Biotechnol Res 2018; Vol 4(4):109-113
eISSN 2395-6763

Copyright © 2018 Viswanath *et al*
This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/2.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

ORIGINAL RESEARCH

Characterization of a Cellulase Producing *Pseudomonas fluorescens* Isolated from Agricultural Waste

Vinod VISWANATH[1*]. A. K BEENA[1] , Jefly D SILVA[2]
[1]Department of Dairy Microbiology, College of Dairy Science and Technology, Mannuthy, Thrissur, Kerala, India-680651
[2]Presentation College of Applied Sciences, Ernakulum, Kerala, India-683594
*Corresponding Author email: vinodkomattil@gmail.com

• Received: 08 August 2018 • Revised: 05 October 2018 • Accepted: 09 October 2018 • Published: 11 October 2018 •

ABSTRACT
Cellulases are a group of hydrolytic enzymes that are capable of degrading all types of cellulosic agricultural waste materials. In this study various agricultural wastes were screened for cellulase producers using CMC (Carboxy methyl cellulose) media. The bacterial isolate from sugarcane bagasse, was found to be the best cellulase producer. This isolate was identified as *Pseudomonas fluorescens* by 16S rRNA typing, the sequence of which is deposited in NCBI with accession number MF508700. The cellulase production potential of the isolate was further assessed quantitatively Dinitro-Salicylic acid (DNS). The isolate performed well at pH 5.0 and at an incubation temperature of 37ºC. Maximum yield was obtained after an incubation period of 48h. The isolate was found to be capable of producing cellulase from sugarcane, banana peel and vegetable waste (cucumber peel) at a level of 0.48U/ml, 0.33 U/ml and 0.23U/ml respectively under the experimental conditions. The crude enzyme extract was found to be a good clarifier of fruit juices. The isolate was found to be capable of utilizing whey, the major byproduct of dairy industry as a carbon source to produce bioethanol. The ability of the isolate to produce proteases and lipases is suggestive of high industrial potential of the isolate obtained in this work.

KEY WORDS: *Agricultural waste, cellulase, protease, lipase, Pseudomonas fluorescens fruit juice clarification, bioethanol*

INTRODUCTION

Cellulose is a renewable resource that can be converted into bio based products and bioenergy. With worldwide concerns over energy crisis and environmental pollution, any process to utilize this rich biomass is of utmost importance. Plant wastes (rice bran and sugarcane bagasse), forestry waste (sawdust, thinning, and mill wastes) and portions of municipal solid waste (waste paper) are very rich in cellulose. Cellulase enzyme hydrolyzes the β-1,4-glycosidic bonds in the cellulose molecule to release glucose units. Glucose so produced from cellulosic substrate could be further used as a substrate for fermentation which could yield valuable end products. Industrial application of cellulase includes textile (used in the formulation of washing powders) and also food industry (extraction of fruit and vegetable juices and in starch processing (Sethi *et al*., 2013). In spite of such industrial potential, cellulosic wastes are getting accumulated and is remaining underutilized. The reaction conditions and production costs has always been major constraints, and search for new organisms capable of producing cellulase with higher specific activities is still important. As cellulase producers are expected in cellulose rich plant based materials, in this study, an attempt was made to isolate and characterize a cellulase producer from sugarcane waste. The study also looked into the potential of

the isolate to produce the enzymes: protease and lipase along with production of ethanol from whey, the major byproduct of dairy which is often very difficult to be disposed because of its high biological oxygen demand (BOD). Utilization of whey as a substrate for fermentation opens up a way for reducing environmental burden.

RESULTS AND DISCUSSION

Cellulose rich plant waste like sugar cane bagasse, banana peel and vegetable waste were screened for isolation of cellulolytic organism. Colonies with hydrolytic zone on CMC agar was taken as cellulase positive. The size of transparent zone (mm) was considered as proportionate to the level of cellulase activity (Khatiwada *et al.*, 2016). Accordingly, maximum cellulase activity was observed for the isolate from sugarcane bagasse. Quantitative estimation also exhibited the same trend. (Table 1). Cellulase production was maximum by the isolate obtained from sugar cane bagasse which contained high cellulose (59%) Rezayati-Charani and Mohammadi-Rovshandeh, 2005; when compared to banana peel (22%) (Singanusong *et al.*, 2013 and cucumber (13%) Gross and Wang, 1984)**.** The higher cellulose content in sugar cane would have triggered the cellulase production of the isolate in a better way.

