ISSN 2278-7763

Optimization of cellulase production by *Bacillus altitudinis* APS MSU and *Bacillus licheniformis* APS2 MSU, gut isolates of fish *Etroplus suratensis*

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ABSTRACT

In the present study two cellulolytic bacterial strains were isolated from the gut of an estuarine fish *Etroplus suratensis*. The strains were identified through basic conventional and also through 16S rRNA gene sequencing methods and they were identified as *Bacillus altitudinis* and *Bacillus licheniformis*. The cellulase production capacity of the candidate strains were optimized through certain nutritional parameters such as carbon sources, nitrogen sources, phosphate sources, surfactants and metal ions. In both the tested strains, Fructose and CMC induced maximum production of cellulase among various carbon sources tested. Among the tested nitrogen sources, ammonium hydrogen carbonate and ammonium sulphate enhanced cellulase production to a maximum extent by *Bacillus altitudinis* and *Bacillus licheniformis*, respectively. Similarly, the cellulase production was maximum in sodium dihydrogen ortho phosphate supplemented medium by both the strains. Tritonx100 was observed to be the best surfactant for inducing cellulase production by *B.altitudinis* and *B.licheniformis*. in the tested metal ions, maximum amount of enzyme production was recorded at magnesium sulphate and manganous sulphate administered media by *B.altitudinis* and *B.licheniformis*, respectively.

Keywords : Cellulase, *Bacillus altitudinis, Bacillus lilcheniformis, Etroplus suratensis*.

1 INTRODUCTION

Cellulose is the most abundant and renewable biopolymer on earth. The enormous potential as a renewable source of energy was recognized only after cellulose degrading enzymes or "cellulases" had been identified (Bhat, 1997). Cellulase is implicated in several food processing, textile, paper, pharmaceutical and other related industries (Hoshino *et al*., 1997; Lynd *et al*., 2002). Several fungi and bacteria are capable of producing multiple group of enzyme named cellulases that act in a synergistic manner to hydrolyse the β-1, 4-D glucosidic bonds within the cellulose molecules (Akiba *et al*., 1995). A Cellulosic enzyme system consists of three major components such as endoglucanases, exoglucanases and β- glucosidases (Parry *et al*., 1983; Gielkenes *et al*., 1999; Kang *et al*., 1999).

The bacterial flora of the gastro intestinal tract of fishes in general, represents a very important and diversified enzymatic potential (Clements, 1997). Bacteria, which has high growth rate as compared to fungi has good potential to be used in cellulase production. However, the application of bacteria in producing cellulase is not widely used. Celluloytic property of some bacterial genera such as *Cellulomonas*, *Cellovibrio, Pseudomonas*, *Sporosphytophaga* spp. (Nakamura and Kappamura, 1982); *Bacillus* and *Micrococcus* (Immanuel *et al*., 2006) were documented well.

Different bacteria and fungi have been used for cellulase production (Bahkali, 1996; Shin *et al*., 2000). Microorganisms of the genera *Trichoderma* sp. and *Aspergillus* sp. are taught to be cellulase producers and crude enzymes produced by these microorganisms are commercially available for agricultural use (Kazuhisa Miyamoto, 1997). Cellulolytic properties of certain bacterial species such as *Pseudomonas* sp, *Cellulomonas* sp, *Cellovibrio* sp and *Sporophytophago* sp. were also reported (Nakamura and Kappamura, 1982). The specific cellulolytic activity shown by the bacterial species was found to be dependent on the source of occurrence (Saxena *et al*., 1993).

Considering the importance and applications of cellulases, the present study was aimed to screen the bacterial isolates from the intestine of fish *Etroplus suratensis* for the cellulolytic ability. Furthermore, to determine the ability of the isolated strains as production of cellulase in varying nutritional conditions.

2 METHODOLOGY

2.1 Isolation and identification of cellulolytic bacteria from fish *Etroplus suratensis*

The fish, *Etroplus suratensis* was collected and the gastro intestine of the fish was aseptically removed and homogenised in saline water. The suspension was serially diluted and from 10^{-4} dilution, 0.1ml of sample was plated on CMC agar plate. Then the plates were incubated at 37°C for 24-48 h.

