Asian Jr. of Microbiol. Biotech. Env. Sc. Vol. 26, No. (4) : 2024 : 466-471
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ISSN-0972-3005
DOI No.: http://doi.org/10.53550/AJMBES.2024.v26i04.003

ISOLATION AND CHARACTERIZATION OF CELLULASE PRODUCING ANOXYGENIC PHOTOSYNTHETIC BACTERIUM FROM PAPER PULP INDUSTRIAL EFFLUENTS

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(Received 7 September, 2023; Accepted 31 October, 2023)

Key words: *Cellulase, CMC, Paper pulp industrial effluents, Photosynthetic purple non-sulphur bacterium, Molecular identification*

Abstract– Cellulose constitutes the primary product of photosynthesis and is the most abundant bio resource produced in the biosphere. It is an important structural component of green plants, many forms of algae, the Oomycetes and some bacterial biofilms. Microorganisms are reported to produce the enzyme cellulase which breaks down cellulose into monosaccharide β-glucose and oligosaccharides. Cellulases are used in paper pulp industry, detergent industry, olive oil extraction; textile industry; clarification of fruit juices and extraction of carotenoids. In the present work, a photosynthetic purple non-sulphur bacterium was isolated from effluents of paper pulp industry, Mahabubnagar, Telangana State, India, by enrichment into Biebl and Pfennig's medium, the culture was streaked onto CMC agar and incubated under anaerobic light conditions. The colonies were examined for the production of cellulase after 2 weeks. Cellulase production in terms of glucose liberated was estimated by DNS method by growing the bacterium in CMC broth. The bacterium is a Gram negative motile rod. The colour of the cell suspension was dark pink. It could utilize fructose, glucose, glycerol, mannitol, tartrate, thiosulphate, galactose, CMC and cellulose as carbon sources. Molecular identification of 16S rRNA sequence has shown similarity to *Rhodopseudomonas sps*. The physicochemical parameters of the sample measured were pH- 7.0, temperature- 37 ^{0}C, BOD- 95.2, COD- 160.4, chlorides- 263.6, TSS- 210 and TDS- 910 mg/lit.

INTRODUCTION

Bacteria present an attractive potential for the exploitation of cellulases and hemicellulases due to their rapid growth rate, enzyme complexity and extreme habitat variability. The development of rapid and reliable methods for the screening of cellulases from microorganisms within inhospitable environments will allow a greater number of novel bacterial cellulases to be isolated for industrial use (Miranda *et al.,* 2009). Cellulases have been commercially available for more than 30 years, and these enzymes have represented a target for both academic as well as industrial research (Singh, 1999; Singh *et al.,* 2007). The commonly described mode of action for cellulases on polymers is either exo- or endo-cleavage, and all cellulases target the specific cleavage of β-1, 4-glycosidic bonds (Wood and Crae, 1979). Anaerobic bacteria like clostridia, few anaerobic fungi like *Neocallimastix*, *Piromyces*, and *Orpinomyces* (Fanutti *et al.,* 1995; Li *et al.,* 1997) possess multienzyme complexes called Cellulosomes. Cellulase-producing photosynthetic bacteria are a fascinating group of microorganisms that possess the ability to degrade cellulose, a complex polysaccharide found in plant cell walls. While the literature on this specific topic may be limited, there are studies that have explored the cellulase production potential of photosynthetic bacteria. A number of microorganisms were reported to produce cellulases and few are listed in Table 1.

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Cellulases are used in paper pulp industries for various treatments of the wood such as refining, grinding of woody raw material and defibrillation (Kuhad *et al*., 2011). They are used in Detergent Industry along with protease (Singh *et al*., 2007); for olive oil extraction (Fantozzi *et al*., 1977) and applied to remove the rough protuberances of fabrics for producing a smoother, glossier, and brighter-colored fabric (Karmakar and Ray, 2011). Use of cellulases for production of carotenoids from orange peel, sweet potato and carrots maintains the natural structure of the pigments in contrast to solvent extraction which dissociates the pigments from protein leading to easy oxidation (Cinar, 2005; Fennema, 1985).