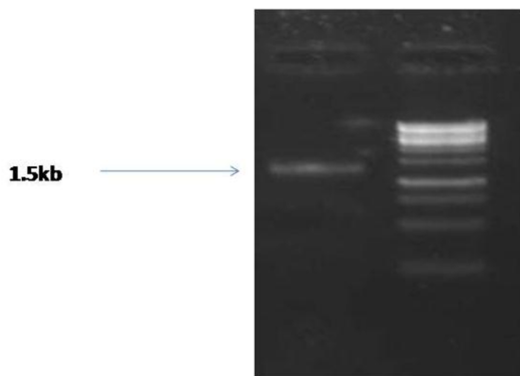
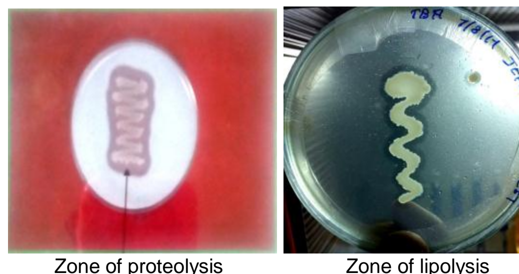


Figure 1: PCR amplified partial cellulase gene from *Pseudomonas fluorescens*

Circular moist translucent surface colonies in nutrient agar on microscopic examination revealed the presence of short gram negative rods .The isolate was found to be catalase , oxidase and citrate positive. The isolate was negative for Indole, Methyl red and Voges proskauer**.** The results were suggestive of *Pseudomonas* sp. Isolation of *Pseudomonas sp.* from agri-waste has been reported by Subathra Devi *et al.*, 2015. The sequence of the partial nucleotide sequence (1.5 Kbp) of 16S rRNA gene amplified from the genomic DNA conformed to *Pseudomonas fluorescens* (99%). The sequence is submitted to NCBI with the accession number MF508700 (Fig.1).



Zone of proteolysis Zone of lipolysis

Figure 2: *Pseudomonas flourecens* on skim milk agar and tributyrin agar

As per the results (Table 1) the maximum enzyme activity of 0.48U/ml was observed when sugar cane used as a substrate. Enzyme activity obtained for Banana peel and Cucumber were 0.33U/ml and 0.23U/ml respectively. Results clearly indicate that the presence of cellulose in the substrate proportionately triggers cellulase production. The observation in this study endorses the reports of Gross and Wang (1984). The plant waste materials are mostly composed of three major units: cellulose, hemi-cellulose and lignin (Facchin *et al.*, 2013). According to Anwar *et al.*, 2014 the cellulose content of plant waste ranges from 40-50%. Heffernan *et al.*, 2009 have already reported the use of *Pseudomonas fluorescens* in waste water treatment.

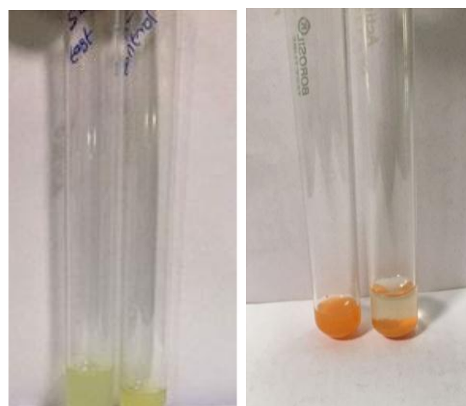


Figure 3: Fruit Juice Clarification of Sugarcane and Carrot Juice

Table 1: Enzyme assay for different substrates:

Substrates used	Volume (ml)	Total protein (mg/ml)	Total activity (U/ml)	Specific activity (U/mg of protein)
Sugar cane bagsse	5.0	0.120	0.48	4.0
Banana peel	5.0	0.125	0.33	2.64
Cucumber	5.0	0.226	0.23	1.02