2.2 Screening bacteria for cellulose activity

After incubation, the morphologically dissimilar colonies were picked up and inoculated in CMC agar plates containing 2% CMC agar and 1% agar agar. The CMC agar plates were incubated at 37°C for 24-48 h. At the end of the incubation, the agar medium was flooded with an aqueous

ISSN 2278-7763

solution of Congo red (0.1% w/v) for 15 min. The excess Congo red solution was poured off, and the plates were further treated by flooding with 1M NaCl for 15 min. Congo red binds with carboxymethyl cellulose and turns into bright red. Cellulase produced by individual bacterium hydrolyzed carboxymethyl cellulose around the bacterial colony and the dye Congo red unable to stain it. Therefore the hydrolyzed zone appears transparent, while the unhydrolyzed regions appear bright red. The ratio of the diameter of the clear zone to colony diameter was measured in order to select the highest cellulase producing bacterium. The largest ratio was assumed to contain the highest activity.

2.3 Identification of highest cellulase producing bacteria by biochemical characters

The bacteria which showed the highest cellulase activity were identified by using the book of classification of bacteria form Bergeys manual of determination bacteriology (Bergey, 1984).

2.4 Identification of the isolated bacteria by sequencing of the amplified 16S rRNA gene

The most powerful tool to identify the unknown bacteria is to sequence the gene (DNA) coding for 16S rRNA, which is present in the genome of the bacteria. The gene coding for the 16S rRNA was amplified using PCR and the amplified product has been subjected to sequencing and the sequence obtained was compared with the sequence obtained from the Nucleotide Database of National Center for Biotechnology Information (NCBI).

2.5 Optimization of culture conditions for the highest cellulase production

Based on the preliminary identification studies, two bacterial strains namely *Bacillus altitudinins* and *Bacillus licheniformis* were identified as the best cellulase producers. Therefore these two strains were further subjected for optimization studies. The strains were individually incubated in the production medium (Carboxymethylcellulose-agar (CMC-agar) medium ($g \cdot L^{-1}$): L-glutamic acid – 0.3g, NH_4NO_3 -1.4g, KH_2PO_4 – 2g, $CaCl_2$ -0.3g, $MgSO_4$ - 0.3g, Proteose peptone -7.5, $FeSO_4$ – 5g, $MnSO_4$ – 1.6g, $ZnSO_4$ – 1.4g, CMC – 30g, Tween80 – 20ml, pH – 5.6) in a shaker at room temperature (~27^{o}C) for 48h.

2.6 Cellulase enzyme assay

The cellulase produced by the strains was measured according to the method of Denison and Koehn (1977). The production of reducing sugar (glucose) from CMC substrate through cellulolytic activity was measured at 540 nm by the dinitrosalicylic acid method using glucose as the standard. One cellulase unit (U) was defined as the amount of enzyme per millilitre culture filtrate that released 1 mg glucose per minute.

2.7 Optimization of cellulase production

2.7a Effect of carbon sources on cellulase production

To identify suitable carbon source for the cellulase production by the test organisms, the following carbon sources were tested. The production medium containing Carboxymethyl cellulose act as a carbon source, this CMC was replaced by adding Glucose, Sucrose, Fructose, Maltose, Cellulose, Lactose, Mannitol and Sarbitol. These carbon sources were tested individually at the concentration of 1% with dry substrate.

2.7b Effect of nitrogen sources on cellulase production

Cellulase production using different nitrogen sources was also analyzed by changing the nitrogen source (NH_4NO_3 and Proteose peptone) of the enzyme production medium. The nitrogen sources used were: Sodium nitrate, Ammonium chloride, Ammonium hydrogen carbonate, Ammonium sulphate, Potassium nitrate, Yeast extract, Soyameal, Beef extract, NH_4NO_3, Proteose peptone and Skim milk. They were incorporated individually into the production medium at the concentration of 0.5%.

2.7c Effect of phosphate sources on cellulase production

The cellulase production by the selected bacteria was also optimized by supplementing different phosphate sources. The different phosphate sources tested for the cellulase production were Sodium dihydrogen ortho phosphate, Tricalcium phosphate, Bismuth phosphate, Disodium hydrogen ortho phosphate, K_2HPO_4, KH_2PO_4 and calcium hydrogen phosphate. They were supplemented individually in the production media at the concentration of 0.5%.

2.7d Effect of surfactants on cellulase production

To identify the surfactants facilitating cellulase production, 6 different surfactants were used for experimentation. They were Tween20, Tween40, Tween60, Tween80, TritonX-100, SDS, and polyethylene glycol. The selected surfactants were tested individually at the concentration of 0.2% in the optimized production medium.

2.7e Effect of metal ions on cellulase production

Normally, the production medium contains $Cacl_2$, $MgSO_4$, $FeSO_4$, $MnSO_4$, and $ZnSO_4$. In the present study to enhance cellulase production Calcium chloride, Magnesium sulphate, Ferrous sulphate, Manganous sulphate, Zinc sulphate, Barium chloride, Copper sulphate, Mercuric chloride and EDTA were tested as the source of metal ions. They were incorporated individually into the production medium at the concentration of 0.02%. The effect was determined after 48 h of incubation.

