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Isolation, identification, screening and optimization of pectinase producing soil fungi (*Aspergillus niger*)

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ABSTRACT

Microbes are well known source for the production of extracellular enzymes and industrially important secondary metabolites. The present study demonstrates the isolation of pectinase producing microbes, screening and optimization of pectinase production from soil born fungi. In order to examine pectinase production from microbes, soil was collected from Porur vegetable market, Chennai, Tamil Nadu, India. Total of 40 fungal isolates were screened for the pectinase production using the selection medium and found three isolates reveals a pectinolytic zone of inhibition in agar plates around the colony. Among three isolates, the best producer was chosen based on their pectinolytic activity (9mm zone of inhibition) for further molecular characterization. The molecular characterization study revealed that, the selected fungi characterized as *Aspergillus niger* with 18s RNA of the organism. Further for the production of pectinase from *Aspergillus niger* the submerged fermentation (SmF) and solid state fermentation (SSF) are used with organic wastes such as wheat bran, rice bran, pomegranate peel and orange peel. Compared with the SmF and SSF the culture with SmF produces higher yields of pectinase (235.04 nkat) at 72 hrs (30°C) on supplemented with wheat bran (20%) and orange peel (10%) in the production medium. In the SmF sucrose (1%) was used as carbon source and physiological pH 5.5 was maintained. The produced enzyme shows the Km value 1.0mg/ml and V_{max} value found to be 500 nkat.

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INTRODUCTION

Pectinase are a major enzyme involved in pectin degradation process in the cell wall of plants and also it act as major cell wall component in plants (Oyewole *et al.,* 2011). Pectin contains high molecular weight, negatively charged, acidic in nature and complex glycosidic macromolecule. In the world market, pectinase accounts for about 10% of total enzyme production. Various sources such as bacteria (Elangovan *et al.,* 2016), fungi (Phutula *et al.,* 2005) and yeasts (Aaish and Barate, 2016) are used for pectinolytic enzyme production. In that, microbial pectinases accounts approximately for 25% of the total worldwide enzyme production and sale.

Microbial production of pectinases has been extensively studied in fungi (Geetha *et al.,* 2012), actinomycetes (Bruhlmann *et al.,* 1994) and in *Aspergillus* sp., (Akhter *et al.,* 2011). Pectinases are classified as pectin esterase, depolymerising enzymes and protopectinases. Polygalactouronase is the depolymerising enzyme which catalyze the hydrolytic cleavage of alpha (1,4) glycosidic bonds in the

D-galacturonic acid moieties of the pectic substances. It catalyze hydrolytic cleavage at substrate nonreducing end producing monogalacturonate or digalacturonate in some cases. Pectinase are produced in several ways including solid state fermentation and submerged fermentation under optimized pH and temperature of the strain.

Pectinases are the most important industrial enzymes. Pectinolytic enzymes from microorganisms have drawn a great deal of attention from various researchers as a biological catalysts in a variety of industrial processes. The largest industrial application of pectinases is found in the fruit juice extraction, clarification, and preparation. Especially, the pectin helps to produce viscosity and turbidity in fruit juice. A mixture of pectinases and amylases are used to clarify the fruit juices and it help to decreases the filtration time up to 50% (Alkorta *et al.,* 1998).

Treatment of fruit pulps with pectinase has showed an increased volume of fruit juice of in banana, grapes and apples (Kaur *et al.,* 2004). Pectinases in combination with other enzymes such as cellulases, arabinases and xylanases are used to increase the pressing efficiency of the fruits for juice extraction (Gailing *et al.,* 2000). Vacuum infusion of pectinases has a commercial application to soften the peel of citrus fruits for removal during preparation instead of hand cutting for the production of canned segments (Baker and Wicker, 1996). Pectinases give various health benefits to human beings like promoting growth of intestinal microbiota, increasing digestibility of plant-foods through absorption. Due to the needs of pectinase in food and pharmaceutical industry, the present research work demonstrates the isolation and screening of the best pectinase producers soil fungi *Aspergillus niger* from Porur vegetable market, Chennai, Tamil Nadu also emphasize the optimization of various parameters including temperature, pH, substrate, process of fermentation, carbon and nitrogen source to obtain high yield of enzyme from *Aspergillus niger* with cost effective manner using organic waste.