Fruit juices are generally cloudy due to the presence of polysaccharides (pectin, cellulose, hemi cellulose, lignin and starch). Clarity of the fruit juice is an important factor that decides consumer acceptability (Vaillant *et al*., 2001). When the crude cellulase was allowed to act on juice for 30 minutes, both the juices became clearer. However in this study residual activity was found slightly lesser in sugarcane juice than carrot juice (Fig 2). Experimental conditions followed and compositional variations in the juice could have contributed to this observation. Under experimental condition, crude enzyme preparation was found to be a better clarifier for carrot juice than sugar cane juice. A further increase in cellulase concentration did not improve the extent of clarification. Subramaniam, (2015) reported the use of cellulase enzyme for clarifying orange juice *Phoma exigua* strain. Cellulase treatment for fruit juice clarification is reported to have a negative impact on the biochemical quality attributes of the fruit juice (Kumar, 2015).

Cellulolytic microorganism isolated in this study was, also found to have the potential to produce protease and lipase (Fig. 3) Production of thermostable proteases and lipases by *Pseudomonas fluorescens* has been reported **(**Christen and Marshall, 1984). The wide application of protease and lipase producers in waste treatment is well supported by Andualema and Gessesse, 2012.

Ethanol production potential of the isolate was also studied .Five percent (v/v) inoculum (*Pseudomonas fluorescens*) was inoculated into whey incorporated media and incubated at 37ºCfor 48 h. The fermentation yielded alcohol 0.718g/100ml. Alcohol production using sugar cane bagasse (Kumar *et al*., 2014) and whey (Sadik and Asmaa,2014) as substrate has been reported Cellulases are highly versatile biocatalysts that can be widely used in textile and food industries This work has just succeeded in isolating a bacteria with multi enzyme production. Well controlled studies are essential for process optimization and scaling up of the process.

CONCLUSION

The study endorses the potential of various agro-wastes to be used as substrates for the production of cellulase by *Pseudomonas fluorescens.* Cellulase production was maximum with sugarcane bagasse followed by banana peel and cucumber peels. The isolate obtained in this study was also found to be capable of producing ethanol utilizing whey as a carbon source. The fact that the cellulase producer used in this work produced protease and lipase enzymes highlights the tremendous potential of the isolate in agro-waste treatment.

MATERIALS AND METHODS

Isolation of cellulolytic bacteria

The cellulose rich plant wastes: sugar cane waste, banana peel and vegetable wastes (cucumber peel) from the various locations of Thrissur district were collected and screened for cellulolytic bacteria. Cellulolytic microorganisms were isolated by pour plate technique in carboxy methyl cellulose (CMC) medium g/L: CMC (10.0), NaCl (6.0), $(NH4)_2SO_4$ (1.0), KH_2PO_4 (0.5), Na_2HPO_4 (0.5), $MnSO_4$ (0.1), $CaCO_3$ (0.1),KCl (0.1)and Yeast extract (1.0) (Sreena *et al.*, 2015).The plates were incubated at 37°C for 24 h. To visualize the zone of hydrolysis (cellulase production), the plates were flooded with an aqueous solution of 0.1% Congo red for 15 minutes and washed with 1 M NaCl. The cellulolytic activity of organisms was assessed by looking into the hydrolytic zone around the growing colony on CMC agar. The most potential isolate was subjected to microscopic examination and biochemical characterization.

Molecular level identification of the isolate was done using 16S rRNA gene typing of the bacterial isolate. Genomic DNA was isolated using NucleoSpin® Tissue Kit (Macherey-Nagel) following manufacturer's instructions. 16S rRNA gene was amplified from the genomic DNA

using specific primers 16sF-5'- GAGTTTGATCCTGGCTCAG – 3' and 16sR 5' – GAATTACCGCGGCGGCTG – 3'. Nucleotide sequences of the PCR amplicon were identified using BLAST software (http://blast.ncbi.nlm.nih.gov). (GeneAmp PCR System 9700 using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems , USA).

Production of cellulase enzyme.