2.8 Statistical analysis

The data obtained in the present study were subjected to Analysis of variance (ANOVA) using SPSS 14.0 version software. *Post hoc* test was employed to test the differences among means. The significance of differences was tested at the level $P = 0.05$.

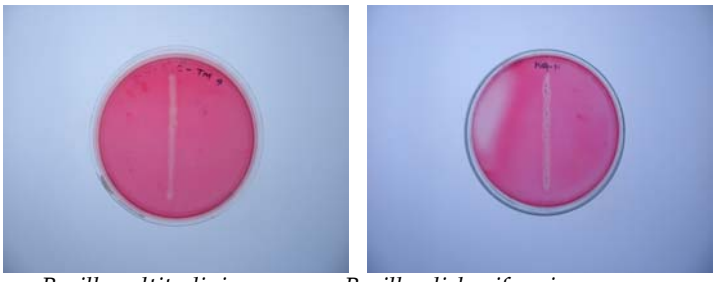
3 Results

3.1 Isolation and screening of cellulase producing bacteria

They were 18 suspected bacterial colonies isolated from the gut of *E. suratensis*. Pure cultures of all the eighteen isolates were prepared and they were tested for cellulase activity on a medium containing CMC as the substrate. As shown in fig 1, the cellulase produced by the bacteria hydrolyzed the substrate and the hydrolyzed regions were highly visible after staining with Congo red dye. After staining, the hydrolyzed regions appeared transparent, while the unhydrolyzed regions appeared intense red. The appearance of a hydrolytic zone is a clear indication that the bacteria had pro-

ISSN 2278-7763

duced the cellulase. After 24-48 h of incubation, all the eighteen isolates showed signs of growth on CMC agar; however only two strains hydrolyzed the CMC very efficiently. These particular bacteria were chosen for identification and further for optimisation of cellulase production at different cultutre conditions. cellulase production. The strains were identified as *Bacillus altitudinis and Bacillus licheniformis* through conventional method of morphological, physiological and biochemical characteristics along with 16S rRNA sequencing (Fig 2,3).



Bacillus altitudinis *Bacillus licheniformis*

Control

Fig 1 Screening for cellulolytic bacteria on CMC agar plate

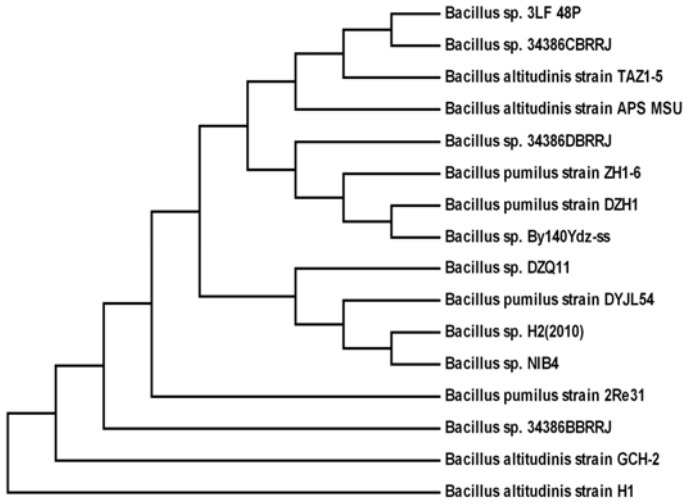


Fig 2 Phylogenetic tree analysis of *Bacillus altitudinis* strain with related sequences obtained from RDB database

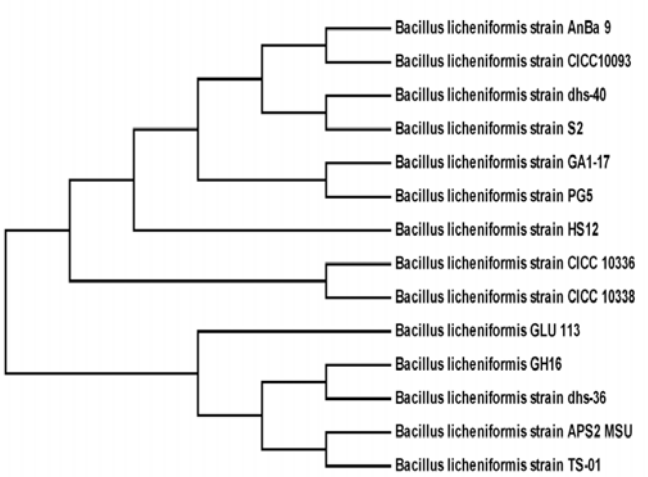


Fig 3 Phylogenetic tree analysis of *Bacillus licheniformis* strain with related sequences obtained from RDB database

3.2 Optimization of culture conditions for cellulase production

3.2a Effect of carbon sources

Carbon sources such as glucose, sucrose, fructose, maltose, cellulose, lactose, mannitol and sarbitol were incorporated individually in the medium at the rate of 1% and the bacteria were grown individually for 48h. The bacterium, *B.altitudinis* APS MSU produced the highest amount (5.48± 0.25 U/ml) of cellulase in the medium supplemented with fructose, whereas lowest (0.96± 0.21U/ml) amount of enzyme production was recorded at maltose supplied medium. Invariably the capability of cellulase production by *B. licheniformis* APS2 MSU was more (2.71±0.18 U/ml) at CMC supplied medium, but it was less at fructose (0.30± 0.05U/ml) supplied medium (Fig.4).

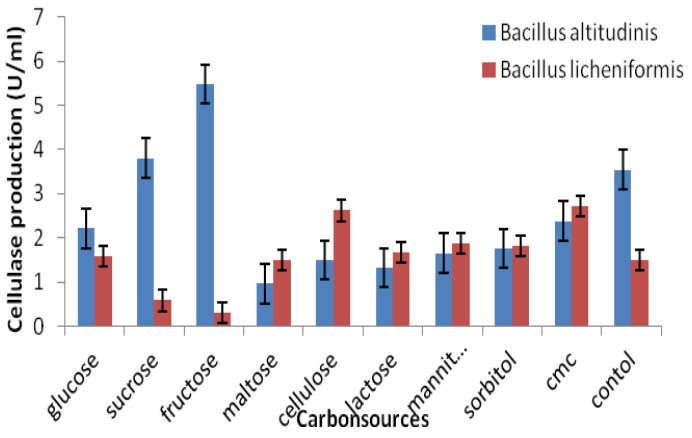


Fig 4 Cellulase production by *B.altitudinis* and *B.licheniformis* in the media supplemented with different carbon sources

3.2b Effect of nitrogen sources

Effect of different nitrogen sources on cellulase production after 48h of incubation by B. *altitudinis* showed the highest level (27.42± 0.11 U/ml) in the medium incorporated with Ammonium hydrogen carbonate, however it was maximum of only 1.52±0.29 U/ml in the medium supplied with

ISSN 2278-7763

ammonium sulphate by *B. licheniformis*. At the same time, minimum amount of cellulase production was recorded in skimmilk (2.91 ± 0.38 U/ml and 0.26 ± 0.41 U/ml) supplied media by both the organisms *B. altitudinis* and *B. licheniformis*, respectively (Fig 5).

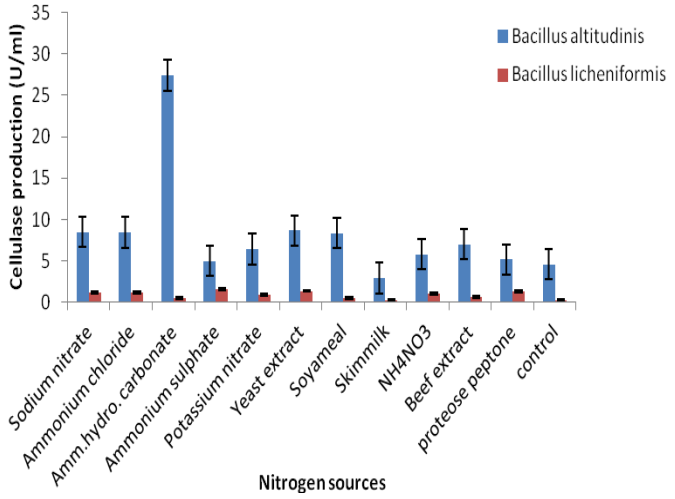


Fig 5 Cellulase production by *B.altitudinis* and *B.licheniformis* in the media supplemented with different nitrogen sources

3.2c Effect of phosphhate sources

Different phosphate sources such as Sodium dihydrogen ortho phosphate, Tricalcium phosphate, Bismuth phosphate, Disodium hydrogen ortho phosphate, K_2HPO_4, KH_2PO_4, and calcium hydrogen phosphate were individually incorporated at 0.5% concentration in the medium and the bacteria were incubated for 48h. The cellulase production was maximum (7.08±0.23 and 8.66±0.59 U/ml) at sodium dihydrogen ortho phosphate supplied media by *B.altitudinis* and *B. licheniformis*, respectively. At the same time, the minimum (2.58±0.57 and 4.97±0.70 U/ml) amount of cellulase production was observed in Disodium hydrogen ortho Phosphate suplplied media by *B.altitudinis* and *B. licheniformis*, respectively (Fig 6).

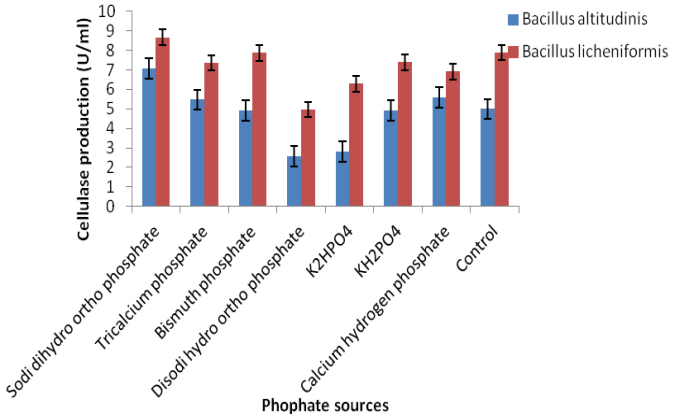


Fig 6 Cellulase production by *B.altitudinis* and *B.licheniformis* in the media supplemented with different phosphate sources

3.2d Effect of surfactants

The effect of different kinds of surfactants (Tween20, Tween40, Tween60, Tween80, TritonX-100, SDS, and polyethylene glycol) was tested for cellulase production by the candidate organisms. Among the tested surfactants, the maximum (7.80±0.73 U/ml and 12.86±0.60 U/ml) amount of enzyme production was recorded in Triton X-100 supplied media by *B.altitudinis* and *B. licheniformis*, respectively. But the minimum amount of cellulase production was recorded in Tween80 (4.36±0.67 U/ml) supplied media by *B.altitudinis* and SDS (1.18±0.29 U/ml) supplied media by *B. licheniformis* (Fig 7).

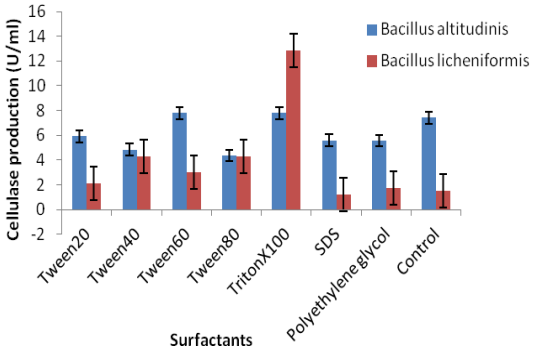


Fig 7 Cellulase production by *B.altitudinis* and *B.licheniformis* in the media supplemented with different surfactants

3.2e Effect of metal ions

Among the tested metal ions, maximum (7.52±0.37 U/ml) amount of enzyme production was recorded in magnesium sulphate supplied medium by *B.altitudinis*, but it was maximum of 1.80±0.42 U/ml in manganous sulphate administered medium by *B. licheniformis*. However, the minimum amount of cellulase production was observed in ferrous sulphate (1.87±0.38 U/ml) and copper sulphate (1.17±0.19 U/ml) supplied media respectively by *B.altitudinis* and *B. licheniformis* (Fig 8).

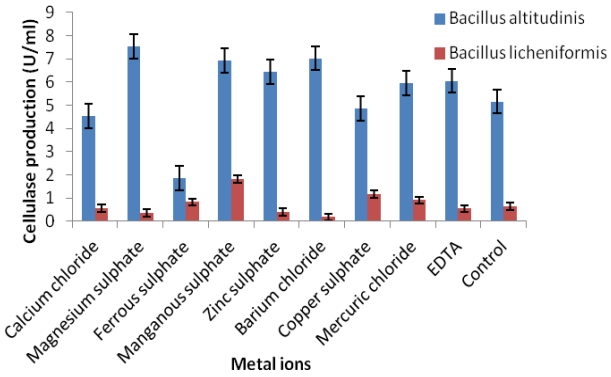


Fig 8 Cellulase production by *B.altitudinis* and *B.licheniformis* in the media supplemented with different surfactants

4 Discussion

The capacity to degrade cellulose is a character distributed among a wide variety of aerobic, facultative aerobic, anaerobic bacteria and fungi. In the present study, two different cellulolytic microbes such as *B.altitudinis* and *B.licheniformis* were isolated and identified from the gut of an

ISSN 2278-7763

estuarine fish *E.suratensis*. Many efforts were taken to generate microorganisms with high ability to produce cellulase that can degrade native cellulose (Aristidou and Penttila, 2000). It has been reported that, physico-chemical factors influence the growth of the organisms and also the cellulase production (Gayal and Khande Parkar, 1998).

Efforts are going on throughout the world to enhance the production and purity of bacterial cellulases. Among the various factors that influence cellulase production during culture, the type of nutritional sources and inducers have a profound effect on production of cellulase. The present study was conducted to evaluate selected carbon sources, nitrogen sources, phosphate sources, surfactants and metal ions as substrates for cellulase production by *B.altitudinis* and *B.licheniformis*. The result revealed that the bacterial isolates utilized all the substrates supplemented with their source for their growth and cellulase production.

The selection and optimization of suitable carbon sources for cellulase enzyme production by *B.altitudinis* inferred that fructose added medium was found to produce maximum (5.48±0.25U/ml) cellulase production and *B.licheniformis* inferred that CMC added medium was found to produce maximum (2.71±0.18U/ml) cellulase than the other carbon sources substituted media. From the result, it was confirmed that fructose and CMC being a simple sugar could be effective for production of cellulase by the candidate organisms. These results are in agreement with those of Narasimha *et al* (2006) and Niranjane *et al* (2007), who have found that CMC was the best carbon source, followed by cellulose for cellulase production. A higher production of cellulase when CMC served as substrate may be a result of induction of the enzyme, since cellulose is known to be a universal inducer of cellulase synthesis. Paul and Verma (1993) had reported the induction of endocellulase by CMC. Some investigators showed that agro-industrial residues such as rice bran, rice straw, sugarcane bagasse and wheat bran could be used as carbon sources for cellulase production. For example, *B.subtilis* CBTK 106, *B. subtilis* BC 62 and *B.pumillus* exhibited their maximum cellulase production when wheat bran, banana fruit stalk and soybean were supplemented to the production media (Heck, 2002; Poorna and Prema, 2007). In the present study fructose was found to be the best inducer and the result substantiates the earlier works of Bagga *et al*. (1989), who reported that fructose, a best inducer of *Aspergillus* sp. as well as it is the best inducer of cellulase in *Clastiridium thermocellum* (Nochure *et al*., 1993).

The optimization of suitable nitrogen sources for cellulase production by test organisms inferred that Ammonium hydrogen carbonate added medium was found to produce maximum (27.42±0.11) cellulase by *B.altitudinis*; whereas, *B.licheniformis* exhibited maximum production in Ammonium sulphate (1.52±0.29U/ml) substituted medium than the other tested organic and inorganic nitrogen sources. Many other workers have found that maximum cellulase productivity was obtained by *B.pumilus* BpCRI6, *Pseudomonas flourescens*, *Monascus purpureus* and *Streptomyces* sp. BRC2, when tryptone was supplemented as an organic source to the production medium (Bakare *et al*., 2005; Chellapandi and Himanshu, 2008; Daniel *et al*., 2008). Moreover, this result was also in correlation with the findings of many other workers, whom found that maximum cellulase productivity was obtained when ammonium sulphate was added to the production media by *B.pumilus, Ruminococcus albus, Bacillus* sp. *Bacillus* sp.B21, *Streptomyces* sp.BRC2, respectively (Wood *et al*., 1982; Heck, 2002; Kotchoni *et al*., 2003; Poorna and Prema 2007; Chellapandi and Himanushu, 2008). Ray *et al*. (2007) suggested that organic nitrogen sources were found to be more suitable for optimizing cellulase production by *B.subtilis* and *B.circulans* than the inorganic sources.

In the present study, the effect of additional surfactants on enzyme yield was tested using production medium with addition of Tween20, Tween40, Tween60, Tween80, Triton X-100, SDS and Polyethylene glycol. The result inferred that, Tritonx100 supplemented medium showed maximum (7.80±0.73 and 12.86±0.60 U/ml) cellulase production by both the organisms, *B.altitudinis* and *B.licheniformis*. Usually surfactants alter the cell membranes of microbes to facilitate enzyme release (Reese and Mangulre, 1969; Pardo, 1996). Also the surfactants improve the cellulase stability and prevent the denaturation of enzymes during hydrolysis by desorbing it from cellulose substrate. Domingues *et al*. (2000) reported that addition of Tween80 enhanced the extracellular protein concentration and FPA of *Trichoderma reesei*. Similarly Bhardwaj (2012) also reported that Tween80 and Tritonx100 enhanced the production of amylase enzyme. Sun and Liu (2010) reported that surfactants increased the hydrolysis of lignocellullosic substances.

In the present study the effect of different metal ions on cellulase production by *B. altitudinis* and *B.licheniformis* were investigated. The results indicated that cellulase production by *B.altitudinis* was maximum of 7.52±0.37U/ml in Magnesium sulphate supplied medium, whereas *B.licheniformis* induced maximum enzyme production (1.82±0.42U/ml) in Manganous sulphate supplemented medium. Metal ions such as Ca, Mg, Fe, Co and Zn were necessary for cellulase synthesis by *Trichoderma viride* QM6a (Mandel and Reese, 1999). Li *et al.* (2012) reported that K^+ and Mn^+ activated cellulase production by *Bacillus thuringiensis.*

5 Conclusion

The present work proved the effect of various substrates on cellulose enzyme production. The results will be further used for large scale production of cellulase under controlled environmental conditions. Further more this work can be extended in aspect of purification and application of cellulose enzyme on large scale.

6 Acknowledgment

The authors wish to thank all the scientists and scholars who helped to complete this project.

References

[1] S. Akiba, Y. Kimura, K. Yamamoto and H. Kumagai, "Purification and characterizationof a protease-resistant cellulase from *Aspergillus niger," J. Ferment. Bioeng*., 79(2):125-130, 1995.

[2] A. Aristidou and M. Penttila, "Metabolic engineering applications to renewable resource utilization," *Current Opinion in Biotechnology*, 11(2): 87-198, 2000.

[3] P.S. Bagga, D.K. Sandhu and S. Sharma, "Catabolite repression of cellulose production in *Aspergillus nidulans," Process Biochemistry,* 24:41-45, 1989.

[4] A.K. Bahkali, "Influence of various carbohydrate on xylanase production by *Verticillium tricopus," Biores. Technol*., 53(3):265-268, 1996.

[5] M.K. Bakare, I.O. Adewale, A.O. Ajayi, A.I. Okoh and O.O. Shonukan, "Regulatory mutations affecting synthesis of cellulase in *Pseudomonas flourescens," African. J. Biotechnol*., 4:838-843, 2005.

[6] D.H. Bergey and J.G. Holt, "Classification of Bacteria Form Bergeys Manual of Determination Bacteriology," 1st edition, 4 vols, 1984.

[7] Bhardwaj, "Isolation and Biochemical characterization of *lactobacillus* species isolated from Dahi," *A journal of Dairy science and technology,* 1(2):18-31, 2012

[8] M. K. Bhat, "Cellulose degrading enzymes and their potential industrial applications," *Biotechnol. Adv.,* 15:583-620, 1997.

[9] P. Chellapandi and M.J. Himanshu, "Production of endoglucanase by the native strains of *Streptomyces* isolates in submerged fermentation," *Braz. J. Microbiol.,* 39(1), 2008.

[10] K.D. Clements, "Fermentation and gastrointestinal microorganisms in fishes," pp. 156-198. In : Mackle, R. I., White, B. A. (eds.), Gastrointestinal Microbiology, Vol. 1, *Gastrointestinal Ecosystem and Fermentations,* Chapman & Hall, Newyork, 1997.

[11] J.D. Daniel, T.S. Silvana, F.H. Plinho and A. Brandelli, "Production of extracellular β-glucosidase by *Monascus pupureus* on different growth substrates," *Process Biochem.,* 42(5):904-908, 2008.

[12] F.C. Domingues, J.A. Queiroz and J.M.S. Carbral, *Enzyme Microb. Technol.,* 26:394-401, 2000.

[13] S.G. Gayal and V.G. Khandeparkar, "Production of cellulase by *Penicillium funiculosum," Indian. J. Microbiol*., 38:167-168, 1998.

[14] M.M.C. Gielkens, E.Dekkers, J.Visser and L.H. Graaff, "Two cellubiohydrolase-encoding genes from Aspergillus niger require D Xylose and the xylanolytic transcriptional activator XlnR for their expression," *Appl. Environ. Microbiol.,* 65(10):4340-4345, 1999.

[15] J.X. Heck, P.F. Hertz and M.A.Z. Ayub, "Cellulase and xylanase productions by isolated amazon *bacillus* strains using soybean industrial residue based solid-state cultivation," *Brazilian. J. Microbiol.,* 33(3), 2002.

[16] E. Hoshino, M. Shiroshi, Y. Amaho, M. Nomula and T. Kanda, "Synergestic action of exotype cellulases in the hydrolysis of cellulases with different crystalllinities," *J. Ferment. Bioengg*., 84(4):300-306, 1997.

[17] G. Immanuel, R. Dhanusha, P. Prema and A. Palavesam, "Effect of different growth parameters on endoglucanase enzyme activity by bacteria isolated from coir retting effluents of estuarine environment," *Marine Biotech. Division*, Vol. 3,pp:25-34, 2006.

[18] S.W. Kang, E.H. Ko, J.S. Lee and S.W. Kim, "Over production of β-glucosidase by *Aspergillus niger* mutant from lignocellulosic biomass," *Biotechnol. Lett*., 21:647-650, 1999.

[19] Kazuhisa Miyamoto, "Renewable biological system for alternative sustainable energy production" (FAO-Agricultural services Bulletin, 28), Osaka University, Osaka, Japan, 1997.

[20] O.S. Kotchoni, O.O. Shonukan and W.E. Gachomo, "*Bacillus pumilus* BPCRI6 a promising candidate for cellulase production under conditions of catabolite repression," *J. Biotechnol.,* 2:140-146, 2003.

[21] W. Li, J. Xu, J. Wang, Y.J. Yan, X.F. Zhu, M.Q. Chen and Z.C. Tan, "Studies of monosaccharide production through lignocellulosic waste hydrolysis using double acids," *Energy fuels*., 22(3):2015-2021, 2008.

[22] L.R. Lynd, P.J. Weimer, W.H. Van zyl and H. Pretorius, "Microbial cellulose utilization. Fundamentals and Biotechnology," *Microbiol. Mol. Biol. Rev*., 66:506-577, 2002.

[23] M. LyndMandels and E.T. Reese, "Fungal cellulases and the microbial decomposition of cellulosic fabric," *J. Ind. Microbiol. Biotechnol.,* 22:22-240, 1999.

[24] K. Nakamura and K. Kappamura, "Isolation and identification of crystalline cellulose hydrolyzing bacterium and its enzymatic properties," *J. Ferment.Technol*., 60(4):343-348, 1982.

[25] G. Narasimha, A. Sridevi, B. Viswanath, S.M. Chandra and R.B. Reddy, "Nutrient effects on production of cellulolytic enzymes by *Aspergillus niger*," *African Journal of Biotechnology,* 5(5):472-476, 2006.

[26] A.P. Niranjane, P. Madhou and T.W. Stevenson, "The effect of carbohydrate carbon sources on the production of cellulase by *Phlebia gigantean," Enzyme and Microbial Technology,* 40(1):1464-1468, 2007.

[27] S.V. Nochure, M.F. Roberts and A.L. Demain, "True cellulose production by *Cellulomonas thermocellum* grown on different carbon sources," *Biotechnology Letter*, 15:641-646, 1993.

[28] A.G. Pardo, *Curr. Microbiol*., 33:275-278, 1996.

[29] J.B. Parry, J.C. Stewart, J. Heptinstall, "Purification of the major endoglucanase from *Aspergillus fumigatus Freseius. Biochem. J*., 213:437-444, 1983.

[30] J. Paul, A.K. Varma, "Hydrolytic enzymes production in *Micrococcus roseus* growing on different cellulosic substrates," *Lett. Appl. Microbiol.,* 16:167-169, 1993.

[31] C.A. Poorna and P. Prema, "Production of cellulase-free endoxylanase from novel alkalophilic thermostable *Bacillus pumilus* by solid-state fermentation and its application in wastepaper recycling," *Bioresource Technol.,* 98:485-490, 2007.

[32] A.K. Ray, A. Bairagi, K.S. Ghosh and S.K. Sen, "Optimization of fermentation condition for cellulase production by *Bacillus subtilis* CY5 and *Bacillus circulans* TP3 isolated from fish gut," *Acta Ichthyologica ET Piscatoria*, 37(1):47-53, 2007.

[33] E.T. Reese and A. Manguire, *Appl.Microbiol.* 17:242-245, 1969.

[34] S. Saxena, J. Bahadur and A. Verma, "Cellulose and hemicelluloses degradation bacteria from termite gut and mound soils of India," *Int. J. Microbiol.,* 33(1):55-60, 1993.

[35] C.S. Shin, J.P. Lee, I.S. Lee and S.C. Park, "Enzyme production of *Trichoderma reesei*. Gut C-30 on various lignocellulosic substance," *Applied Biotechnology and Biotechnology*, 84-86 (1-9) : 237-245, 2000.

[36] Z. Sun and S. Liu, "Production of n-butanol from concentrated sugar sample hemicellulosic hydrolysate by *Clostridia acetobutylicum* ATCC824," *Biomass & Bioenergy*, 2010, doi: 10.1016/ j.biombioe .2010.07.026.

[37] T.M. Wood, C.A. Wilson and C.S. Stewart, "Preparation of the cellulase from the cellulolytic anaerobic rumen bacterium *Ruminococcus albus* and its release from the bacterial cell wall," *Biochem. J.,* 205:129-137, 1982.

