Wood yards in the Kallai river belt of Kerala State of India as a rich source of microbes producing industrial enzymes

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Abstract: *The centuries old wood yards on the Kallai river banks in the suburbs of Kozhikode city of Kerala state of India are famous for timber based industries. Enormous microbes thriving on wood and producing industrial enzymes would have evolved in this environment. With this focus, a large number of cultures were isolated from these sites which included bacteria and fungi. The isolates were grown in mineral salt medium (MSM) supplemented with carboxy methyl cellulose (CMC) as carbon source. Among these, Penicillium verruculosum BS3 showed maximum cellulase activity in CMC medium and this culture was selected for fermentation studies. A comparative study of submerged and solid state fermentation was done using MSM supplemented with a mixture of 1% CMC and natural substrates like banana powder, potato powder, tapioca powder and saw dust. In comparison, cellulase production by P. verruculosum BS3 strain was very less in submerged fermentation (SmF) (2.3 U/mL), but the yield by solid state fermentation (SSF) was enhanced over hundred fold (252.93 U/gds) in the presence of banana powder (30%) in the MSM. Likewise, 10% tapioca powder also supported substantial production of cellulase (161.615 U/gds). From this, it is evident that SSF offers better choice over SmF for increasing the production.*

Key words: wood yard, microbes, industrial enzymes, *Penicillium verruculosum*

INTRODUCTION

Cellulases are inducible enzymes, which are synthesized by many microorganisms during their growth on cellulosic materials. A large number of microorganisms including fungi, bacteria and actinomycetes have the ability to produce cellulose degrading enzymes, cellulases. Of these, fungi are particularly active as cellulose decomposers and may be responsible for as much as 80% of the cellulose breakdown (Landecker, 1996). Enzymatic hydrolysis of cellulosics, the most abundant renewable resource on earth, offers an attractive alternative for the generation of sugars which can serve as the raw materials for the production of various products of commercial interest such as ethanol (Qu *et al*., 2006), organic acids (Shen and Xia, 2006; Lee *et al*., 2004) and other chemicals (Cao *et al*., 1997), if the process can be made economically competitive. Aiming towards reducing the cost of enzyme production, the use of various industrial wastes such as sawdust hydrolysate (Lo *et al*., 2005), corn cob (Liming and Xueliang, 2000), bagasse (Bigelow and Wyman, 2002) and agricultural waste (Jiang *et al*., 1998) have been successfully used as substrates for cellulase fermentation by microorganisms. Solid state fermentation (SSF) for the production of cellulase is rapidly gaining interest as a cost effective technology for the production of enzyme and higher yields of cellulase is reported under SSF compared to liquid cultures (Singhania *et al*., 2006). The isolation of potent strain has an important role in enzyme production.

The centuries old wood yards on the Kallai river banks in the suburbs of Kozhikode city of Kerala state of India are famous for timber based industries and allied business. Due to this an enormous quantity of lignocellulolytic wastes is being created day by day in the form of carpentry waste, sawdust, wood chips, etc. It is expected that, by natural processes, enormous microbial wealth (bacteria, fungi and yeast) thriving on wood have evolved in this environment. Considering this fact, an effort has been carried out presently to isolate cellulolytic microbes from this river belt with the following objectives:

1. Cellulase production by SmF using carboxymethyl cellulose and various natural carbon sources.
2. Cellulase production by SSF using natural carbon sources and determination of dry biomass in the culture after SSF.

MATERIALS AND METHODS

1. Selection of organism

Even though numerous cellulolytic bacteria and fungi were isolated from the wood yards of Kallai *Penicillium verruculosum* BS3 which showed maximum cellulolytic activity upon primary screening was selected for fermentation study. Pure fungal culture (*Penicillium verruculosum* BS3) isolated from the decayed wood samples collected from Kallai river belt and were maintained on potato dextrose agar (PDA) slants at 4^{o}C. in the Enzyme Technology Laboratory of University of Calicut, Kerala was used for the present study.