Application of exogenous cellulase improves soil fertility and plant growth due to acceleration of straw decomposition (Han and He, 2010). Cellulases also have an important application as a part of macerating enzyme complex (cellulases, xylanases, and pectinases) used for extraction and clarification of fruit and vegetable juices to increase the yield of juices (Minussi *et al*., 2002; Carvalho *et al*., 2008). Use of cellulases and hemicellulases improve feed value and performance of animals (Dhiman *et al*., 2002). Babykin *et al*., (1988) reported production of cellulase by genetically altered *Rhodobacter sphaeroides*. Apart from this, no literature is available on cellulolytic photosynthetic bacteria. So, we have taken up this work.

MATERIALS AND METHODS

Collection of the sample

Effluent water (Fig. 1) is collected from Paper pulp industry in a flask filled completely to the neck and stoppered. The pH and temperature of the sample was noted to be 7.0 and 37^0C respectively. The physicochemical parameters of the sample Biological Oxygen Demand (BOD), Chemical Oxygen Demand (COD), Chlorides, Total suspended Solids (TSS) and Total Dissolved Solids (TDS) were estimated by the methods of American Public Health Association (APHA), 2005.

Table 1. Cellulase producing microorganisms

Bacteria	Reference
Cellulomonas flavigena	Amtul, *et al*., 1988
Pseudomonas fluorescens	Sonia Sethi *et al*., 2013
Clostridium thermocellum	Hirano *et al*., 2016
Thermotoga maritima	Zhiwei Chen *et al*., 2013
Ruminococcus flavefaciens	Marco *et al*., 2001
Fungi	
Trichophyton terrestre	Marques *et al*., 2011
Cladosporium cladosporoides	Isaie and Padmavathi, 2013
Aspergillus niger	Namita *et al*., 2012
Penicillium funiculosum	Marcelle *et al*., 2014
Trichoderma reesei	Bischof *et al*., 2016

Fig. 1. Effluent water collected from paper pulp industry, Mahabubnagar

Isolation of the bacterium

One ml of the sample was inoculated into Biebl and Pfennig's medium (Biebl and Pfennig, 1981) containing carboxy methyl cellulose as organic carbon in screw cap vials filled with the medium. The vials were incubated at room temperature under anaerobic light conditions. After growth, the culture was inoculated onto agar plates and incubated at room temperature under anaerobic light conditions. Pure cultures were obtained by repeated streaking onto agar plates and again sub cultured into liquid medium.

Screening for Cellulase production

The bacterium was inoculated onto CMC agar ($NH_4H_2PO_4$ - 1g; KCl - 0.2g; $MgSO_4$. $7H_2O$ - 1g; Yeast extract - 1g; Carboxymethyl cellulose - 26g; Agar – 3g and Distilled Water – 1000ml) and incubated under anaerobic light conditions. Colonies were developed by 2 weeks. To check for cellulase production, the plate was stained with 1% Congo red in Distilled water for 30 minutes and destained with 1M NaCl solution. The enzyme activity was measured by DNS method after growing the bacterium in CMC broth for one week.

Identification of the bacterium

The culture was studied for its morphological properties, Gram's staining and utilization of carbon

sources. Molecular identification was carried out at NCIM, Pune using BLAST (NCBI) using the 16S rRNA gene. The genomic DNA was isolated using a Gen Elute DNA extraction kit, as per manufacturer's guidelines (Sigma Aldrich). The PCR reaction was performed in a 20 µl reaction volume containing 10 µmol L-1 primers, 40 ng template DNA, and TaqReadyMix (JumpStart, Sigma-Aldrich, India). The PCR was performed onto Applied BioSystem (Thermo Scientific, USA). The obtained PCR product was purified and sequenced at Agile Life science Technology Ind. Pvt. Ltd. Thus obtained nucleotide sequence was performed for BLAST analysis and a Phylogenetic tree was constructed using the Neighbour-Joining method. 27 F and 1492 R primers are used for the identification (Table 2).