MATERIALS AND METHODS

Isolation and screening of pectinase producing fungi from soil

Fungi and bacteria are the major source of organism to decompose the dead leaves and other organic matter in the soil, hence soil sample was collected from the Porur vegetable market in Chennai, Tamil Nadu, India. After collection, sample was stored at 4°C. A 1g of soil was taken and homogenised in sterile distilled water and 10-fold serial dilutions were prepared. 0.1 ml aliquots from each dilution were spread on sterile potato dextrose agar (PDA) (effective substrate for fungi) plates and incubated at 37 °C for 96 hrs. A total of 40 isolates with active growing mycelia were isolated and sub-cultured agar plates supplemented with PDA for further production of pectinase.

Then, the isolated pectinase producer were inoculated in pectinase production enhancing medium containing pectin (1%), diammonium orthophosphate (0.3%), KH_2PO_4 (0.2%), K_2HPO_4 (0.3%), $MgSO_4$ (0.01%), agar (2.5%) with pH 4.5 and incubated for 48 hrs for identification of high yield pectinase producer. After 48 hrs of incubation, three isolates showed clear zone around the margins of the colony and detected as pectinase producers upon flooding with 50mM I_2 solution. The fungal positive producers of pectinase were sub cultured on potato dextrose agar slants.

Pectin as substrate for production of pectinase

For pectinase production enhancing medium, the pectin was used as substrate in submerged fermentation supplemented with $NaNO_3$ (2%), K_2HPO_4 (1%), $MgSO_4$ (5%), KCl (5%), $FeSO_4$ (0.001%), Pectin (15%) and pH 3.8. The spore suspension from selected fungi 10^7 spores/mL^{-1} was inoculated in prepared pectinase enhancing medium and agitated (200 rpm) for 5 days at room temperature (RT) (30°±1°C). Every 24 hrs of time interval 5ml fermented medium was taken and centrifuged at 10,000 rpm for 10 mins to remove mycelium and the supernatant was served as crude enzyme source for further study.

Organic waste used as substrate

SmF medium of 100 ml was prepared with wheat bran 20% and orange peel 10%, pH 5.5 (Adjusted using acetic acid) and sterilized at 15 psi (121°C). The spore suspension (10^7spores.mL^{-1}) was inoculated and incubated at RT for 8 days under agitation (200 rpm). The culture medium (5 ml) was centrifuged (10,000 g, 10 min, at 30°C) and supernatant was taken for further analysis. In the same way other organic waste material such as sugarcane buggass, pomegranate peel and rice bran was used along with 10% orange peel.

Optimization of bacteria growth

Aspects such as time, pH, and temperature, were taken into account for fungal growth optimization. In this study, Design expert software 11 was used to investigate fungal growth through optical density. The collaborations between these factors were recognized through contour plots. To evaluate the coordination between time, pH, and temperature, a three dimensional response surface plots were created and their cooperative effects on the response factors were assess during pectinase

activity (nkat) of organism. Almost twenty trial runs were carried out with pH ranging from 2 to 9, temperature from 20 to 50 °C, and incubation time from 24 to 120 h (Ushani *et al.,* 2016). There was a rise in pectinase activity at the central points when they were observed with the response of pH, temperature and time as variable parameters. The model revealed that the central point offered the definite process optimization region.

Optimisation process for high titre of pectinase

The high titre pectinase production from *Aspergillus niger* was optimized with various physical, chemical and physiological parameters such as suitable substrate, suitable carbon sources and suitable nitrogen sources. For the optimization process, organic substrate of wheat bran (WB), sugarcane baggas (SB), rice bran (RB), pomegranate peel (PM)(dry powder) (20% each) with combination of 10 % orange peel (OP), carbon source such as sucrose, glucose, galactose, starch and nitrogen source also used along with substrate (WB + OP) such as ammonium nitrate, peptone, and yeast extract were used for optimization of pectinase production from spore suspension (10^7spores.mL^{-1}) of *Aspergillus niger*. The pectinase production was estimated using enzyme assay through DNS method (Miller 1959).

Determination of pectinolytic activity

The quantitative assay for pectinase was made possible using citrus pectin (0.1%) as the substrate through Di-nitrosalicylic acid method (DNS method) (Miller 1959). The tubes containing 1 ml of substrate was incubated at 37°C for 3 min followed by addition of 1 ml of enzyme extract. The mixture was incubated for 15 min at 37°C. 1 ml of DNS reagent was added into the tubes and seethed for 10 min and cooled immediately by adding 3ml of distilled water. The reaction mixture was centrifuged at 10,000 rpm for 5 min. The absorbance was read at 540 nm using a UV/Vis spectrophotometer (UNICO). One unit of the pectinolytic enzyme was defined by the amount of enzyme that catalyses the formation of 1µmol galacturonic acid under the assay conditions.

Molecular characterization and Identification of higher pectinase producer strain

The fungal isolate (F-4, maximum pectinase producer) was grown in potato dextrose Broth (Himedia) for 48 h under agitation (200 rpm). The genomic DNA was isolated according to the Sambrook *et al.,* (1989) method. The 2 days old mycelium was allowed to drain for about 3 min after being placed on a filter paper. The mycelium was then macerated in pestle and mortar with 500µl of the extraction buffer containing: 200 mMTris - Hcl (pH 7.5), 25 mM EDTA, 250 mMNacl and 0.5% SDS. The squashed sample was transferred to a microfuge tube and again one ml of extraction buffer was added to the tube, vortexed and incubated at 55^o C for 1 h. The clear lysate was transferred to another fresh tube, to which 100 µl KCl solution was added and incubated on ice for 5 min.

The content was centrifuged at 10,000 rpm for 5 min and the supernatant was transferred to another microfuge tube and 750 µl of Phenol: Chloroform: Isoamylalcohol (25:24:1) mixture was added. The tubes were centrifuged for 10 min at 10,000 rpm. The Upper aqueous phase was mixed with an equal volume of absolute ice-cold isopropanol and incubated for 10 min at RT. The tubes were centrifuged at 10,000 rpm for 10 min. The pellet was washed with 70% (v/v) ethanol. The pellet was air-dried and dissolved in 100 µl TE buffer. The DNA samples were stored at -20°C for further use and the ITS region was amplified using forward primers (5'- CTTGGTCATTTAGAGGAAGTAA 3') and reverse primer (5'- TCCTCCGCTTATTGATATGC – 3' with PCR reaction. The PCR reaction mix contained 100ng of genomic DNA, 1x *Taq* buffer, 0.2mM dNTPs, 0.3 µM of each primer, and 1U *Taq* DNA polymerase in a final volume of reaction mixture was 20µl. The following PCR conditions were initial denaturation at 94°C for 3 min, denaturation at 94°C for 45 sec, annealing at 50°C for 1 min (35 cycles) and extension at 72°C for 80 sec, final extension at 72°C for 7 min for amplification of ITS region. The amplified product was analyzed with 0.8% agarose gel electrophoresis and using marker further, the selected band was further purified using Qiagen gel extraction kit.

RESULTS AND DISCUSSION

Isolation and screening of microorganisms

Soil sample from vegetable market, Porur, Chennai was examined for the presence of pectinolytic microbes. A total of 40 fungal isolates obtained from soil was subjected to screen pectinase production. The pectinase producers were identified by flooding the plates with iodine solution. The degradation was observed around fungal growth at varying diameters. Three different strains F-3, F-4, F-P were chosen based on the diameter of zone of clearance and further screened. Among three strains isolate F-4 produced the maximum clearance zone (09 mm) around the colony (Figure. 1). In earlier study Patil *and* Dayanand (2006) had mentioned about hydrolysis around the colony indicating the zone of clearance.

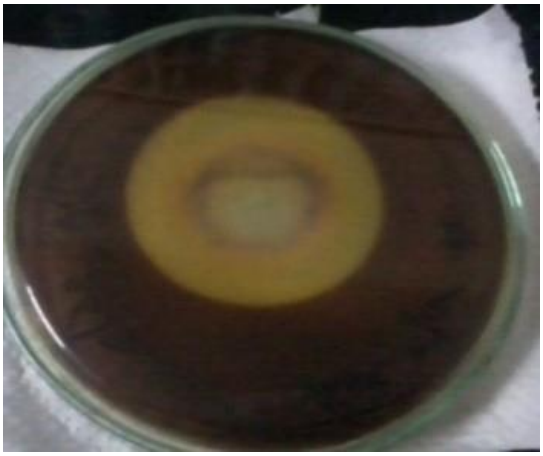

Figure 1: Pectinolytic activity (Clear Zone of isolate F-4)

Fermentation process

Submerged fermentation was performed with two different substrates and the enzyme activity was tabulated (Table 1). Initially F-4 strain was found to have maximum titre (120.02 nkat) through submerged fermentation using wheat bran and orange peel as nutritional supplement compared with the pectin as substrate (15nkat). Ultimately the strain showed the maximum diameter in the zone of clearance (09mm; Figure 1).

Table 1: Comparison of submerged fermentation using pectin and wheat bran with orange peel as substrates

Isolates	Wheat bran and orange peel Enzyme activity (nkat)	Pectin Enzyme activity (nkat)
F-3	30.00	05
F-4	120.02	15
F-P	41.67	05

Optimization of Fungal growth by response surface

From the response surface quadratic model, the pectinase activity showed R^2 value of 0.94 which specified that the detected and predicted values had good correlation. The adjusted R^2 was 0.89 and practiced R^2 was 0.93 which indicates that predicted and adjusted values are in good coordination (Ushani *et al.,* 2017). The interactions between time, pH, and temperature were aided by contour plots. The three dimensional response surface plots were made to examine the collaborations between pH, temperature and time and to assess their interactive effects on the response of pectinase activity of fungi as shown in Figures 5-7. The RSM outcomes specify that the three variables studied have a sensible impact on fungal growth (pectinase activity (nkat)). There was an increase in pectinase activity at the central points of about 231.3nkat when they were observed with the response of pH, temperature and time as variable parameters. The model exposed that the central point offers the definite method optimization region. The central point corresponds to pH 5.5, temperature of 30°C and time of 72 h.

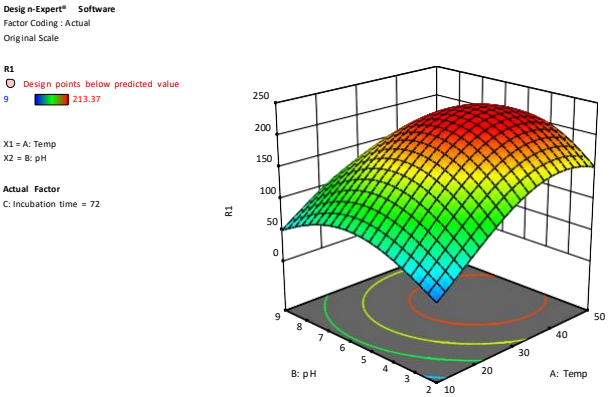

Figure 2: Pectinase activity of *Aspergillus niger* in different time intervals

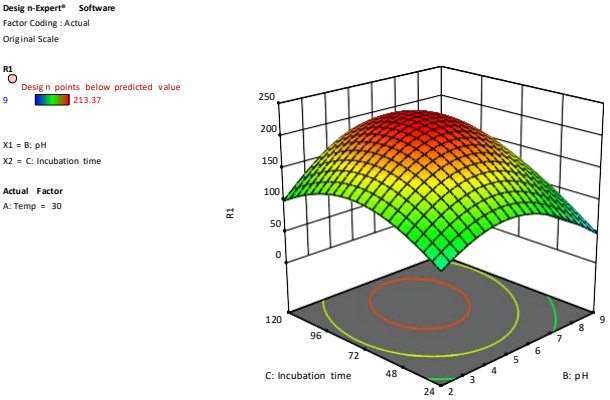

Figure 3: Effect of temperature on pectinase activity from *Aspergillus niger*

Pectinase production was evaluated up to 8 days. At 72 h of incubation, the enzyme activity was found higher (213.37 nkat). The steady increase in the enzyme activity corresponding to the incubation time was shown in the Figure 2. In an earlier study incubation of 84 h was found to be optimum for the production of pectin lyase by *A.niger* in solid state fermentation (SSF) (Kashyap *et al.,* 2001).

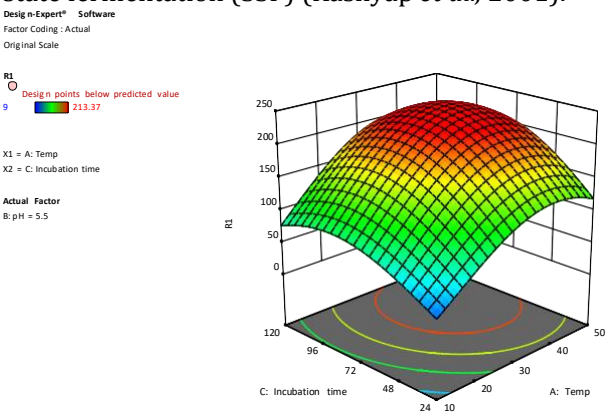

Figure 4: Effect of pH on pectinase activity from *Aspergillus niger*

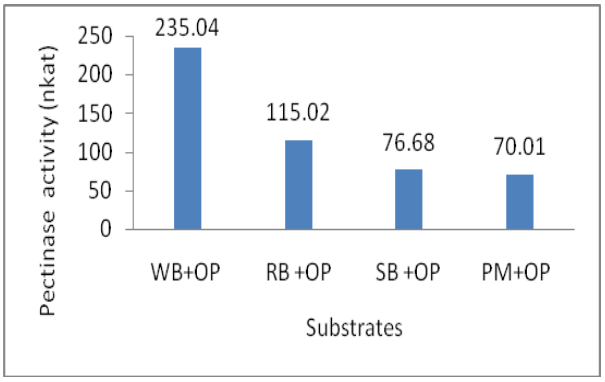

Figure 5: Effect of substrates on pectinase activity from Aspergillus niger

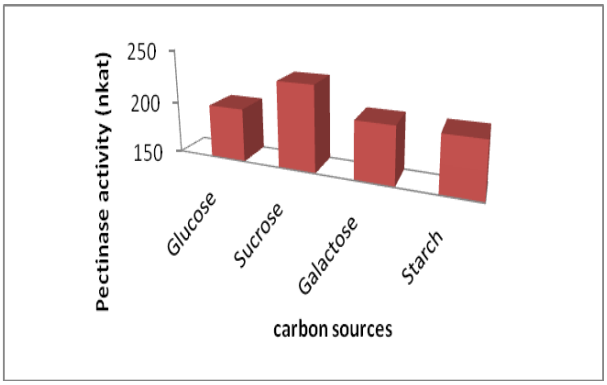

Figure 6: Effect of carbon sources on pectinase activity of *Aspergillusniger*

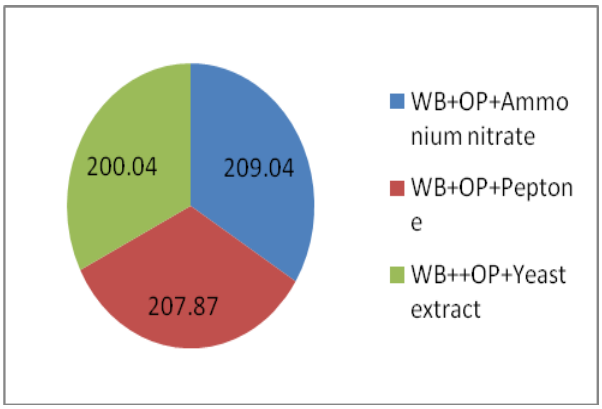

Figure 7: Effect of Nitrogen sources on pectinase activity from *Aspergillusniger*

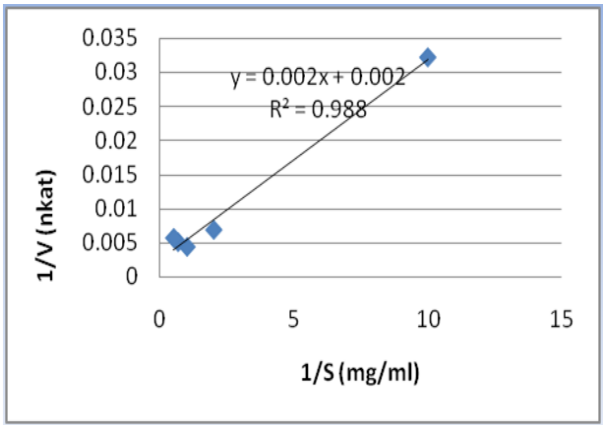

Figure 8: Effect of Nitrogen sources on pectinase activity from *Aspergillus niger*

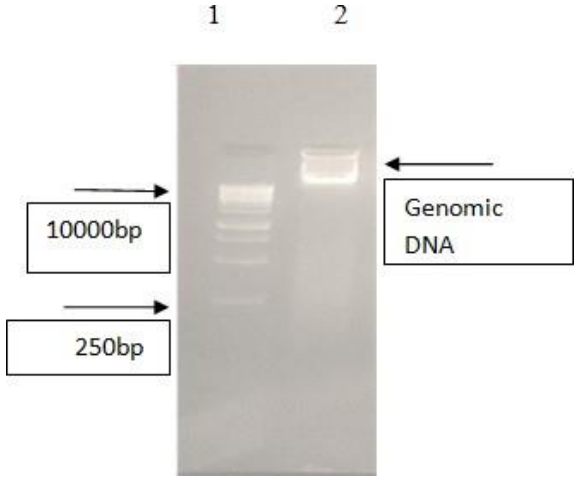

Figure 9: Isolated genomic DNA from Soil fungi (*A.niger*)

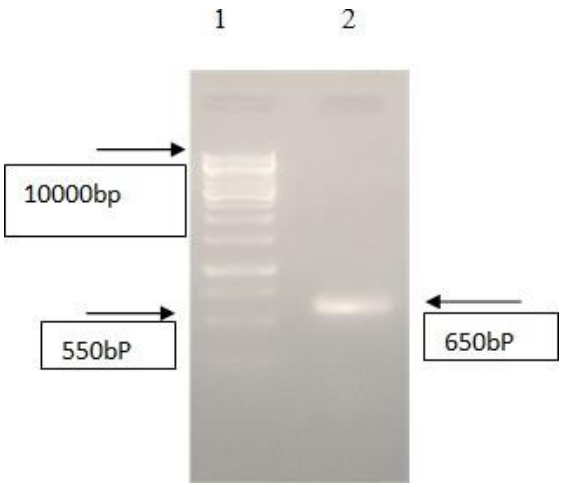

Figure 10: Amplified ITS region

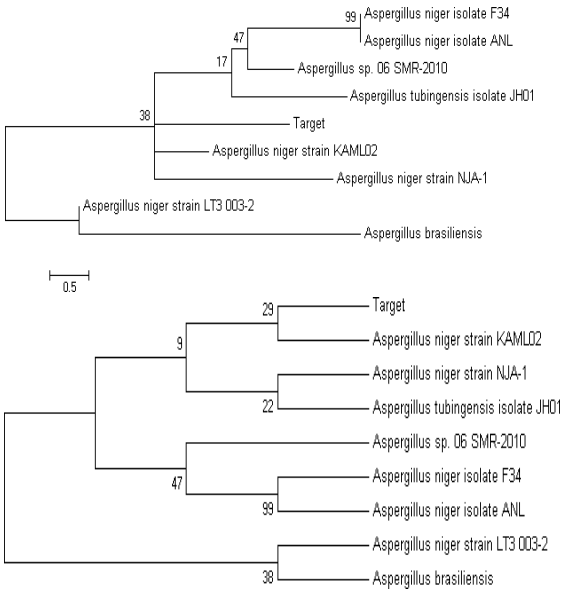

Figure 11: Phylogenetic pattern of the isolates

The influence of temperature was depicted in the Figure 3. At 30°C, the enzyme activity was at high titre (209.04 nkat). Similar studies indicated that the optimum temperature for the production of pectinase was at 30°C (Ramanujam *et al.,* 2008)

whereas Frietas *et al.,* (2006) reported that exo-polygalactouronase from *Monascus* and *Aspergillus* sp., showed maximum activity at 60°C and 50°C, respectively.