2. Cuitivation of *P. verruculosum* BS3 for cellulase production

For the production of extracellular cellulase, *P.verruculosum* strain BS3 was cultivated in various media combinations as described in the succeeding sessions. Czapek- mineral salt medium (g/l): $NaNO_3$-2; K_2HPO_4-1; $MgSO_4$-0.5; KCl-0.5 and proteose peptone was the basal medium used throughout the study. During the study, it was supplemented with soluble/ raw carbon source. Prior to inoculation, all the media were autoclaved at 15 ψ, 121 ^oC for 15 min.

3. Submerged fermentation (SmF)

To investigate the cellulase activity in liquid medium *P. verruculosum* BS3 was cultured in MSM with 1% carboxy methyl cellulose sodium salt (CMC) 1% tapioca powder (TP), banana powder (BP), potato powder (PP) and jackseed powder (JP) as carbon source in 100 mL flasks. 15 mL of MSM with 1% CMC was taken in each 100 mL flasks and sterilized at 121^oC for 15 min. After cooling, inoculated the flasks with 100µL of spore suspension (~ 15 x 10^7 spores/mL= colony forming unit, CFU) in aseptic condition. All flasks were incubated in a rotary shaker at 200 rpm, 28^oC for 7 days. The culture broth was centrifuged at 9000xg for 10min. at 4^oC temp. and the clear supernatant was used for enzyme assay.

4. Solid state fermentation (SSF)

To find out the effect of natural carbon source on cellulase production by SSF, *P. verruculosum* BS3 was cultured in 5mL of MSM with raw materials like tapioca powder (TP), banana powder (BP), potato powder (PP) and jack seed powder (JP) of different percentages (10%, 30%, 50% and 100%) on Petri plates. All Petri plates were sterilized at 120^oC for 15 min. Inoculated all plates with 100 µL spore suspension (15 x 10^7/ mL) and incubated at 28^oC for 7 days.

5. SSF with sawdust

The lignocellulosic substrate, sawdust was collected freshly from one of the saw mills at Farook, Kozhikode, Kerala. It was sun-dried and stored in plastic bags. This was used as such as carbon source for SSF. Solid state fermentation of sawdust was carried out in 100 mL conical flasks. 2g of sawdust was taken in individual flasks and added MSM (pH 7) to give moisture content and nutrients. The flasks were inoculated with 100µL spore suspension (15 x 10^7/ mL) in sterile condition. All flasks were incubated at 28^oC for 15 days under static condition. The samples were withdrawn after 3rd, 6th, 9th, and 15th day of incubation and were extracted by adding 10mL of citrate buffer and stirred for 5 min. under chilled condition followed by centrifugation at 9000xg for 10 min. at 4^oC temp. and the clear supernatant was used for enzyme assay.

6. Effect of moisture level on cellulase production by SSF

To investigate the effect of moisture level on cellulase production, different moisture levels were used to compare the enzyme activity. For the purpose, added MSM into 2g sawdust in the ratio of 1:1, 1:2 and 1:3 in 100mL flasks. Then mixed well and sterilized at 121^oC for 15 min. After cooling the flasks were inoculated with 100µL of spore suspension (~ 15 x 10^7 spores/mL) and the contents, after mixing, were incubated at 28^oC for 15 days. The samples were withdrawn after 3rd, 6th, 9th and 15th day of incubation and were extracted by adding 10mL of citrate buffer and stirred for 5 min. under chilled condition, followed by centrifugation at 9000xg for 10 min at 4^oC temp. and the clear supernatant was used for enzyme assay.

7. Determination of dry biomass

The dry fungal biomass was determined by measuring the solid dry weight. By this method, the mycelial weight was calculated from the difference between the total dry weight of the solids (comprising mycelium and substrate) and that of the substrate (control). The dry weight of the solids was determined by drying 1g sample overnight at 100^oC in a hot air oven.

