Cellulolytic Enzymes Production from Submerged Fermentation of Different Substrates by Newly Isolated *Bacillus* spp. FME

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Newly isolated strains *Bacillus* sp. FME 1 and FME 2 were evaluated for the cellulolytic enzymes production during submerged fermentation (SmF) of different substrates including rice husk, Whatman filter paper and cellulose powder CF 11. Extracellular enzyme assays for CMCase, FPase and β-glucosidase were examined up to 8 days of submerged fermentation. Among the three substrates, rice husk was the most suitable substrate for higher production of cellulolytic enzymes. Maximum titers of 100, 45, and 3.5 U/mL in respect of CMCase, FPase and β-glucosidase in *Bacillus* sp. FME 2 were recovered as against 45, 12, and 0.39 U/mL in *Bacillus* sp. FME 1 respectively, at their respective peak time intervals. *Bacillus* sp. FME 2 was found to produce higher cellulolytic enzyme activities than *Bacillus* sp. FME 1.

Key words: Bacillus *sp., cellulolytic enzymes, cellulose powder CF 11, rice husk, submerged fermentation, Whatman filter paper*

Rice husk, Whatman filter paper, and cellulose powder CF-11 are lignocellulosic materials containing cellulose, hemicellulose, and lignin [Mishra *et al.,* 2007]. Free living cellulolytic bacteria such as *Bacillus* sp. have been studied from the point of understanding the enzyme systems involved in the cellulose degradation [Srinivasan and Lakshman, 1988; Mishra *et al.,* 2007]. Cellulases act synergistically to convert the complex carbohydrates present in the lignocellulosic biomass into glucose, which can be subsequently fermented into ethanol, butanol, acetone or 2,3-butanediol on a large scale [Gadgil *et al.,* 1995; Hoshino *et al.,* 1997; Van Wyk, 2001] for use as biofuels. In spite of the huge coverage of research for finding more active enzyme preparations from a large variety of microorganisms, the enzymatic saccharification of lignocelluloses so far has not been reached to the level of conversion of starch into glucose by the microbial enzymes [Gomes *et al.,* 2006]. Thus, much work and research is needed to produce enzymes capable of saccharifying the lignocelluloses.

The cellulases have attracted considerable attention in recent years due to their great biotechnological and industrial potentials. Cellulases have a wide range of applications, and the main potential applications are in food, animal feed, textile, fuel, and chemical industries. Other areas of application include the paper and pulp industry, waste management, medical/pharmaceutical industry, protoplast production, genetic engineering, and pollution treatment [Coughlan, 1985a; Coughlan, 1985b; Mandels, 1985; Beguin and Aubert, 1994].

The promising cellulolytic microorganisms can be employed for the production of cellulolytic enzymes such as CMCase, FPase, and β-glucosidase by using different ago-residues as the carbon source during submerged

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Abbreviations: CMC, carboxymethyl cellulose; CMCase, carboxymethyl cellulase; DNS, dinitrosalicylic acid; FME, flour mill effluent; FPase, filter paperase; PNPG, *p*-nitrophenyl-β-D-glucopyranoside

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fermentation [Mishra *et al.,* 2007]. Large amount of agro-residues, rich in lignocelluloses, are generated in bulk throughout the world. In India substantial amount of crop residues (376 million tons) are produced every year [FAO, 1998]. To date, the production of cellulolytic enzymes has been intensively studied in the submerged fermentation with different microorganisms in comparison to the solid-state fermentation [Lynd *et al.,* 2002]. Keeping in view the above essentials, the present investigation was under taken to assess the cellulolytic enzymes activity of the selected microbial strains during the submerged fermentation.

Materials and Methods

Isolation and identification of bacterial cultures. Bacterial cultures were isolated from the flour mill effluents around Tirupati, India. Traditional serial dilution agar plating method was used for the isolation of cellulolytic bacteria. Isolated bacterial cultures were tested for the cellulase production by the plate assay method. The promising isolates were exploited for further characterization. The bacterial cultures were initially identified by means of morphological examination and some biochemical characterizations. The parameters investigated included colony morphology, Gram staining, pigment production, spore staining, motility, starch hydrolysis, and casein and gelatin hydrolyses (Table 1).

Fermentation medium. Fifty milliliters of Nutrient solution medium (yeast extract 0.1 g, sucrose 0.2 g, substrate 1.0 g, K_2HPO_4 0.1 g and $FeSO_4$ 0.001 g, distilled water 100 mL) containing 0.5 mL of the basal salt solution ($NaNO_3$ 10.0 g, KCl 2.5 g, $MgSO_4$ 2.5 g and distilled water 50 mL) in 250-mL Erlenmeyer flasks were inoculated at 0.5% (v/v) bacterial culture after 3 days growth on the Nutrient solution medium and incubated at 30°C and 120 rpm for 24 h in an orbital shaking incubator.

Table 1. Biochemical and growth characteristics of the bacterial isolates

Characteristics/ Biochemical tests	Bacterial isolates	
	Bacillus sp. FME 1	*Bacillus* sp. FME 2
Pigment production	-	-
Colony size	0.2 mm	0.2 mm
Spore staining	+	+
Gram's staining	Gram positive	Gram positive
Motility	+	+
Starch hydrolysis	+	+
Casein hydrolysis	+	+
Gelatin hydrolysis	+	+

+, positive reaction; -, negative reaction

The flasks were withdrawn every 2 days over a period of 8 days and centrifuged at 10,000 rpm for 10 min at 4°C. The clear centrifugal supernatant obtained was used for the enzyme assay.

Assay of CMCase. The CMCase activity was measured according to the method of Ghosh [1987] using a reaction mixture containing 1 mL of 1% CMC in 0.05 M citrate acetate buffer (pH 5.0) and aliquots of the suitably diluted centrifugal supernatant. The reaction mixture was incubated at 50°C for 30 min, and the reducing sugar produced was determined by the DNS method [Miller, 1959]. One unit (U) of endoglucanase activity was defined as the amount of enzyme releasing 1 μmole of reducing sugar from CMC per min.

Assay of FPase. The activity of FPase in the centrifugal supernatant was determined according to the method of Mandels *et al.* [1976]. Aliquots of the appropriately diluted centrifugal supernatant as enzyme source was added to 50 mg of Whatman No. 1 filter paper strip immersed in 1 mL of 0.05 M sodium citrate buffer, pH 5.0. After incubation at 50°C for 30 min, the reducing sugar released was estimated by the DNS method [Miller, 1959]. One unit of filter paper (FPU) activity was defined as the amount of enzyme releasing 1 μmole of reducing sugar from filter paper per milliliter per min.

Assay of β-glucosidase. The β-glucosidase activity was assayed by the method of Herr [1979]. β-Glucosidase activity was measured in 1 mL of 5 mM PNPG in 0.05 M citrate buffer (pH 5.0) and aliquots of the appropriately diluted centrifugal supernatant and incubated at 50°C for 30 min. The reaction was terminated by the addition of 4 mL of 0.1 M NaOH-glycine buffer, and the released *p*-nitrophenol was read at 420 nm. One unit of the enzyme activity was defined as the amount of enzyme producing 1 μmole of *p*-nitrophenol per min.

Statistical analysis. Data presented are the averages of three replicates. The statistical analysis for standard deviation was carried out using Instat+v3.33 and SPSS 10.0 soft ware packages.

Results and Discussion

Production of CMCase. The selected promising bacterial cultures were tentatively identified as *Bacillus* sp. FME 1 and FME 2 based on the characteristics and the microscopic observation of the cultures. The production of CMCase in the lignocellulosic substrates was monitored for 8 days in submerged fermentation (Fig. 1). Maximum CMCase activity was recorded on day 6 of incubation in rice husk and Whatman filter paper, whereas that of the cellulose powder CF 11 was recorded on day 4. Growth of *Bacillus* sp. FME 2 in the rice husk

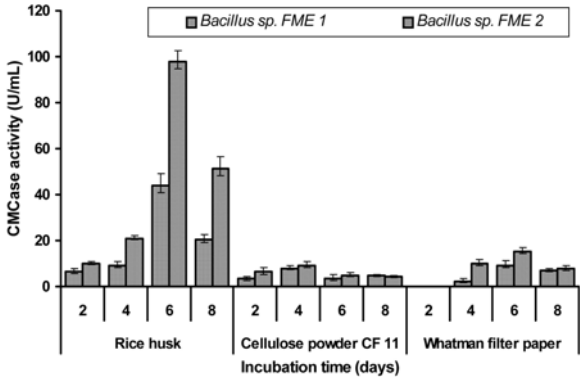

Fig. 1. Effect of different cellulosic substrates on CMCase production. Values in the figure are means of three replicates with standard deviation.

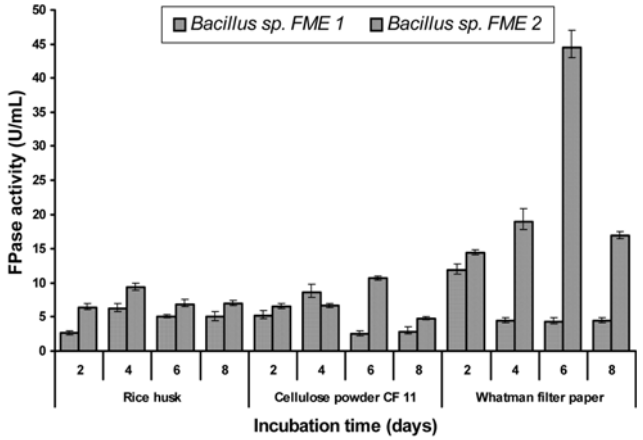

Fig. 2. Effect of different cellulosic substrates on FPase production. Values in the figure are means of three replicates with standard deviation.

gave the highest CMC activity of 100 U/mL as against 50 U/mL from *Bacillus* sp. FME 1, while Whatman filter paper and cellulose powder CF 11 gave 17 and 11 U/mL by FME 2 as against 10 and 8 U/mL by FME 1, respectively. Among all lignocellulosic substrates used in the present study, rice husk was the best substrate for the production of CMCase, and *Bacillus* sp. FME 2 was a better culture for CMCase production than *Bacillus* sp. FME 1.

Production of FPase. Growth of *Bacillus* sp. FME 2 on the Whatman filter paper yielded highest titres of 45 U/mL of FPase as against 11.6 U/mL on cellulose powder CF 11 and 10.2 U/mL on rice husk. Whereas the growth of *Bacillus* sp. FME 1 on Whatman filter paper yielded highest titres of 12 U/mL of FPase as against 10 U/mL on cellulose powder CF 11 and 7 U/mL on rice husk (Fig. 2). Whatman filter paper was the best substrate for FPase production, and *Bacillus* sp. FME 2 was a more suitable culture for FPase production than *Bacillus* sp. FME 1.

Production of β-glucosidase. Maximum titres, 3.5 U/mL, of β-glucosidase was produced by *Bacillus* sp. FME

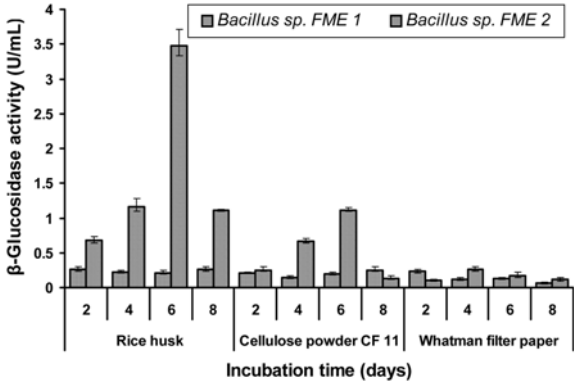

Fig. 3. Effect of different cellulosic substrates on β-glucosidase production. Values in the figure are means of three replicates with standard deviation.

2 at 6th day of incubation from rice husk as against 1.2 U/mL from cellulose powder CF 11 and 0.3 U/mL from Whatman filter paper. Whereas *Bacillus* sp. FME 1 showed higher â-glucosidase activity (0.39 U/mL) at 1st day of incubation from rice husk as against 0.3 U/mL from cellulose powder CF 11 and 0.21 U/mL from Whatman filter paper (Fig. 3). Maximum production of β-glucosidase was found on rice husk than other substrates used in the present study by *Bacillus* sp. FME 2. Thus, rice husk is the most suitable substrate for β-glucosidase production in submerged fermentation.

Lignocellulosic materials containing cellulose, hemicellulose, and lignin are the most abundant organic resources on earth. The utilization of renewable resources for energy and chemicals is expected to increase in the near future. Agricultural wastes and, in fact, all lignocellulosics can be converted into products of commercial interest such as ethanol, glucose, and single cell protein [Solomon *et al*., 1999]. The bioconversion of cellulosic materials has been receiving attention in recent years. It is now a subject of intensive research as a contribution to the development of a large-scale conversion process beneficial to mankind [Kumakura, 1997]. Differences in the enzyme production in the agro-residues by microorganisms depend on many factors-chemical composition of the agro-residues (cellulose, hemicellulose, lignin, nitrogen, and minerals), presence of an activator or an inhibitor in the agro-residues, diffusion of the catabolite, and type of organisms for fermentation [Damaso *et al.,* 2000; Souza and Peralta, 2001; Chinn *et al.*, 2006]. Release of the cellulolytic enzymes will lead to the initiation of attack on the cellulosic components of lignocellulosic substrates [Chandra *et al.,* 2007]. Titres of cellulolytic enzymes at the peak production time in the submerged fermentation were higher on the rice husk than on the other substrates

used in the present study. The yields of cellulolytic enzymes were higher (without optimization of conditions) when compared to those reported by most of the recent studies [Apun *et al.,* 2000; Gomes *et al.,* 2006; Mishra *et al.,* 2007; Peciulyte, 2007]. According to Mishra *et al.* [2007], the yields of CMCase, FPase and β-glucosidase by *Cellulomonas cellulans* MTCC23 in the paddy straws were 0.384, 0.720, and 1.053 IU/mL at day 15 of incubation, respectively. Removal and degradation of the hemicelluloses and the lignin by pre-treatments break up the cell wall structure, thereby increasing the accessibility of the cellulases to the celluloses [Ortega *et al.,* 2000]. Pretreatment process may improve substrate utilization by the microbes and enhance the enzyme yields [Pandey *et al.,* 2000; