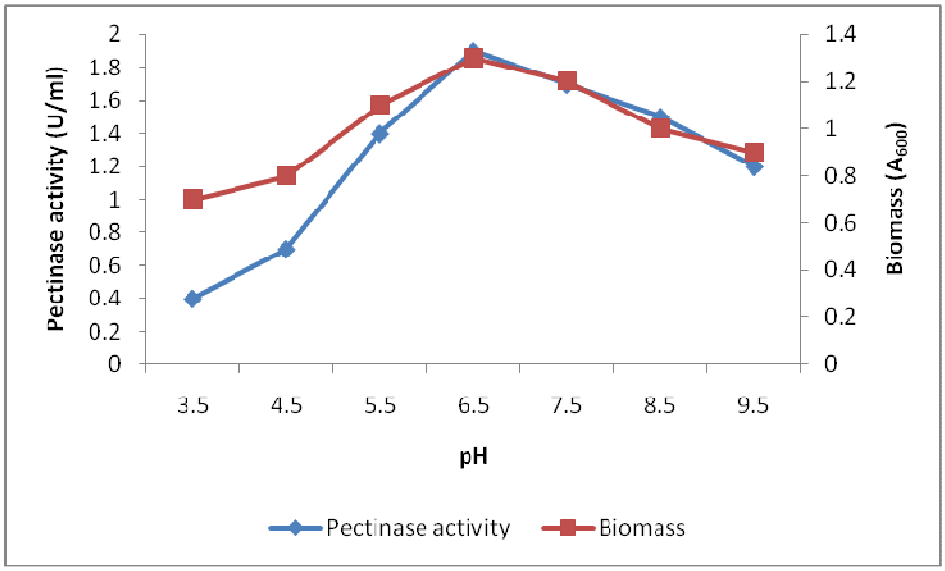

Fig. 3: Growth and enzyme activity profile of *Bacillus* sp. MFW7 With respective to different pH

The cassava waste at the concentration of 1% resulted in maximum enzyme productivity (Fig. 4). Below and above the optimal concentration showed decrease in productivity. This reduction in the enzyme production was due to the decrease/limited inoculums concentration [10].

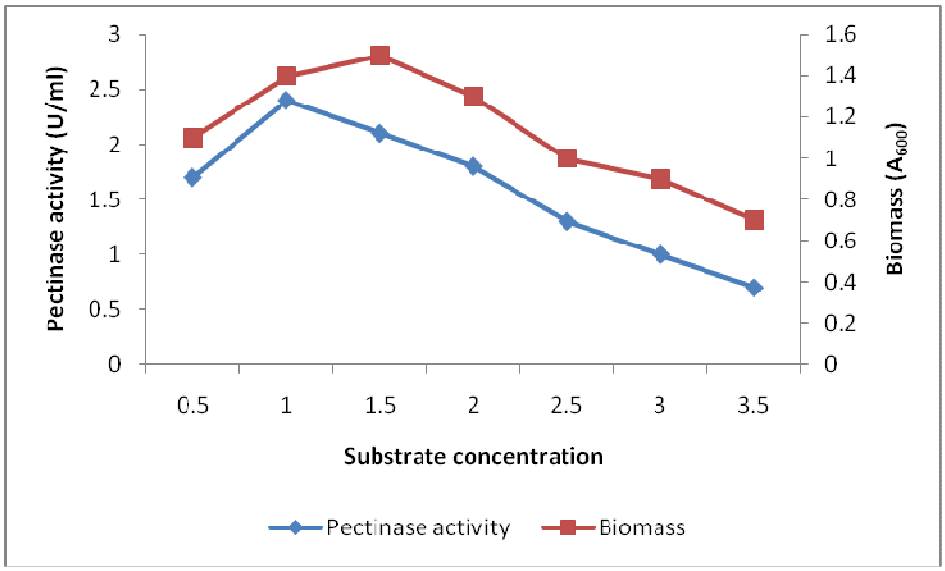

Fig. 4: Growth and enzyme activity profile of *Bacillus* sp. MFW7 With respective to different substrate concentration

Supplementation of carbon sources in the form of carbohydrates resulted in marginal increase in pectinase production by *Bacillus* sp. MFW7 during fermentation using cassava waste (Fig. 5). Highest production was observed with lactose. The synthesis of pectinase was greatly suppressed when the bacterium was grown either on glucose, maltose, or sucrose, but pectinase production was found to be good when the bacterium was grown on lactose. In concurrent to our results, citrus pectin and xylose were reported as best carbon source for the pectinase production by *Bacillus sphaericus* [18].