8. Cellulase assay*s*

Cellulase was assayed by employing the 3, 5– dinitrosalicylic acid (DNS) method (Miller, 1959). The reaction mixture contained 0.5mL extracted enzyme solution (supernatant) and 0.5mL 1% CMC in 0.1M citrate buffer (pH4.8) solution and it was incubated for 30min. at 50^oC. Stopped the reaction by adding 3mL DNS reagent. Heated the solution in a boiling water bath (100^oC) for 5 min. and cooled. Absorbance was measured at 540nm using spectrophotometer (ELICO DOUBLE BEAM BL 200 BIO SPECTROPHOTOMETER).

9. Calculation of cellulase activity

One unit (U/mL) of cellulase activity is defined as the amount of protein (cellulase) required to liberate 1µmol of reducing sugar (D- glucose) from CMC/min. under the assay condition. Protein content was estimated using Lowry's method (Lowry *et al*., 1951) with bovine serum albumin (BSA) as the standard.

RESULTS AND DISCUSSION

RESULTS

1. Molecular characterization of the fungus

The service of Xcelris Labs Ltd. Ahmedabad , India was employed for the sequencing of the PCR product for the identification of the fungus used in this study at molecular level. Upon sequencing and alignment, the culture was identified as *Penicillium verruculosum* strain BS3 (GenBank Accession Number: HQ876770).

2. Culturing of *P. verruculosum* BS3 on PDA medium

In PDA medium, *P. verruculosum* BS3 started to grow within 24h of inoculation with spores. Sporulation was visible after 2 days of incubation at 28^{o}C. Maximum growth occurred within 5- 6 days (Fig. 1).

3. Cellulase production by *P. verruculosum* BS3 in MSM with 1% CMC

Presence of 1% (w/v) CMC in MSM enhanced the cellulase activity of *P. verruculosum* BS3 by shake flask culture at 200rpm, 28^{o}C in a rotary shaker. The result was observed at 24h intervals. The maximum production was at 5th and 6th day of incubation and it was 2.192U/mL and 2.301U/mL respectively (Fig. 2).

4. Cellulase activity of *P. verruculosum* BS3 in MSM supplemented with 1% natural carbon sources

The raw carbon sources used were TP, BP, PP and JP at a concentration of 1% (w/v) supplement in MSM. The effect of natural carbon sources on cellulase production by *P. verruculosum* BS3 was observed on 5th day of incubation, at 200rpm and 28^{o}C. From the results of cellulase assay all of the four supplements showed almost similar cellulase activity. Maximum production was exhibited by the organism within 1% banana powder (1.98U/mL) and the lowest activity was shown by 1% tapioca powder (1.69 U/gds) (Fig. 3). The results suggest that presence of natural carbon sources in the medium can induce production of cellulases as 1% CMC by this organism.

5. Cellulase production by *P. verruculosum* BS3 on MSM supplemented with different natural raw carbon sources by SSF

Cellulase activity of *P. verruculosum* BS3 was studied by replacing sawdust with four locally available natural raw carbon sources, *viz*., TP, BP, JP and PP. The MSM was supplemented with one of these carbon sources at 10%, 30%, 50% and 100% (w/v) concentrations at a time. Cellulase activity observed on the 7th day of incubation varied in each carbon source. Maximum cellulase activity was observed in BP supplemented medium than others.

In BP supplemented MSM, maximum activity (252.929 U/gds) observed at 30% concentration of BP (Fig. 4).This was the only carbon source which induced maximum activity at 30% concentration. But the activity was much higher, compared to other supplements. In MSM with TP, maximum cellulase activity was 161.6 U/gds which was observed at the concentration of 10% TP (Fig. 4). This was the second carbon source which showed some more cellulase production. At 100% there was no production. Among the four ones, this was the only one natural carbon source which provided this much maximum activity by *P. verruculosum* BS3 at 10% of its concentration.

