World Applied Sciences Journal 12 (11): 2120-2128, 2011
ISSN 1818-4952

Sachaarification of Banana-agro Waste and Clarification of Apple Juice by Cellulase Enzyme Produced from *Bacillus pumilis*

[1]*R. Kanmani,* [1]*P. Vijayabaskar and* [2]*S. Jayalakshmi*

[1]CAS in Marine Biology, Annamalai University, Parangipettai-608 502, Tamilnadu, India
[2]Deptarment of PG Research Microbiology, Ayya Nadar Janaki Ammal (Autonomous), Sivakasi - 626 124, Tamilnadu, India

Abstract: Production of extracellular cellulase by a marine bacterium *Bacillus pumilis* isolated from Vellar estuary was studied. Cultural conditions were optimized in batch culture. Totally 36 morphologically distinct strains were isolated using cellulose agar medium. 28 strains were isolated from sediment sample and 8 strains from water sample. Screening of cellulase producers was made with the Congo red test as a preliminary study. The potential strains were selected by measuring the clear zone formed around the colony. The most potential strain was found to be the *Bacillus pumilis* with the highest zone formation of about 17mm. Temperature 30°C, pH 7 and 24 hours of incubation, 3% salinity were the optimal conditions for the maximum bacterial growth and the enzyme activity. The present study was also aimed to Sachaarification of banana-agro waste and Clarify the of apple juice. Sachaarification of banana-agro waste using cellulase enzyme in which the release of sugar at different incubation period was carried out and the reducing sugar was calculated using DNS method. At the end of 8th hr the maximum amount of reducing sugar obtained was 3.4 mg/ml and the sachaarification percentage was found to be 13%. Clarification of apple juice was carried out using cellulase enzyme and the clear juice was obtained indicating the effect of cellulase enzyme on juice clarification.

Key words: *Bacillus pumilis* · Cellulose · Enzyme activity · Sachaarification · SDS PAGE

INTRODUCTION

Enzymes play crucial roles in producing the food we eat, the clothes we wear, even in producing fuel for our automobiles. Enzymes are also important in reducing both energy consumption and combating environmental pollution. Cellulases refer to a group of enzymes which act together to hydrolyze cellulose into soluble sugars. They are distributed throughout the biosphere such as plants, animals and microorganisms. Cellulases from higher plants such as *Lantana camara* and *Cuscuta reflexa* are mostly involved in fruit ripening and senescence [1]. Few animals such as the blue mussel *Mytilus edulis* [2], the green mussel [3], the edible snail *Helix pomatia* [4], termites and protozoa [5] were reported as cellulase producers. Protozoa such as *Epidinium caudatum* and *Eudiplodinium ostracodinium*. Archaea such as *Sulfolobus solfataricus* [6] and *Pyrococcus furiosus* [7, 8] are also cellulase producers. However, microorganisms are considered to be the main source for cellulases with novel and high specific activities. Microbial cellulases are the most economic and available sources, because microorganisms can grow on inexpensive media such agriculture and food industries by-products. In the most familiar case of cellulase activity, the enzyme complex breaks down cellulose to beta-glucose. This type of cellulase is produced mainly by symbiotic bacteria in the ruminating chambers of herbivores. Aside from ruminants, most animals (including humans) do not produce cellulase in their bodies and are therefore unable to use most of the energy contained in plant material. Cellulose is the most abundant organic biopolymer on earth with an estimated annual production of 180 billion tons in nature [9].

Cellulose can be converted into glucose by either chemical, physical treatments or enzymatic hydrolysis. Acid or high temperature degradation is unsatisfactory, because the resulting sugars are partly decomposed. Cellulosic materials, its derivatives and polymers with glycosidic linkages are substrates of cellulolytic enzymes. Also, the cellulosic wastes contain impurities that may generate unwanted by-products under these harsh conditions [10].

Corresponding Author: R. Kanmani, CAS in marine Biology, Annmalai University, Parangipettai-608502, Tamilnadu, India.
E-mail: kanmanir80@yahoo.in / baski_bos@yahoo.co.in.

Biological degradation of cellulose by cellulases is preferred for industrial purposes due to the high yields of desired hydrolytic products with minimal by-products [11]. Effective utilization of cellulosic material through bioprocesses will be an important key to overcome the shortage of foods, feed and fuels, which the world may face in the near future, because of the explosive increase in human population [12]. Cellulases have a wide range of applications. The main potential applications are in food, animal feed, textile, fuel and chemical industries. Other areas of application include the paper and pulp industry, waste management, medical and pharmaceutical industry, protoplast production, genetic engineering and pollution treatment. The cellulase enzymes have attracted considerable attention in recent years due to their great biotechnological and industrial potential. Conversions of food industries and agriculture wastes to valuable sugars are the great uses of cellulase enzymes [13].

This work aimed to optimize the cellulase enzyme production and also to check the purification and clarification ability of cellulase enzyme produced by *Bacillus pumilis*.

MATERIALS AND METHODS

Collection of Samples: Water and sediment samples were collected from Uppanar Estuary (Cuddalore District) and samples were transferred to the laboratory in an ice box maintained at 4°C for further study.

Isolation and Screening of Potential Strains: Serially diluted samples were spreaded on each cellulolytic agar plates containing (g/L) $MgSo_4$-0.01; $(NH4)_2So_4$-0.02; KH_2Po_4-0.7; K_2HPO_4-0.05; Cellulose-0.1; Agar-1.5; pH-7.0 ± 0.2 at 25°C. To visualize the hydrolysis zone, the plates were flooded with an aqueous solution of 0.1% Congo red for 15 min and washed with 1 M NaCl [14]. To indicate the cellulase activity of the organisms, diameters of clear zone around colonies on cellulose agar plates were measured. The cellulase activity of each culture was measured by determining the amount of reducing sugars liberated by using a dinitrosalicylic acid (DNS) method [15]. A bacterial isolate with the highest cellulase activity was selected for optimization and mass scale of cellulase production.

Bacterial Identification: The bacterial isolates were presumptively identified by means of morphological examination and biochemical characterization. The parameters investigated included colonial morphology, Gram reactions, endospore formation, catalase production, MR-VP reaction, Indole production, motility, glucose fermentation, starch hydrolysis, citrate utilization and gelatin hydrolysis. The results were compared with Bergey's Manual of Determinative Bacteria [16].

Process of Optimization: The factors like temperature, pH, salinity, different substrates and substrate concentration affecting the production of cellulolytic enzyme were optimized by adopting search techniques i.e. varying parameters one at a time. The experiments were conducted in 250ml Erlenmeyer flasks containing production medium. After sterilization by autoclaving the flasks were cooled and inoculated with cultures at 30°C for 72hrs under various experimental conditions as described below. The fermentation was carried out at various temperatures like 25°C- 45°C to study their effect on enzyme production, the pH of the aqueous solution was varied from 3 to 11 with 1N HCL and 1N NaOH. The medium was prepared with different salt concentration from 1 to 5%. The effect of different substrates on cellulase production was studied with different substrates like corn cobs, rice bran, wheat bran, cellulose and carboxymethylcellulose. The effect of different substrate concentrations on cellulase production was studied for maximum enzyme activity ranging from 1 to 5%.

Mass Scale Culture of Cellulase Producing Bacteria: Based on the results obtained through the optimization, mass scale culture of the cellulase producing bacteria was carried out. Inoculation was made with 500ml of production media by adding 1% (v/v) of inoculum. The fermentation was carried out in 1000ml Erlenmeyer flasks on a rotary shaker (300rpm). The biomass and the enzyme activity were tested at every 6hrs interval. At the end of the 36th hour the culture was harvested for the recovery of cellulase enzyme.

Estimation of Enzyme Activity: Cellulase activity was measured by a DNS method, through determination of reducing sugars liberated [17]. 0.5 ml of CMC solution, 0.5 ml of crude enzyme and 0.5 ml of 0.05 M citrate buffer pH 4.8 were incubated for 30 minutes at 50°C before adding 2 ml of DNS solution. The treated samples were boiled for 15 min prior to cool down in cold water for color stabilization. The optical density was read at 540 nm against reagent blank by a spectrophotometer (Thermo Spectronic, USA). Protein concentrations were determined by using a Lowry's method with bovine serum albumin (BSA) as a standard [18].

Fig. 1: Sachaarification of banana agro-waste using cellulase enzyme

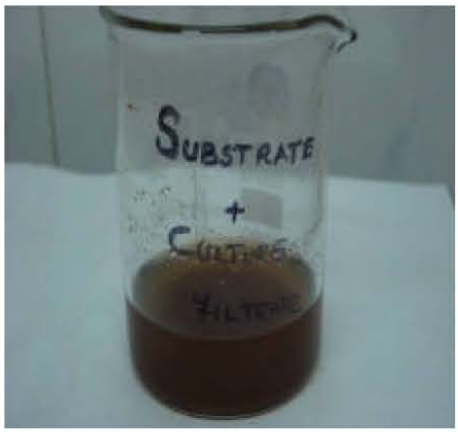

Fig. 2: Beaker containing the substrate along with culture filtrate

Extraction and Purification: The various steps of enzyme purification were carried out at 4°C unless otherwise stated. In the initial purification step, the supernatant containing the extracellular enzyme was treated with different saturation levels of solid ammonium sulphate (40, 60 and 80% saturation levels), with continuous overnight stirring. The precipitated enzyme was collected by centrifugation (10,000 rpm for 15 mins) and dissolved in 0.1M citrate phosphate buffer (pH 5.0). The enzyme solution was dialyzed in a dialysis membrane No.150 (HIMEDIA) against the same buffer for 48hrs with several intermitter buffer changes. The partially purified enzyme obtained was lyophilized in to a powder and the cellulase activity was assayed.

Determination of Molecular Weight by SDS-PAGE: Samples were solubilized in reducing sample buffer and equal amount of protein was loaded into 12% SDS-PAGE (Polyacrylamide gel and electrophoresis) was carried out at constant current (30mA).

Application Study

Sachaarification of Banana Agro-waste Using Cellulase Enzyme: The banana agro waste like pseudo stem and leaves were freshly collected and washed thoroughly with water and air dried (Fig.1). It was ground to powder and used as a substrate for sachaarification. A suspension of substrate (1g/100ml) was prepared in 50mM acetate buffer (pH5.0) and autoclaved at 15 lbs for 20 mins for sterilization and also heat treatment prior to release of sugar. 15ml of substrate suspension was taken and 5ml of culture filtrate was added to it (Fig. 2). Sachaarification was done in water bath at 27 ± 2°C for 24 hrs and centrifuged at 2500 rpm for 15 mins. The resultant supernatant was assayed for total reducing sugars using DNS method [15]. Different incubation period (1st to 8th hr) was carried out for the release of sugar. The release of sugar was expressed as equivalent to glucose. The percentage sachaarification was calculated as: Sachaarification (%) = Glucose (mg/ml) × 100/substrate (mg/ml).

Clarification of Apple Juice Using Cellulase Enzyme: Cellulase is one of the most widely used enzymes in clarifying fruit juices. Clarification of the apple juice was carried out in this work. Generally the cell wall of the apple fruit is made up of pectin. In order to produce clear apple juice it is absolutely necessary to remove pectin from it. This is achieved using cellulase enzyme. The activity takes about 50 minutes. Cloudy pure apple juice (5ml) and cellulase enzyme (1ml) was used in this experiment. Two test tubes were used one as a control and other as test. 1ml of water and 5ml of cloudy apple juice was taken in a first tube. Similarly 1ml of cellulase enzyme and 5ml of cloudy apple juice was placed in the second tube and the contents of the tubes were stirred well in order to mix the enzymes throughout the juice and kept in boiling water bath at 50°C and clarification of the apple juice was observed.

Test for starch and pectin was carried out to test the effectiveness of the enzyme treatment. In the starch test 10ml of the juice was heated in boiling water bath at 70°C and it was cooled down. Then 2 to 3 drops of 1% iodine in 10 % potassium iodide solution was added. Blue colour indicates the presence of starch, brown indicates that the starch is partially broken and yellow colour indicates the absence of starch. In the pectin test 1 part of juice was added to 1.5 parts of acidified ethanol and it was tested for flocculation after 15 minutes. If flocculation occurs the pectin is still present.

RESULTS

Isolation and Screening of Potential Strains: The bacterial density was found to be maximum in sediment sample ($4.3X10^9$ CFU/g) when compared to the density in water sample ($4.5X10^6$ CFU/g). Totally 36 morphologically distinct strains were isolated using cellulolytic agar medium of which 28 strains were from sediment sample and 8 strains from water samples. Screening of bacteria was conducted by using the Congo red test as a preliminary study for identifying cellulase producers. The potentiality of the strains was checked by measuring the clear zone formed around the colony. Among all 10 strains were identified as most potential strains. They belonged to the Genus *Bacillus, Klebsiella* and *Pseudomonas* (Fig. 3). The most potential strain was found to be the *Bacillus pumilus* with the highest zone formation of about 17mm (Fig. 4).

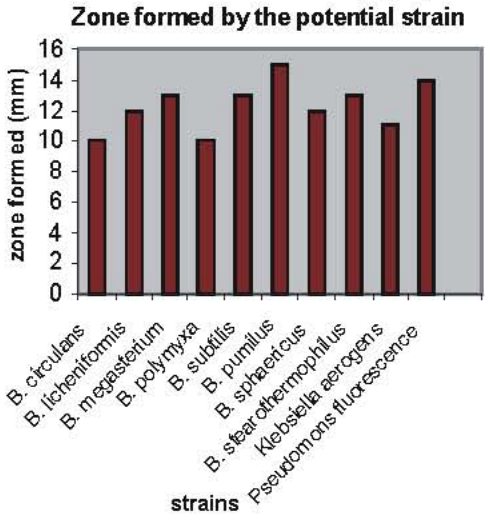

Fig. 3: Identified potential strains and their ability of zone formation

Fig. 4: Zone of clearance in the cellulose agar medium indicating the hydrolysis of cellulose

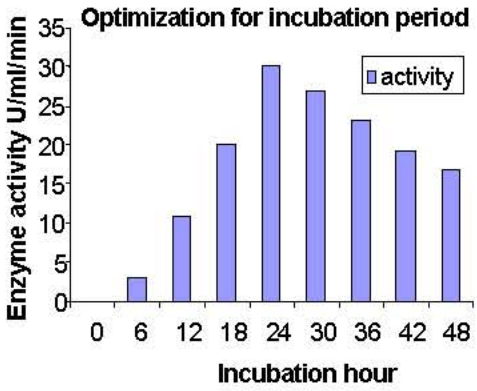

Fig. 5: Enzyme activity at different incubation period

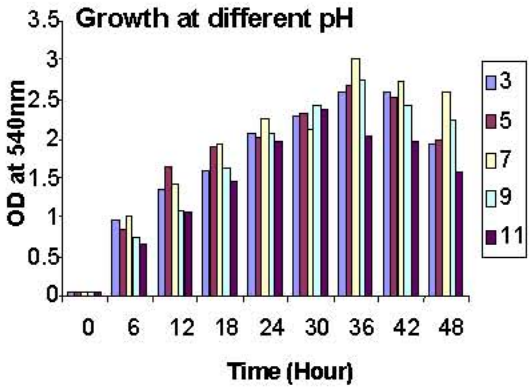

Fig. 6: Showing bacterial growth at different pH

Bacterial Identification: The isolated potential strains were identified by biochemical tests following the method of Bergy's manual and they were identified as *Bacillus circulans, Bacillus licheniformis, Bacillus megaterium, Bacillus polymyxa, Bacillus subtilis, Bacillus stearothermophilus, Bacillus pumilus, Bacillus sphaericus, Klebsiella aerogens and Pseudomonas fluorescence*.

Process of Optimization: Among the different incubation period studied, the optimum incubation period for the bacterial growth was observed at 36th hr and the maximum cellulolytic activity occurred at 24th hour of incubation (Fig. 5). For cellulase production the pH and the enzyme activity was found to be 7.0 and the enzyme activity was about 40 U/ml/min (Figs. 6,7). The optimum temperature for growth was found at 35°C. The enzyme activity was found to be 35 U/ml/min at 35°C (Figs. 8, 9). From the different salinity conditions studied, the growth and the enzyme activity was found to be maximum at 3%. The enzyme activity was about 39 U/ml/min (Figs. 10, 11). Among the different substrates used, the maximum growth and the activity was found in corn cobs.