Effect of pH on the production of pectinase was evaluated (Figure 4). The optimum pH revealed that the enzyme was highly active at pH 5.5 in which the enzyme activity was 225.04nkat. This is in accordance with the result that the optimum pH for pectinase activity from thermo tolerant *Aspergillus* sp. N12 was 5.5 (Sayem *et al.,* 2006). The effect of pH on enzyme synthesis has been studied and pH 4.0-5.5 was reported for *Aspergillus terreus* and *A. niger* (Frietas *et al.,* 2006).

Optimization of Medium

The fermentation period was depended upon the composition of the medium, its concentration and the organism grown (Patil *and* Dayanand 2006). All the agro-industrial waste materials contain pectin as the cell biomass and could be used as substrate for the production of the pectinase (Thangaratham and Manimegalai, 2014). Substrates should provide required nutrients to the microorganism. Various substrates that are being used include sugarcane bagasse (SB), wheat bran (WB), rice bran (RB), pomegranate peel (PP) at the concentration of 20% along with orange peel (OP)10% (Figure 5). The medium containing wheat bran (20%) along with orange peel (10%) had showed the maximum activity of 235.04nkat.

The influence of various carbon sources such as glucose (1%), sucrose (1%), galactose (1%) and starch (1%) in the medium containing orange peel 10% and wheat bran 20% were analyzed (Figure 6). Among the different carbon sources that tested the medium containing sucrose and produced higher activity of 230.04nkat. These results were similar to earlier reports for pectinase production by *A. niger* in submerged and solid state fermentation (Patil and Dayanand, 2006). The influence of various nitrogen sources such as ammonium nitrate, yeast extract and peptone in the medium were examined (Figure 7). The addition of ammonium nitrate in the medium yielded the maximal activity of 209.04 nkat.

Km value of Pectinase

The Km value as an indicator of the affinity of pectinase to substrate was determined by using double reciprocal Line weaver and Burk plot. Pectinases share high degree of sequence homology but their rate of pectin hydrolysis differ, hence their kinetic constants also can differ. The graphical data presentation (Figure. 8) revealed that apparent Km value was calculated from the line weaver Burk plot. The Km was found to be 1.0 $mg.mL^{-1}$and V max was found to be 500 nkat. Nitinkumar *et al.,* (2010) reported a Km of 1.0 $mg.mL^{-1}$and Vmax of 85U.mg^{-1}protein for pectinase isolated from *Penicillium chrysogenum.* Also Km values less than 0.15 and up to 5.0 $mg.mL^{-1}$(<0.15-5.0 $mg.mL^{-1}$) were reported for some fungal pectinases (Banu *et al.,* 2010).

Molecular characterization of the strain

Based on the colony morphology and molecular analysis, the fungal strain was confirmed as *Aspergillus niger.* Genomic DNA was isolated from *A.niger* (Figure 9) and strain was identified by 18s RNA by amplification of ITS region using PCR (Figure 10). Nucleotide sequences of amplified ITS region was used for similarity analysis using Nucleotide Blast (BLASTN). The results showed greatest (99%) similarity with *A. niger* isolates (Buga *et al.,* 2010). The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura 3-parameter model (Tamura, 1992). The bootstrap consensus tree inferred from 500 replicates (Felsenstein, 1985) was taken to represent the evolutionary history of the taxa analyzed (Tamura *et al.,* 2011). Evolutionary analyses were done in MEGA5 (Tamura, 1992) (Figure 11).

CONCLUSION

Pectinase producer *Aspergillus niger* (soil fungi) isolated from soil sample collected at Porur Vegetable Market, Chennai, Tamil Nadu using submerged fermentation suplimented with abundant bio mass wheat bran (20%) and orange peel (10%) as substrate was achieved on first time in this study. Microbial pectinases has much of commercial value and also has a positive effect on human health too. From the present study, it is concluded that, *Aspergillus niger* can be used for pectinase production using organic waste. This is a cost effective method with simple culture preparation and cheapest organic waste which was used as substrate. However, further investigative studies such as purification and pilot scale production of pectinase are needed to make use of the full potential of *Aspergillus niger*.

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Conflict of interest

There is no conflict of interest

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