The maximum cellulase activity observed in MSM with PP was 107.319 U/gds, which was observed at 10% of concentration (Fig. 4). PP provides comparatively low cellulase production. There was no much activity in other concentrations. In the case of JP supplemented MSM the maximum cellulase activity observed was also at 10% (35.209 U/gds) (Fig. 4). But as compared to all others this carbon source had low contribution to cellulase production by *P. verruculosum* strain BS3. The result shows that, the presence of natural carbon sources in MSM at different ratios can enhance cellulase production by *P. verruculosum* BS3 than the presence of sawdust. The moisture content plays an important role in SSF.

6. Cellulase production by *P. verruculosum* BS3 on sawdust with initial moisture content (MSM) at different ratios, by SSF

Cellulase production of *P. verruculosum* BS3 was studied by culturing on sawdust as carbon source with different percentage of initial moisture content, MSM (1:1, 1:2 & 1:3 ratios). Within 3 days of incubation fungal mycelia started to visualize. Enzyme production was observed at 3 days interval by enzyme assay. Maximum production was observed at 1:3 ratio of sawdust and MSM compared to others. In the case of 1:1 and 1:2 concentrations maximum activity was shown on 9th (16.486 U/gds) and 6th (17.837 U/gds) day of incubation. At 1:3

concentration more activity started within three days when compared to others. Increase in enzyme production was observed until the 12th day (29.739 U/gds) and after it, it started to reduce (Fig. 5).

7. Determination of dry biomass after SSF with sawdust

Dry biomass of *P. verruculosum* BS3 by SSF with sawdust and MSM in the ratio of 1:1, 1:2 and 1:3 were determined by calculating fresh weight and dry weight of samples. As a result an increase in dry biomass was observed at each culture condition (Fig. 6). Maximum dry biomass was observed at 1:1 ratio than others and it was about 0.251g /1g sample on 15th day. On the day, the other combinations 1:2 and 1:3 showed dry biomass as 0.102g/1g and 0.086g/1g respectively.

DISCUSSION

The prime objective of this study was to check cellulase production by *P. verruculosum* strain BS3 at different culture conditions and its partial purification. Researchers have strong interests in cellulases because of their applications in industries of starch processing, grain alcohol fermentation, malting, brewing and extraction of juice from fruits and vegetables and pulp from wood for paper industries (Sipos *et al*., 2010). Many microorganisms including fungi, bacteria and actinomycetes have the ability to produce cellulases. Among them fungi are predominant in industrial applications of cellulase production. Several studies have been carried out to produce cellulolytic enzymes in bio-waste degradation process by several fungi such as *Trichoderma sp*., *Penicillium* sp. and *Aspergillus* sp. (Hoffman and Wood, 1985; Brown *et al.,* 1987; Lakshmikant and Mathur, 1990). Based on the morphological characteristics, *Jin et al.* (2008) isolated different cellulolytic bacteria and fungi from water logged woods.

The type of strain, culture conditions, nature of the substrate and availability of nutrients are the important factors affecting cellulase production by microbes (Pandey et al., 2001). Sankar and Isaiarasu (2011) showed that agro-industrial substrates are considered best for enzyme production. Cellulase production by microorganisms is conventionally made by submerged fermentation process (SmF). But now-a-days solid state fermentation (SSF) is being practised, because it offers some apparent economic and engineering advantages over the classical SmF (Karmakar and Ray, 2011). SSF is advantageous to produce more stable products, requiring less energy, in smaller fermenters, with easier downstream processing measures (Robinson *et al.,* 2001).

The core objective of this study was to bioprospect the microbial wealth in this environment and to make it available for lignocelluloses based bioprocess industry. Many lignocellulolytic fungal and bacterial cultures were purified to homogeneity from this site and among them, the fungus *Penicillium verruculosum* BS3 seems to be a moderately good producer of cellulase.