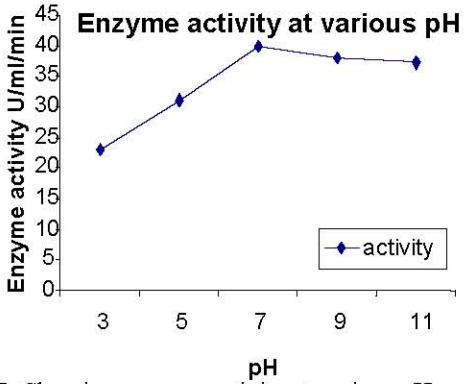

Fig. 7: Showing enzyme activity at various pH

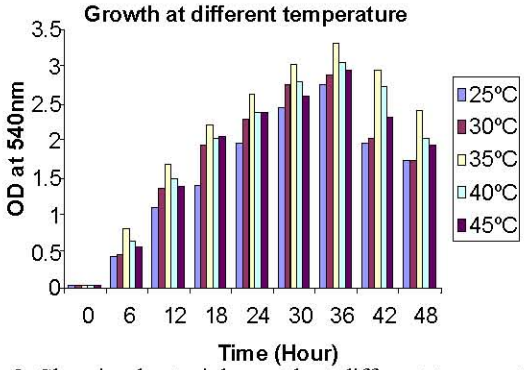

Fig. 8: Showing bacterial growth at different temperature

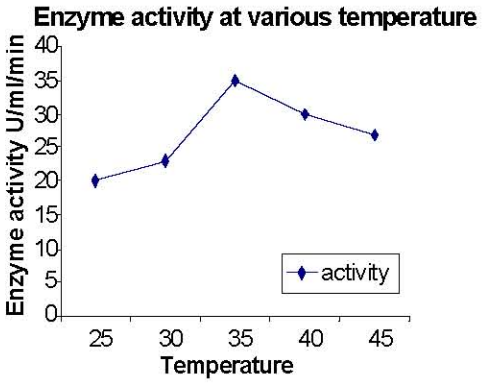

Fig. 9: Showing enzyme activity at various temperature

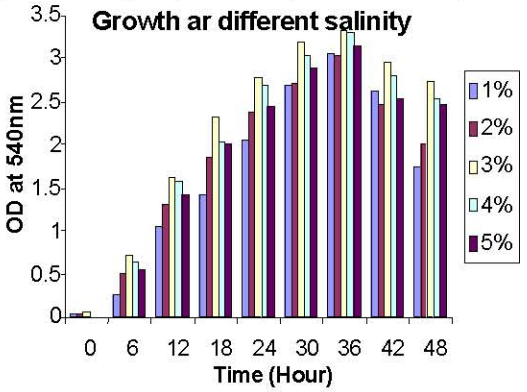

Fig. 10: Showing bacterial growth at different salinity

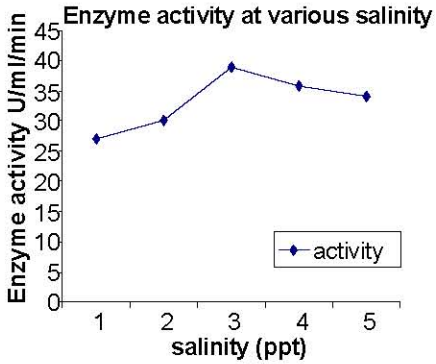

Fig. 11: Showing enzyme activity at various range of salinity

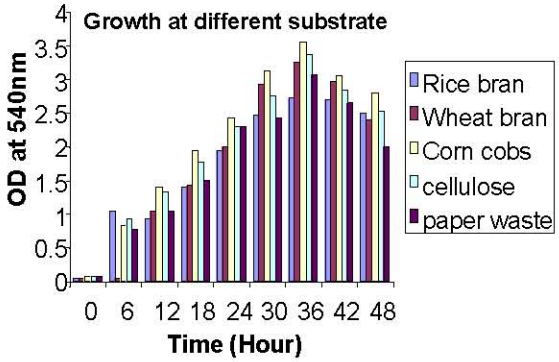

Fig. 12: Showing bacterial growth at different substrates

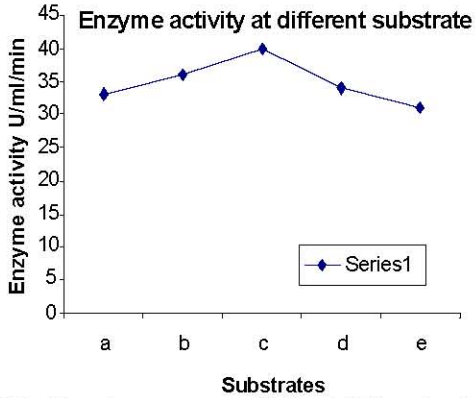

Fig. 13: Showing enzyme activity at different substrates

The enzyme activity was about 40 U/ml/min (Figs. 12,13). Among the different substrate concentrations used, the maximum growth and the enzyme activity was found at 3%. The enzyme activity was found to be 43 U/ml/min (Figs. 14, 15). The most potential strain *Bacillus pumilus* was selected for mass culture using the optimized condition. The 500ml of cell free supernatant was obtained. The extracellular cellulose enzyme was precipitated with varying amount of ammonium sulphate (40, 60 and 80%). The 80% gave the maximum precipitation of protein (0.84g) followed 60% (0.56g) and

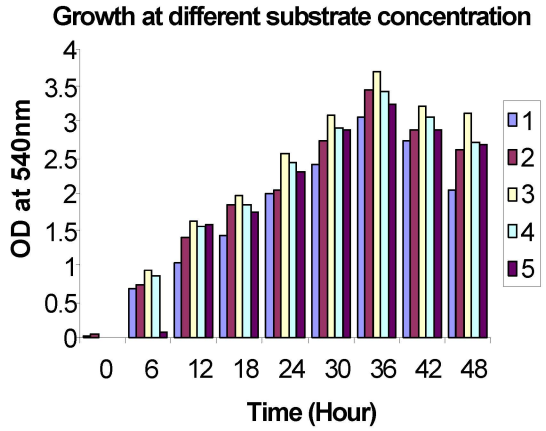

Fig. 14: Showing bacterial growth at different substrate concentration

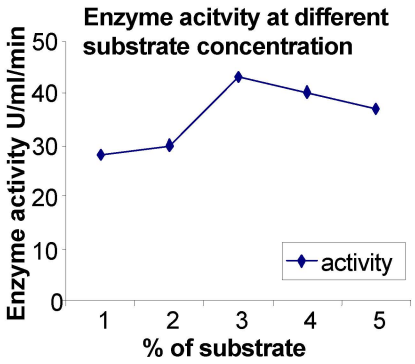

Fig. 15: Showing enzyme activity at different substrate concentration

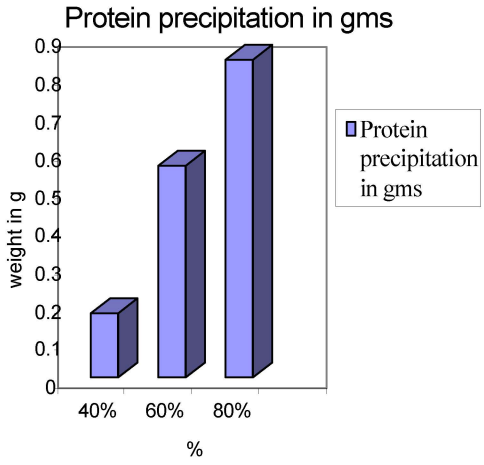

Fig. 16: Showing Protein Precipitation

40% (0.17g) (Fig. 16). The purification started with the dialysis in a regenerated seamless cellulose tube against phosphate buffer for 24-48 hours. The dialysis membrane is partially permeable and has a molecular weight cut off between 12,000-4000. The pore size is 2.4 nm meters.

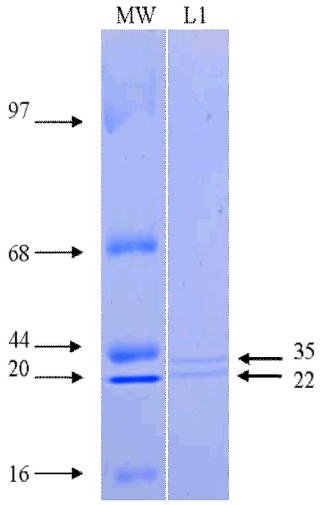

Fig. 17: SDS- PAGE of purified cellulase MW - Standard molecular weight marker, L1 - Purified cellulase.

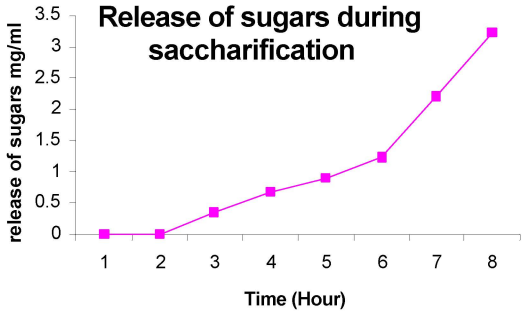

Fig. 18: Showing the time course for release of sugar during sachaarification

SDS-PAGE: The results exhibited that the purified cellulase enzyme has a molecular weight range of 22-35 KDa. Protein concentrations were determined by using a Lowry's method and it was found to be 0.33U/mg protein (Fig. 17).

Sachaarification of Banana Agro-waste Using Cellulase Enzyme: Synthesized cellulase form *Bacillus pumilus* was used for saccharification of banana agro waste. The cellulolytic enzyme complex when incubated with the agro waste released sugars. The degree of sachaarification was assayed on the basis of release of reducing group. The amount of reducing sugars increased with time of incubation in the presence of the enzyme. The maximum amount of reducing sugars was released at the end of 8thhr. The steam treated agro-waste yielded 3.4 mg/ml of reducing sugars after 8th hr. Likewise the percentage of sachaarification was calculated and it was found to be 13% at the end of 8th hr (Figs.18, 19).

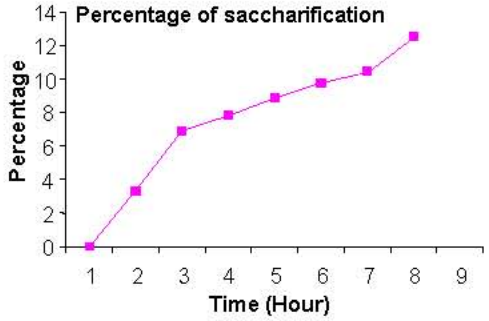

Fig. 19: Showing the Sachaarification (%)

Fig. 20: Clarification of the apple juice using cellulase enzyme

Clarification of the Apple Juice Using Cellulase Enzyme: The first tube was the control tube (Left) and the second was the culture tube in which the clarification of the apple juice was clearly observed. A clear apple juice was obtained by the removal of pectin. The activity takes about 50 minutes. Starch test showed negative result by forming yellow colour indicating the absence of starch after the addition of iodine solution. Likewise pectin test was also carried out and the flocculation was not observed after 15 minutes which indicates that the pectin is broken down (Fig. 20).

DISCUSSION

In the present study, a total of 36 bacterial strains were isolated from Uppanar estuary, in which 10 most potential cellulase producing bacterial strains were identified. Similarly, a total of 125 bacterial isolates including 62 Gram positive rods, 10 Gram positive cocci, 32 Gram negative rods and 24 Gram negative cocci were isolated from 39 samples of soils, decomposing logs and composts. When applied the Congo red test, 62 bacterial isolates showed positive results with clear zone ranging from 1 to 7 mm [17].

Likewise a total of 9 strains of bacteria and 2 strains of actinomycetes were obtained from MARDI (Malaysian Agricultural Research and Development Institute). The strains were isolated from oil palm empty fruit bunch (EFB) heap meant for composting. As they were isolated from rotten lignocellulose, there is high chance that the strains are cellulase producer. Therefore, a preliminary qualitative analysis for cellulolytic activity was conducted by using Congo red dye [19]. Vetriselvi *et al*. [20] isolated five species of bacteria namely *Pseudomonas, Proteus, Micrococcus, Serratia and Bacillus* and five species of fungi viz. *Aspergillus niger, Trichoderma harzianum, Myrothecium roridum, Curvularia lunata* and *Fusarium oxysporum* isolated from agricultural crop and their cellulolytic activity was also studied.

Production of cellulase from *Aspergillus niger* and *Bacillus subtilis* using pine apple peel as substrate by solid state fermentation was carried out in which the maximum enzyme yield was obtained in *B. subtilis* when compared to *A. niger* [21]. Nitrogen fixing *Paenibacillus* strains were isolated from soil in N-free media. Three of these cellulase positive isolates with CMCase were identified as *Paenibacillus* strain E, H and SH [22].

In the present study a preliminary qualitative analysis for cellulolytic activity was conducted by using Congo red dye. The bacteria were grown on cellulose agar plates. The plates were flooded with Congo red solution (1% w/v) for 15 min and the solution was poured off and further it was flooded with 1M NaCl for 15 min. The formation of a clear zone of hydrolysis indicated cellulose degradation. The ratio of the clear zone diameter to colony diameter was measured in order to select for the highest cellulase activity producer. The largest ratio was assumed to contain the highest activity. Ariffin [19] described, the screening of bacteria was conducted by using the Congo red test as a preliminary study for selecting cellulase producers. About 9 bacterial strains (EB1-EB9) showed positive result in congo red test,of which EB3 showed the highest ratio of clear zone diameter to colony diameter indicating more cellulose degradation in CMC agar plates. *Salinivibrio* sp. NTU-05, isolated from *Szutsau saltern* exhibited evident clear zones around the colonies on CMC agar plates following staining with 1% Congo red solution, indicating that it secretes prominent amounts of cellulose [23].

Media optimization for cellulase production was carried out using different parameters like pH, temperature, salinity, substrate and substrate concentration. The optimum pH for cellulase production was found to be 7 and the same was observed in purification and characterization of a novel halostable

cellulase from *Salinivibrio sp.* strain NTU-05 which retained the maximum enzyme activity at pH 7 [23]. In the purification and characterization of CMC isolated from a marine bacterium, *Bacillus subtilis A-53* the optimal pH for the CMCase activity was found to be at 6.5 [24].

Optimum temperature for cellulase production was found at 35°C. The same optimal temperature was observed for the cellulase production using pineapple peel as a substrate [21]. Effect of temperature on activity and stability of CMCase produced by a marine bacterium *Bacillus subtilis* was found to be at 50°C [24]. The optimal temperature of the purified cellulose was found to be at 70°C in *Salinivibrio* sp. strain NTU-05 [23].

The optimal salinity in the present study was found to be 3%, whereas in another study the optimal concentration of NaCl was 5% in purification and characterization of a novel halostable cellulase from *Salinivibrio* sp. strain NTU-05 [23]. Among the different substrates used corncobs showed increased enzyme activity in the study and the substrate concentration was about 3%. In contrast Sunitha *et al.* [21] found, *Bacillus subtilis* produced the peak enzyme activity when grown on the increased pineapple peel substrate concentration (10%).

Purification of cellulase was carried out using SDS-PAGE analysis. The present study results were exhibited that, the purified cellulase enzyme had a molecular weight range of 22-35 kDa. In another study purified CMCase from *Bacillus pumilus* has a molecular weight range of 30-65 kDa [19]. The molecular mass of the bacterial strains, CH43 and HR68 estimated from SDS-PAGE was 40kDa [25]. A halostable cellulase with a molecular mass of 29kDa was purified using SDS-PAGE [23].

Sachaarification of banana agro waste using *Bacillus pumilus* synthesized cellulolytic enzyme was carried out in which the maximum amount of reducing sugar 3.4mg/ml was obtained at the end of 8th hr. and the sachaarification (%) was found to be 13%. The steam treated agro waste yielded 1.34mg/ml of reducing sugar by the cellulases of *T. lignorun* [26]. The clarification of the apple juice was carried out using cellulase enzyme. Similarly a clear juice was obtained by the removal of pectin indicating the presence of cellulase enzyme. The activity takes about 50 minutes. Starch and pectin test was performed to test the effectiveness of enzyme treatment and both showed negative results. Fatma *et al.* [27] made an experiment on production of bioethanol via enzymatic sachaarification of rice straw by cellulase produced by *Trichoderma Reesei* under solid state fermentation. In their observation, the maximum glucose yield was obtained after 16 hrs and it was found 1.07%.

It was concluded that from economic point of view *Bacillus pumilis* was optimized in various production parameters like pH, temperature, NaCl concentration and substrate concentration. So it can be used for cellulase production on cheaper and more easily available recourses than on expensive and refined substrates and also to check the purification and clarification ability of cellulase enzyme produced by *Bacillus pumilis.*

REFERENCES

1. Chatterjee, U. and G.G. Sanwal, 1999. Purification and properties of a protein from *Lantana camara* activating *Cuscuta reflexa* cellulose. Phytochem. 52: 361-366.
2. Bingze, X., U. Hellman, B. Ersson and J. Janson, 2000. Purification, characterization and amino acid sequence analysis of a thermostable, low molecular mass endo-ß-1,4-glucanase from blue mussel, *Mytilus edulis.* Eur. J. Biochem. 267: 4970-4977.
3. Marshall, J.J., 1973. Preliminary report on the digestive enzymes present in the digestive gland of *Penrna viridis.* Comp. Biochem. Physiol., 44: 981-988.
4. Maeda, I., Y. Shimohigashi, H. Kihara and M. Ohno, 1996. Purification and characterization of a cellulase from the giant snail *Achatina fulica.* Biosci. Biochem. 60: 122-124.
5. Konig, H., J. Frohlich, M. Brechtold and M. Wenzel. 2002. Diversity and micro habitats of the hindgut flora of termites. Recent Res. Devel. Microbiol., 6: 125-156.
6. Moracci, M., A. Trincone, C. Ponzano, G. Perugino, M. Ciaramella and M. Rossi. 2001. Enzymatic synthesis of oligosaccharides by two glycosyl hydrolases of Sulfolobus solfataricus. Extremophil., 5: 145-152.
7. Voorhorst, W.G.B., Y. Gueguen, A.C.M. Geerling, G. Schut, I. Dahlke, M. Thomm, J. Van der oost and W.M. De vos, 1999. Transcriptional regulation in the hyperthermophilic archaeon *Pyrococcus furiosus*: coordinated expression of divergently oriented genes in response to ß-linked glucose polymers. J. Bacteriol., 181: 3777-3783.
8. Anandapandian, K.T.K., D. Christybapita, and A.K. Chandrika, 2002. Optimization studies on the extracellular Carboxy methyl Cellulase (CMCase) producing fungal cultures. Indian J. Environ. and Ecoplan. 6(3): 467-472