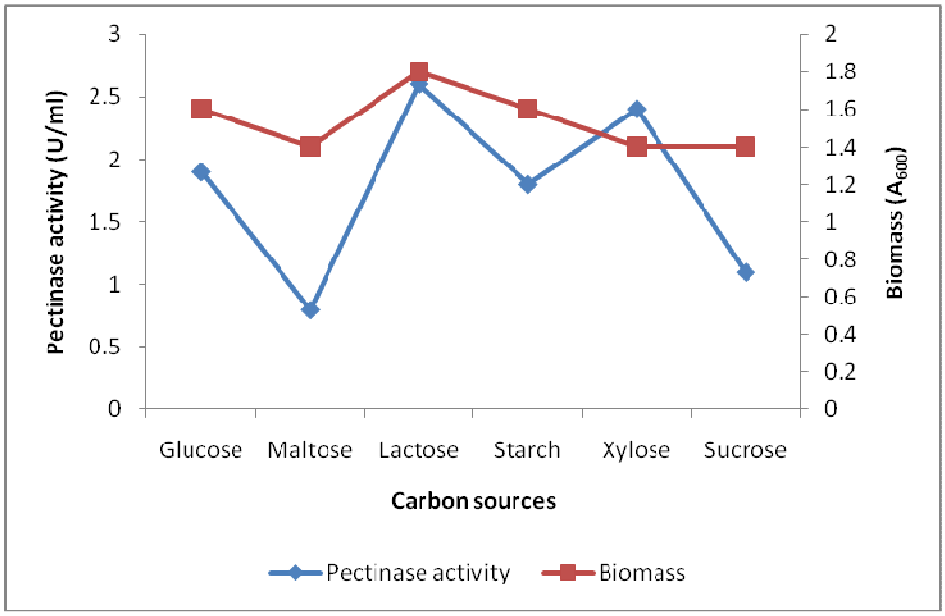

Fig. 5: Growth and enzyme activity profile of *Bacillus* sp. MFW7 With respective to different carbon sources

The Organic sources like peptone and inorganic sources like NH_4Cl were found to stimulate the pectinase production (Fig. 6). Peptone was considered as the most potent nitrogen source for pectinase production amongst the tested nitrogen sources. Tryptone also served as better nitrogen source for pectinase production [17].

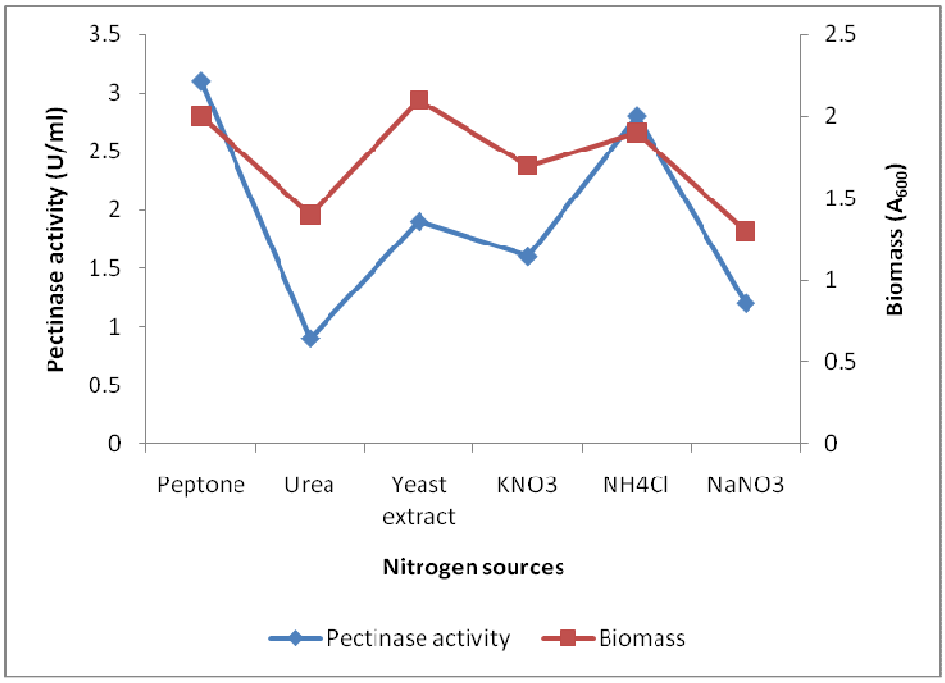

Fig. 6: Growth and enzyme activity profile of *Bacillus sp.* MFW7 With respective to different nitrogen sources

From the culture filtrate, pectinase was precipitated out with the help of ammonium sulphate and the further purification was done using stepwise chromatography using sepharose column [19]. The enzyme was precipitated with ammonium sulphate followed by DEAE-Sephacel and Sephadex G-150 column chromatographies.

The purified pectinase was characterized by SDS-PAGE analysis in denatured conditions. The molecular weight of the pectinase was known by the formation of single band in gel adjacent to marker and was found to be 37 kDa. The microbial pectinases possesses the molecular weight ranges between 35 and 79 kDa [20].

CONCLUSION

The bacterium *Bacillus* sp. MFW7 produced significant amount of pectinase after 96 h of incubation in fermentation medium at 35 °C and pH 6.5. Maximum enzyme production was with lactose as carbon source, peptone as nitrogen source and cassava waste as substrate. This study illustrated that the usage of cassava wastes as a substrate for pectinase production. To conclude, this enzyme may be further scaled up for juice production after careful investigations.

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