To analyse the cellulase activity by SmF in the 1% CMC (w/v) supplemented MSM (at 200 rpm, pH 7, and temperature ~28° C), crude supernatant obtained after 24 h fermentation was used. The result showed that the presence of 1% CMC in the MSM could enhance cellulase production. Maximum production was observed on the 6th day of incubation (2.3 U/mL). Of different natural carbon sources (1% w/v) such as tapioca powder, banana powder, potato powder and jack seed powder tested under SmF, 1% banana powder supplement was found to be better (2U/mL, on 5th day), which was comparable to the effect of CMC. Alam *et al.* (2004) investigated cellulase production by *Streptomyces omiyaensis* in liquid Winsted's medium having 1.2% CMC as substrate and beef extract as nitrogen source. CMC-ase activity was 269.44 U/mL at pH 6.5 and temperature 45°C. They concluded that CMC was the best cellulosic substrate for inducing the synthesis of extracellular cellulolytic enzymes. Milala *et al*. (2005) used millet, guinea corn straw, rice husk and maize straw as substrates for the production of cellulolytic enzymes by *Aspergillus niger* in SmF. The result showed that optimal cellulase secretion by A*spergillus niger* was achieved at a time (growth period) of 72 hours in maize straw and rice husk media respectively.

In order to check whether water restricted environment (SSF) would enhance cellulase production by *P. verruculosum* BS3, sawdust was supplemented with MSM or MSM was supplemented with different percentages (w/v) of natural raw carbon sources (TP, BP, PP and JP at 10%, 30%, 50% and 100%, all w/v). In the MSM supplemented medium, sawdust to MSM (liquid) ratios were 1:1, 1:2 or 1:3. The results at 3 day intervals showed that, compared to others, maximum cellulase activity was observed in 1:3 ratio. From the analysis of the effect on cellulase production by various natural supplements, BP supported the maximum production (252.93U/gds at 30% concentration, on 7th day of incubation). According to Benjamin and Pandey (1998), SSF holds tremendous potential for the production of enzymes and has special interest in those processes where the crude fermented product may be used directly as enzyme source. Sawdust is considered as a good substrate for cellulase production

by microorganisms. Sawdust (wood) is composed of complex plant cell wall polymers which include cellulose, hemicellulose and lignin (Grant and Long, 1981). Chinedu *et al.* (2007) reported that *P. chrysogenum* PCL501 produced extracellular proteins with significant cellulase activity in media containing cellulose and sawdust but not in glucose containing medium. The waste cellulosic material like wood waste can be used as low cost carbon source for commercial cellulase production.

Kostov *et al*. (1991) investigated the decomposition of coniferous sawdust and bark by treating with cellulose degrading microorganisms, *viz*., strains of *Bacillus* sp., *Cephalosporium* sp. and *Streptomyces* sp. Chandra *et al.* (2007) compared the production of cellulolytic enzymes by *Aspergillus niger* on lignocellulosic substrates such as groundnut fodder, wheat bran, rice bran and sawdust by SSF at laboratory level. Czapek Dox liquid broth with 0.5% cellulose was used to moisten the substrates. The result showed that wheat bran was the best solid support followed by groundnut fodder in cellulolytic enzyme production. Wheat bran yielded 3.24 U/g CMCase against 1.36U/g by ground nut fodder.

In conclusion, the isolated *P. verruculosum* BS3 has proved to be a potent strain with high cellulolytic activity. Cellulase production by *P. verruculosum* BS3 strain was 2.3U/mL)in SmF. In SSF it was enhanced over hundred fold (252.93 U/gds) in the presence of banana powder (30%) in the MSM. Likewise, 10% tapioca powder also supported substantial production of cellulase (161.615 U/gds). From this, it is evident that SSF offers better choice for the commercial production of cellulase. Sawdust mixed with the aforesaid natural carbon sources would enhance the production still at higher levels.

Fig. 1. Growth of *P. verruculosum* BS3 on potato dextrose agar medium on 5th day of incubation at 28^{0}C on Petri plate.