Table 2. Primers used for identification of the bacterium

Target Gene	Primer Sequence (5′ to 3′)	Tm (°C)
27 F	AGAGTTTGATCM(A/C) TGGCTCAG	56
1492 R	TACCTTGTTACGACTT	

Cellulase Activity

The cellulase activity was measured by DNS method involving action of the enzyme on carboxymethylcellulose followed by determination of reducing power of resulting sugars (glucose) with the aid of Di-Nitro Salicylic acid (Miller, 1959).

RESULTS AND DISCUSSION

The physicochemical parameters of the effluent were measured and given in the Table 3.

The growth of the bacterium was indicated by pigment production in the liquid medium (Fig. 2) and production of colonies in the agar plate. The agar plates have shown clear zones of cellulase production by the bacterial colonies when stained with Congo red (Fig. 3).

The bacterium was found to be Gram negative, rod shaped (Fig. 4) and motile.

Out of the various carbon sources tested, the bacterium utilized cellulose, fructose, galactose, glucose, glycerol, maltose, mannitol, tartarate and thiosulphate (Table 4). Fructose and glycerol supported more pigment production (+++) by the organism followed by cellulose and glucose (++) whereas maltose, mannitol, tartarate and thiosulphate induced less pigment production (+) by the bacterium. The organism could not utilize acetate, benzoate, lactose, malate, methanol and sodium sulphide.

The cellulase activity is expressed in terms of glucose liberated in mg/ml of the substrate. The

Fig. 2. Culture of the Photosynthetic bacterium

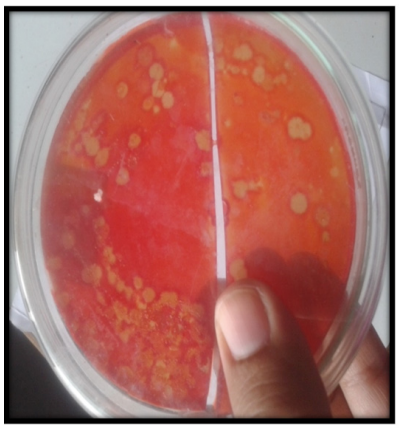

Fig. 3. Colonies stained with Congo red showing cellulolytic activity as clear zone around the colonies

Table 3. Physicochemical parameters of the sample

pH	Temperature	BOD (mg/Lit)	COD (mg/Lit)	Chlorides (mg/Lit)	TSS (mg/Lit)	TDS (mg/Lit)
7.0	37^0C	95.2	160.4	263.6	210	910

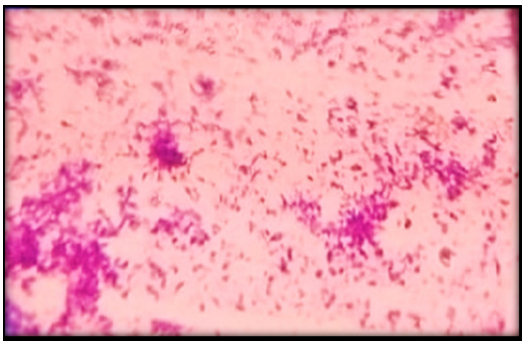

Fig. 4. Gram negative rods as seen under the microscope (oil immersion)

Table 4. Utilization of Carbon sources

Sl. No.	Carbon Source	Utilized/not (Pigment production)
1.	Acetate	-
2.	Benzoate	-
3.	Cellulose	++
4.	Fructose	+++
5.	Galactose	++
6.	Glucose	++
7.	Glycerol	+++
8.	Lactose	-
9.	Malate	-
10.	Maltose	+
11.	Methanol	-
12.	Mannitol	+
13.	Tartarate	+
14.	Sulphide	-
15.	Thiosulphate	+

activity of cellulase produced by the bacterium increased with increasing concentration of carboxy methyl cellulose in the medium (Table 5).

Molecular identification of the bacterium done using BLAST (Basic Local Alignment Search Tool) with 16S rRNA partial sequence is shown in the Table 6 and Taxonomic Tree (Fig.5).

Table 5. Cellulase activity in terms of glucose liberated (mg/ml)

Concentration of CMC (mg/ml)	Glucose liberated (mg/ml)
1.0	0.1
2.0	0.21
4.0	**0.45**

CONCLUSION

The photosynthetic purple non-sulphur bacterium isolated in the present work was found be Gram negative motile rod and has sequence similarity of 16S rRNA with *Rhodopseudomonas sps*. The bacterium could be utilized for the production of the enzyme cellulase which can be used in various industries. The enzyme may also be applied for bioremediation of cellulosic wastes like paper pulp effluents.

ACKNOWLEDGEMENTS

The authors acknowledge Sri P. Srinivas Rao, Director, Adarsh Degree & P. G. College, Mahabubnagar, Telangana State, India, for his encouragement and providing facilities for performing this work and NCL Pune, for performing the Molecular Analysis.

Conflicts of Interest: Authors express No Conflicts of interest.

REFERENCES

Amtul, J. S., Waheed, A.M., Malik, N. N. and Bashir, A. N. 1988. Production of free and substrate-bound cellulases of *Cellulomonas flavigena. Enzyme and Microbial Technology*. 10 (10) : 626-631.

APHA. 2005. Standard Methods for the Examination of Water and Wastewater, 21st Edition, American Public Health Association (APHA), Washington, D.C.

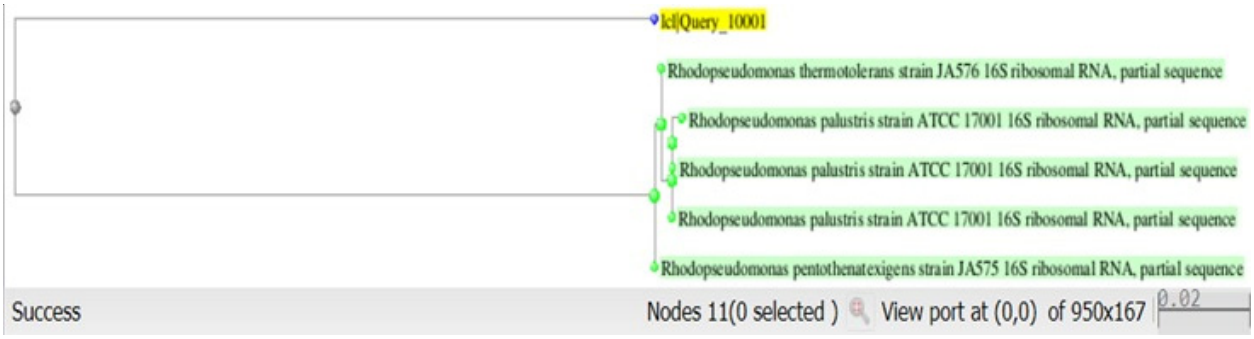

Fig. 5. Taxonomic Tree based on MUSCLE Multiple Alignment computed for Mole BLAST

Table 6. Taxonomy Report

Organism Report Lineage Report

Taxonomy Report

Taxonomy	Number of hits	Number of Organisms	Description
Bacteria	100	14	
. Proteobacteria	97	13	
.. Alphaproteobacteria	95	11	
... Rhodopseudomonas	78	10	
.... uncultured *Rhodopseudomonas* sp.	66	1	uncultured *Rhodopseudomonas* sp. hits
.... *Rhodopseudomonas* sp. JA904	1	1	*Rhodopseudomonas* sp. JA904 hits
.... *Rhodopseudomonas* sp. EGAT-KU 1/3	1	1	*Rhodopseudomonas* sp. EGAT-KU 1/3 hits
.... *Rhodopseudomonas* sp. S8-1	1	1	*Rhodopseudomonas* sp. S8-1 hits
.... *Rhodopseudomonas* sp.	3	1	*Rhodopseudomonas* sp. hits
.... *Rhodopseudomonas thermotolerans*	2	1	*Rhodopseudomonas thermotolerans* hits
.... *Rhodopseudomonas pentothenatexigens*	1	1	*Rhodopseudomonas pentothenatexigens* hits
.... *Rhodopseudomonas* sp. S16-VOGS3	1	1	*Rhodopseudomonas* sp. S16-VOGS3 hits
.... *Rhodopseudomonas* sp. MV9(ext sed)	1	1	*Rhodopseudomonas* sp. MV9(ext sed) hits
.... *Rhodopseudomonas palustris*	1	1	*Rhodopseudomonas palustris* hits
... uncultured *Rhodobacter* sp.	17	1	uncultured *Rhodobacter* sp. hits
.. Betaproteobacteria	2	2	
... uncultured *Comamonas* sp.	1	1	uncultured *Comamonas* sp. hits
... uncultured *Thauera* sp.	1	1	uncultured *Thauera* sp. hits
. uncultured bacterium	3	1	uncultured bacterium hits

Babykin, M. M., Velikodvorskaya, G. A. Mogutov, M. A. Pirazyan E. S. and Shestakov S. V. 1988. *Academia Nauk USSR Seriya Biologii*. 15: 186-190.

Biebl, H. and Pfennig, N. 1981. Isolation of members of the family Rhodospirillaceae. *The Prokaryotes*. Eds. M. P. Starr, H. Stolp, H. G. Truper, A. Balows and H. G. Schlegel, Springer-Verlag, Berlin. 267-273.

Carvalho de L. M. J., Castro de, I. M. and Silva da C. A. B. 2008. A study of retention of sugars in the process of clarification of pineapple juice (*Ananas comosus*, L. Merril) by micro- and ultra-filtration. *Journal of Food Engineering*. 87 (4): 447–454.

Cinar, I. 2005. Eects of cellulase and pectinase concentrations on the colour yield of enzyme extracted plant carotenoids. *Process Biochemistry*. 40 (2): 945–949.

Dhiman, T. R., Zaman, M. S., Gimenez, R. R., Walters, J. L. and Treacher, R. 2002. Performance of dairy cows fed forage treated with brolytic enzymes prior to feeding. *Animal Feed Science and Technology*. 101 (1–4): 115–125.

Fantozzi, G. Petruccioli, and Montedoro, G. 1977. Trattamenti con additivi enzimatici alle paste di oliva sottoposte ad estrazione per pressione unica: inuenze delle cultivars, dell'epoca di raccolta e della conservazione. *Grasse.* 54: 381–388.

Fanutti, C., Ponyi, T., Black, G.W., Hazlewood, G.P. and Gilbert, H.J. 1995. The conserved noncatalytic 40-residue sequence in cellulases and hemicellulases from anaerobic fungi functions as a protein docking domain. *Journal of Biological Chemistry*. 270 (49): 29314–29322.

Fennema, O. W. 1985. *Food Chemistry*, Marcel Dekker, New York, NY, USA.

Han, W. and He, M. 2010. The application of exogenous cellulase to improve soil fertility and plant growth due to acceleration of straw decomposition. *Bioresource Technology*. 101 (10): 3724–3731.

Hirano, K., Kurosaki, M., Nihei, S., Hasegawa, H., Shinoda, S., Haruki, M. and Hirano, N. 2016. Enzymatic diversity of the *Clostridium thermocellum* cellulosome is crucial for the degradation of crystalline cellulose and plant biomass. *Scientific Reports*|6:35709| DOI: 10.1038/srep 35709. www.nature.com/scientificreports.9 pages.

Isaie, M. and Padmavathi, T. 2013. Optimization of process parameters for biosynthesis of cellulase by *Cladosporium cladosporioides* using agro wastes. *International Journal of Pharma and Bio Sciences*. 4(4): (B) 1129 – 1138.

Karmakar, R. and Ray, R. 2011. Current trends in research and application of microbialcellulases. *Research Journal of Microbiology*. 6 (1): 41–53.

Kuhad, R.C., Gupta, R. and Singh, A. 2011. Microbial Cellulases and their Industrial Applications. *SAGE-Hindawi* Access to Research *Enzyme Research*. 2011, Article ID 280696, 10 pages doi:10.4061/2011/280696.

Li, XL, Chen, H. and Ljungdahl, L. G. 1997. Two cellulases, CelA and CelC, from the polycentric anaerobic fungus Orpinomyces strain PC-2 contain N-terminal docking domains for a cellulase-hemicellulase complex. *Applied Environmental Microbiology*. 63 (12): 4721–4728.