9. Amor. Y., C. Haigler, S. Johnson, M. Wainscott and D. Delmer, 1995. A membrane associated form of sucrose synthase and its potential role in synthesis of cellulose and calose in plants. Proc. Natl. Acad. Sci. USA., 92: 9353-9357.
10. He, D., L. Bao, Y. Long, W. Wei and S. Yao, 2000. A new study of the enzymatic hydrolysis of carboxymethyl cellulose with a bulk acoustic wave sensor. Talanta. 50: 1267-1273.
11. Parry, N.J., D.E. Beever, E. Owen, I. Vandnberghe, J.V. Beeumen and M. Bhat, 2001. Biochemical characterization and mechanism of action of a thermostable ß-glucosidase purified from *Thermoascus aurantiacus*. Biochem. J., 353: 117-127.
12. Ohmiya, K., K. Sakka, S. Karita and T. Kimura, 1997. Structure of cellulases and their applications. Biotechnol. Genetic Engg. Rev., 14: 365-413.
13. Bothast, R.J. and B.C. Saha, 1997. Ethanol production from agricultural biomass substrates. Adv. Appl. Microbiol., 44: 261-286.
14. Apun, K., B.C. Jong and M.A. Salleh, 2000. Screening and isolation of a cellulolytic and amylolytic *Bacillus* from sago pith waste. J. Gen. Appl. Microbiol., 46: 263-267.
15. Miller, G.C., 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Analytical Chem., 31: 426-428.
16. Buchanan, R.E., N.E. Gibbons, S.T. Cowan, T.G. Holt, J. Liston, R.G.E Murry, C.F. Niven, A.W. Ravin and R.Y. Stainer, 1974. Bergy's Manual of Determinative Microbiology, eds; Williams an Wilkins Co: Baltimore.
17. Krootdilaganandh, J., 2000. Isolation and selection of thermotolerant bacteria capable of producing cellulase. Chiang Mai: Chiang Mai University Press, pp: 20-21.
18. Lowry O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall, 1951. Protein measurement with the folin-phenol reagent. Biol Chem. pp: 265-275.
19. Ariffin, H., N. Abdullah, M.S.U. Kalsom, Y. Shirai and M.A. Hassan, 2006. Production and characterization of cellulase by *Bacillus pumilus* EB3. Inter. J. Engg. Tech., 3(1): 47-53.
20. Vetriselvi, J., C. Muthukumar, G. Jayanthi and R. Saravanamuthu, 2007. Studies on the potentiality of cellulose degradation by the micro organisms associated with crop residues in the soil. Asian J. Microbiol. Biotech. 9(1): 91-94.
21. Sunitha, M., E. Rani and K. Devaki. 2006. Production of cellulase from *Aspergillus niger* and *Bacillus subtilis* using pine apple peel as a substrate by solid state fermentation. Asian J. Microbiol. Biotech. 8(3): 577-580.
22. Emtiazi, G., M. Pooyan and M. Shamalnasab, 2007. Cellulase activities in nitrogen fixing *Paenibacillus* isolated from soil in N- free media. W. J. Agri. Sci., 3(5): 602-608.
23. Wang, C.Y., Y.R. Hsieha, C.C. Nga, H.Chanb, H. Linc, W.S. Tzenga and Y.T. Shyua, 2009. Purification and characterization of a novel halostable cellulase from *Salinivibrio* sp. strain NTU-05. Asian J. Microbiol. Biotech. 7: 311-315.
24. Kim, B.K., B.H. Lee, Y.J. Lee, I.H. Jin, C.H. Chung and J.W. Lee, 2009. Purification and characterization of carboxymethylcellulase isolated from a marine bacterium, *Bacillus subtilis subsp. subtilis*. Asian. J. Microbiol. Biotech. 53: 220-224.
25. Mawadza, C., R.H. Kaul, R. Zvauya and B. Mattiasson, 2000. Purification and characterization of cellulases produced by two bacillus strains. J. Biotech. 83: 177-187.
26. Baig, M.M.V., M.L.B. Baig, M.I.A. Baig and M. Yasmeen, 2004. Saccharification of banana agro waste by cellulolytic enzymes. Afri. J. Biotechnol., 3(9): 447-450.
27. Fatma, H., A.E. Zaher and M. Fadel, 2010. Production of bioethanol via enzymatic saccharification of rice straw by cellulase produced by *Trichoderma Reesei* under solid state fermentation. New York Science J., 4: 176-182.