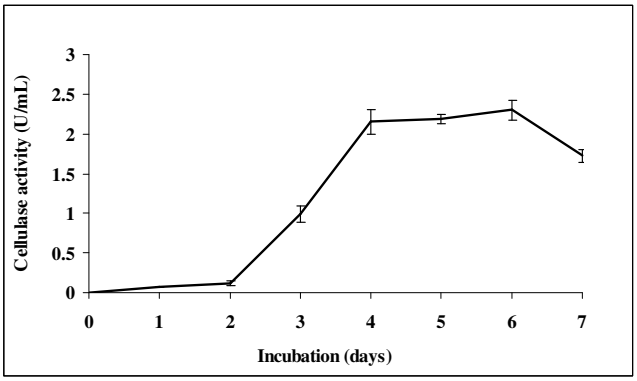

Fig. 2. Cellulase production profile of *P. verruculosum* BS3 on MSM supplemented with 1% CMC at 24h intervals (pH 7) at 200rpm and 28^{o}C incubation.

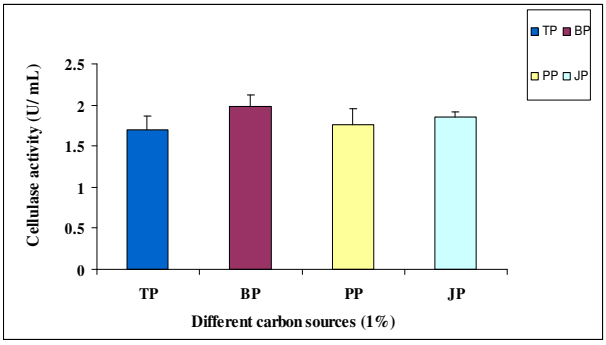

Fig. 3. Cellulase production profile of *P. verruculosum* BS3 on MSM supplemented with different natural carbon sources (1%, w/v) on 5th day of incubation, 200rpm and 28^{o}C: TP (tapioca powder), BP (banana powder), PP (potato powder) and JP (jack seed powder).

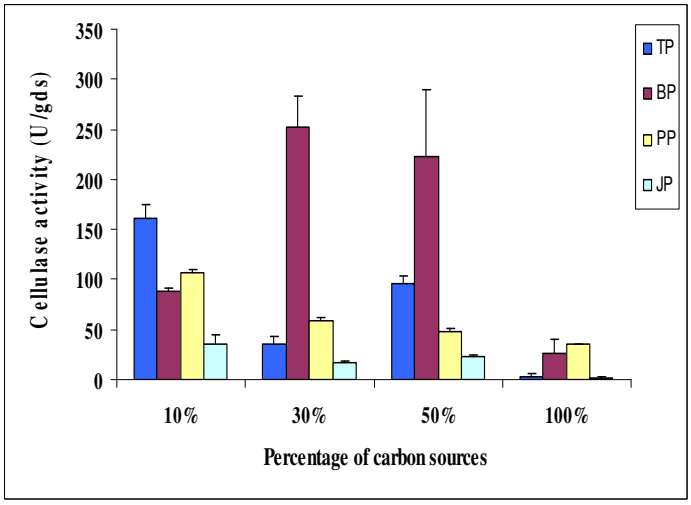

Fig. 4. Cellulase production of *P.verruculosum* BS3 on MSM supplemented with different percentages (10%, 30%, 50% and 100%) of natural carbon sources on 7th day at 28^{o}C of incubation: TP (tapioca powder), BP (banana powder), PP (potato powder) and JP (jack seed powder), all (w/v) .

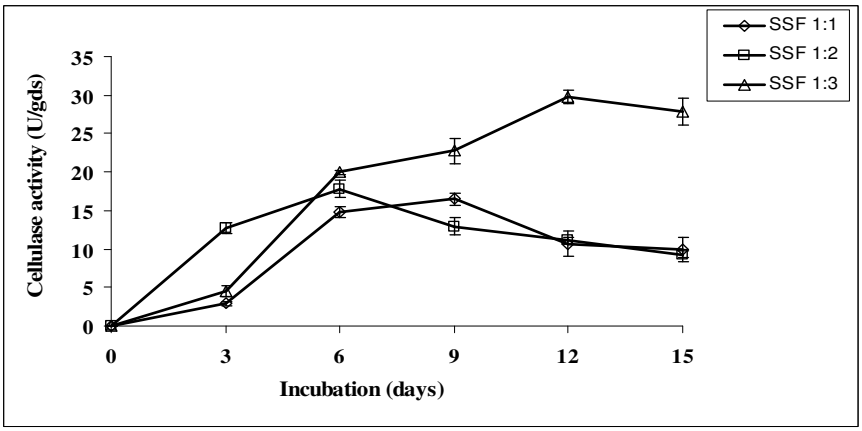

Fig. 5. Cellulase production profile by *P. verruculosum* BS3 on sawdust supplemented with MSM at different moisture content levels, sawdust: water (MSM) at 1:1, 1:2 and 1:3 ratios by SSF at 3 days interval and 28^{o}C of incubation.

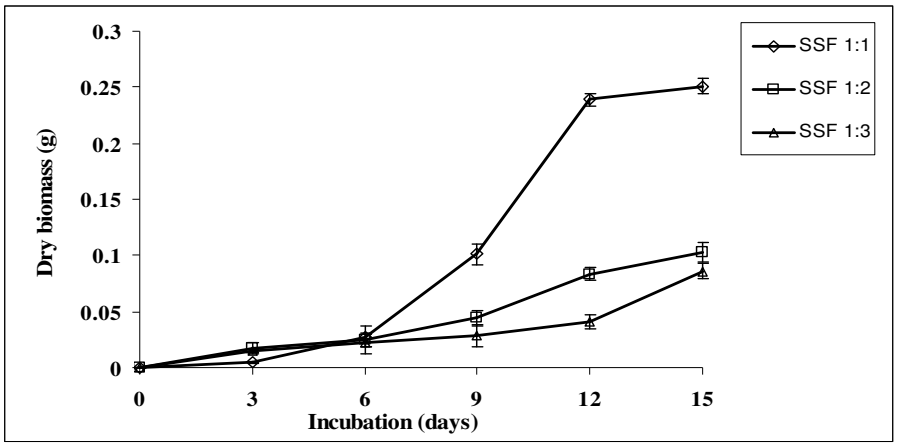

Fig. 6. Dry biomass profile of *P. verruculosum* BS3 after SSF on sawdust: water (MSM) of different ratios (1:1, 1:2 and 1:3) at 3 days interval and 28^{o}C of incubation.

REFERENCES

Alam M.Z., Manchur M.A. and Anwar M.N., 2004. Isolation, purification and characterisation of cellulolytic enzymes produced by the isolate *Streptowyces omiyaensis. Pakistan Journal of Biological Sciences* 7: 1647 - 1653.

Archana P.I. and Mahadevan A., 2002. Lignin degradation by bacteria. *Progress in Industrial Microbiology,* 36: 311-330.

Benjamin S. and Pandey A., 1998. *Candida rugosa* lipases: molecular biology and versality in biotechnology. *Yeast* 14: 1069- 1087.

Bigelow M. and Wyman C.E., 2002. Cellulase production on bagasse pretreated with hot water. *Applied Biochemistry and Biotechnology* 98-100: 921–934.

Brown J.A., Collin S.A. and Wood T.M., 1987. Development of a medium for high cellulase, xylanase and β-glucosidase production by a mutant strain (NTG1116) of the cellulolytic fungus *Penicillium pinophillum*. *Enzyme Microbial Technology* 5: 425-429.

Cao N.J., Xia Y.K., Gong C.S. and Tsao G.T., 1997. Production of 2, 3- butanediol from pretreated corn cob by *Klebsiella oxytoca* in the presence of fungal cellulase. *Applied Biochemistry and Biotechnology* 63- 65:129–139.

Chandra M.S., Viswanath B. and Reddy B.R., 2007. Cellulolytic enzymes on Lignocellulosic substrates in solid state fermentation by *Aspergillus niger*. *Indian Journal of Microbiology* 47: 323-328.

Chinedu S.N., Okochi V.I., Smith H.A., Okafor U.A., Okerenta B.M.O. and Omidiji O., 2007. Effect of carbon sources on cellulase (EC 3. 2. 1. 4) production by *Penicillium chrysogenum* PCL501. *African Journal of Biochemistry Research* 1: 006-010.