Marcelle L de A de Carvalho, D. F. Carvalho, E de Barros Gomes, R. N. Maeda, L.M. M. S. Anna, Machado, A. de C. and Nei P., Jr. 2014. Optimisation of Cellulase Production by *Penicillium funiculosum* in a Stirred Tank Bioreactor Using Multivariate Response Surface Analysis. *Enzyme Research*. 2014, Article ID 703291, 8 pages http://dx.doi.org/10.1155/2014/703291.

Marco, T. Rincón, Sheila, I. McCrae, James, K., Karen, P. Scott and Harry J. Flint. 2001. EndB, a Multidomain Family 44 Cellulase from *Ruminococcus flavefaciens*17, binds to Cellulose via a Novel Cellulose-Binding Module and to Another *R. flavefaciens* Protein via a Dockerin Domain. *Applied and Environmental Microbiology.* 67(10): 4426–4431.

Marques, M. P., Monti, R. and Sponchiado, S. R. P. 2011. Cellulolitic Activity of the Fungus *Trichophyton terrestre* Cultivated in Carboximetilcellulose or Mycrocrystalline Cellulose. *XL Annual Meeting of Brazilian Biochemistry and Molecular Biology Society Foz do Iguaçu*, PR, Brazil, April 30th to May 3rd.

Miller, G.L. 1959. Use of Di-Nitro Salicylic acid reagent for determination of reducing sugar. *Analytical Chemistry*. 31: 426-428.

Miranda, M., Kam, T. L. and Qin, W. 2009. The prospects of cellulase- producing bacteria for the bioconversion of lignocellulosic biomass. *International Journal of Biological Sciences.* 5 (5): 500–516.

Minussi, R.C., Pastore, G. M. and Duran, N. 2002. Potential´ applications of laccase in the food industry. *Trends in Food Science and Technology*. 13(6-7): 205–216.

National Center for Biotechnology Information, U.S. National Library of Medicine 8600 Rockville Pike, Bethesda MD, 20894 USA.

Namita, B., Rupinder, T., Raman, S. and Sanjeev, K. S. 2012. Production of cellulases from *Aspergillus niger* NS-2 in solid state fermentation on agricultural and kitchen waste residues. *Waste Management.* 32 (7): 1341-1346.

Robert, H. Bischof, Jonas R. and Bernhard, S. 2016. Cellulases and beyond: the first 70 years of the enzyme producer *Trichoderma reesei*. *Microbial Cell Factories*. 15: 106, DOI 10.1186/s12934-016-0507-6.

Singh, A. 1999. Engineering enzyme properties. *Indian Journal of Microbiology*. 39 (2): 65 –77.

Singh, A., Kuhad, R. C. and Ward, O. P. 2007. Industrial application of microbial cellulases. in *Lignocellulose Biotechnology: Future Prospects*. R. C. Kuhad and A. Singh, Eds., pp. 345–358, I. K. International Publishing House, New Delhi, India.

Sonia, S., Aparna, D., B., Lal, G. and Saksham, G. 2013. Optimization of Cellulase Production from Bacteria Isolated from Soil. *ISRN Biotechnology*. 2013, Article ID 985685, 7 pages http://dx.doi.org/10.5402/2013/985685.

Wood, T. M. and Mc Crae, S.I. 1979. Synergism between enzymes involved in the solubilization of native cellulose. Advances in Chemistry Series. 181:181–209.

Zhiwei Chen, Jose H. Pereira, Hanbin Liu, Huu M. Tran, Nathan S. Y. Hsu, Dean Dibble, Seema Singh, Paul D. Adams, Rajat Sapra, Masood Z. Hadi, Blake A. Simmons and Kenneth L. Sale, 2013. Improved Activity of a Thermophilic Cellulase, Cel5A, from *Thermotoga maritima* on Ionic Liquid Pre-treated Switchgrass. PLOS ONE https://doi.org/10.1371/journal.pone.0079725.