Cellulase production was determined on solid state substrate comprising of sugar cane bagasse and mineral media in the ratio 1:1 The residue of sugar cane after juicing was collected freshly from the market .It was dried in hot air oven at 80°C for 2h and then ground to a fine powder .This was used as the carbon source in the media for production of cellulase (Sreena *et al.*, 2015). All measures were taken to avoid extraneous contamination. Medium (Sugarcane substrate (powder) and culture mineral media containing (g/l)-$NaNo_3$-2g, K_2HPO_4-0.5g, $MgSO_4.7H_2O$-0.02g, $MnSO_4.7H_2O$-0.02g, $FeSO_4.7H_2O$-0.02 g, $CaCl_2.2H_2O$-5 g) was prepared and autoclaved at 121°C at 15 lbs for 20 minutes. Standardized inoculam (OD at 0.4 at 600nm) of the isolate was inoculated into the media at level of five percent and incubated at 37°C for 48 h. The fermented substrate was manually pressed for getting the extract. The extract was filtered through whatmans No.1 filter paper and the filtrate was subjected to refrigerated centrifugation at 10,000rpm for 20mins at 4°C. The supernatant served as the crude enzyme

Enzyme activity was determined as per Miller assay using 0.1 M sodium citrate buffer (pH-7) having one percent starch. One milliliter of this was mixed with equal quantity of crude enzyme and incubated 50ºC for 15 min. The reaction was terminated by addition of 2 ml of 3, 5 dinitrosalicylic acid (DNS) reagent. The color of the final solution was stabilized by the addition of one ml of 40% potassium sodium tartarate. The reducing sugar present in the final mixture was determined spectrophotometrically at 540 nm. The amount of reducing sugar released was determined by DNS method (Miller 1959). One unit (U) of cellulase activity was defined as the amount of enzyme that is required to release one μmol of reducing sugar per minute under standard assay conditions. The specific activity was determined as the number of units of enzyme activity per mg of enzyme protein. Total protein content present in crude enzyme was determined using Bovine Serum Albumin (BSA) as the standard (Lowry *et al.*, 1951).

Cellulases are widely used in juice clarification. The clarification potential of cellulase obtained in this work was determined for juice collected from sugar cane and carrot .For doing the test,10 ml of fruit juice was mixed with one milliliter of crude enzyme. Allowed the enzyme to act at 37°C for 30 minutes. The reaction was terminated by subjecting the sample to 50°C for 15 minutes. The reduction in the turbidity of the juice was taken as an indicator of clarification. Residual enzyme activity was assayed by DNS method to evaluate the clarification potential of the enzyme. Lesser residual activity was suggestive of better clarifying efficiency.

Production of protease and lipase enzyme

The ability of the isolate to produce protease and lipase enzyme was also assessed qualitatively by streaking on skim milk agar and tributyrin agar (TBA) medium (Harrigan 1998). Zone of clearance around the growth was suggestive of proteolytic and lipolytic potential.

Production of Bioethanol

Media for ethanol production comprised of 0.5% $(NH_4)_2SO_4$; 0.3% Yeast extract; 0.5% KH_2PO_4; 0.1% $MgSO_4$; and 0.01% $CaCl_2$ and 15.2% glucose in water.(Demuyakor and Ohta, 1993). To evaluate the potential of the isolate to utilize whey, glucose was replaced by 12.5 ml of clarified whey. Standardized inoculum of *Pseudomonas flourescens* was added at a level of five percent. After incubation at 37°C for 48 h. alcohol in the medium was assessed qualitatively using potassium dichromate (Salim *et al.*, 2015) and quantitatively by a spectrophotometric method using Tri-n-butyl phosphate (Sayyad and Chaudhari, 2015).

DISCLOSURE STATEMENT

There is no conflict of interest.

ACKNOWLEDGMENTS

The authors acknowledge Dean College of Dairy science and technology, Mannuthy, Thrissur for providing the facilities to carry out the research work.

REFERENCES

Sethi, S., Datta, A., Gupta, B. L., *and* Gupta, S. (2013). Optimization of cellulase production from bacteria isolated from soil. *ISRN biotechnology*, *2013*.

Sreena, C. P., Resna, N. K., *and* Sebastian, D. (2015). Isolation and Characterization of Cellulase Producing Bacteria from the Gut of Termites (Odontotermes and Heterotermes Species). *British Biotechnol J*, *9*(1), 1-10.

Harrigan, W. F. (1998). Schemes for the Identification of Microorganisms. *Laboratory Methods in Food Microbiology.(3rd Edn). Academic Press, London.*

Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical chemistry*, *31*(3), 426-428.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., *and* Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of biological chemistry*, *193*(1), 265-275.