Grant W.D. and Long P.E., 1981. The Carbon Cycle. In 'Environmental Microbiology, Tertiary Level Biology, Thomas Litho Ltd., Scotland: 91-116.

Hoffman R.M. and Wood T.M., 1985. Isolation and partial characterisation of a mutant *Penicillium* for the saccharification of straw. *Biotechnology and Bioengineering* 27: 81- 85.

Jiang Y., Qu Y. and Chen S., 1998. Screening of alkaline B-glycanases producer and optimization of enzyme production. Proc. VII International Conference on Biotechnology in the Pulp and Paper Industry, 1998, Canada: 177–180.

Karmakar M, and Ray R.R., 2011. A statistical approach for optimization of simultaneous production of β-Glucosidase and Endoglucanase by *Rhizopus oryzae* from solid state fermentation of water hyacinth using central composite design. *Biotechnol Res Int*. 2011. doi: 10.4061/2011/574983.

Kostov O., Rankov V., Atanacova G. and Lynch J.M., 1991. Decomposition of sawdust and bark treated with cellulose decomposing microorganisms. *Biology and Fertility of Soils* 11: 105-110.

Lakshmikanth K. and Mathur S.N., 1990. Cellulolytic activities of *Chaetomium globosum* on different cellulosic substrates. *World Journal of Microbiology and Biotechnology* 11: 23-26.

Landecker E.M., 1996. Fundamentals of the Fungi, Fourth Edition. *Simon and Schuster / A. Viacom Company, Prentice Hall, Upper Saddle River.*

Liming X. and Xueliang S., 2000. High yield cellulase production by *Trichoderma reesei* ZU-02 on corn cob residue. *Bioresource Technology* 91:259–262.

Lo C.M., Zhang Q., Lee P. and Ju L.K., 2005. Cellulase production by *Trichoderma reesei* using sawdust hydrolysate. *Applied Biochemistry and Biotechnology* 121–124: 561–573.

Lowry O.H., Rosebrough N.J., Farr A.L. and Randall R.J., 1951. Protein measurement with folin-phenol reagent. *Journal of Biological Chemistry* 193: 265-275.

Milala M.A., Shugaba A., Gidado A., Ene A.C. and Wafar J.A., 2005. Studies on the use of agricultural wastes for cellulase enzyme production by *Aspegillus niger. Research Journal of Agriculture and Biological Sciences* 1: 325-328.

Miller G., 1959. Use of dinitrosalicylic reagent for the determination of reducing sugars. *Analytical Chemistry* 31: 426- 428.

Qu Y., Zhu M., Liu K., Bao X. and Lin J., 2006. Studies on cellulosic ethanol production for sustainable supply of liquid fuel in China. *Biotechnology Journal* 1: 1235–1240.

Robinson T., Singh D. and Nigam P., 2001. Solid state fermentation: a promising microbial technology for secondary metabolite production. *Applied Microbial Biotechnology* 55:284-289.

Shankar T. and Isaiarasu L., 2011. Cellulase production by *Bacillus pumilus* EWBCM1 under varying cultural conditions. *Middle East Journal of Scientific Research* 8: 40-45.

Shen X. and Xia L., 2006. Lactic acid production from cellulosic material by synergistic hydrolysis and fermentation. *Applied Biochemistry and Biotechnology* 133: 252–262.

Singhania R.R., Sukumaran R.K., Pillai A., Prema P., Szakacs G. and Pandey A., 2006. Solid state fermentation of lignocellulosic substrates for cellulase production by *Trichoderma reesei* NRRL 11460. *Indian journal of Biotechnology* 5: 332-336.

Sipos B., Benko Z., Dienes D., Reczey K., Viikari L. and Siika-aho M., 2010. Characterisation of specific activities and hydrolytic properties of cell wall degrading enzymes produced by *Trichoderma reesei* Rut C30 on different carbon sources. *Applied Biochemistry and Biotechnology* 161: 347-364.