Demuyakor, B., *and* Ohta, Y. (1993). Characteristics of single and mixed culture fermentation of pito beer. *Journal of the Science of Food and Agriculture*, *62*(4), 401-408.

Salim, T., Ratnawati, L., *and* Agustina, W. (2015). Bioethanol production from glucose by thermophilic microbes from Ciater hot springs. *Procedia Chemistry*, *16*, 503-510.

Sayyad, S. A. F., Chaudhari, S. R., & Panda, B. P. (2015). Quantitative determination of ethanol in arishta by using UV-visible spectrophotometer. *Pharmaceutical and Biological Evaluations*, *2*(5), 204-207.

Khatiwada, P., Ahmed, J., Sohag, M. H., Islam, K., *and* Azad, A. K. (2016). Isolation, screening and characterization of cellulase producing bacterial isolates from municipal solid wastes and rice straw wastes. *Journal of Bioprocessing and Biotechniques*, *6*, 280-285.

Rezayati-Charani, P., *and* Mohammadi-Rovshandeh, J. (2005). Effect of pulping variables with dimethyl formamide on the characteristics of bagasse-fiber. *Bioresource technology*, *96*(15), 1658-1669.

Singanusong, R., Tochampa, W., Kongbangkerd, T., *and* Sodchit, C. (2014). Extraction and properties of cellulose from banana peels. *Suranaree J Sci Tech*, *21*, 1-19.

Gross, K. C., *and* Wang, C. Y. (1984). Compositional changes in cell wall polysaccharides from chilled and non-chilled cucumber fruit. *Phytochemistry*, *23*(8), 1575-1578.

Subathra Devi, C., Mohanasrinivasan, V., Chetna, M., Nikhil, A. S., *and* Jemimah Naine, S. (2015). Thermostable lipase from novel *Pseudomonas sp.* VITSDVM1 isolated from bovine milk. *Frontiers in Life Science*, *8*(2), 165-171.

Facchin, S., Alves, P. D. D., de Faria Siqueira, F., Barroca, T. M., Victória, J. M. N., *and* Kalapothakis, E. (2013). Biodiversity and secretion of enzymes with potential utility in wastewater treatment. *Open Journal of Ecology*, *3*(1), 34.

Anwar, Z., Gulfraz, M., *and* Irshad, M. (2014). Agro-industrial lignocellulosic biomass a key to unlock the future bio-energy: a brief review. *Journal of radiation research and applied sciences*, *7*(2), 163-173.

Heffernan, B., Murphy, C. D., Syron, E., *and* Casey, E. (2009). Treatment of fluoro acetate by a *Pseudomonas fluorescens* biofilm grown in membrane aerated biofilm reactor. *Environmental science and technology*, *43*(17), 6776-6785.

Vaillant, F., Millan, A., Dornier, M., Decloux, M., *and* Reynes, M. (2001). Strategy for economical optimization of the clarification of pulpy fruit juices using cross flow microfiltration. *Journal of Food Engineering*, *48*(1), 83-90.

Subramaniam, G. (2015). The Potential of Hydrolytic Enzymes from *Phoma exigua* for Fruit Juice Clarification.

Kumar, S. (2015). Role of enzymes in fruit juice processing and its quality enhancement. *health*, *6*(6), 114-124.

Christen, G. L., & Marshall, R. T. (1984). Thermostability of Lipase and Protease of *Pseudomonas fluorescens* 27 Produced in Various Broths1. *Journal of Dairy Science*, *67*(8), 1688-1693.

Andualema, B., & Gessesse, A. (2012). Microbial lipases and their industrial applications. *Biotechnology*, *11*(3), 100-118.

Kumar, M. R., Kumaran, M. B., Balashanrnugam, P., Rebecca, A. I. N., Kumar, D. M., *and* Kalaichelvan, P. T. (2014). Production of Cellulase Enzyme by *Trichoderma reesei* Cefl9 and its. *Pakistan Journal of Biological Sciences*, *17*(5), 735-739.

Sadik, M. W., & Asmaa, A. H. (2014). Production of ethanol from molasses and whey permeate using yeasts and bacterial strains. *Int. J. Curr. Microbiol. Appl. Sci*, *3*(3), 804-818.

.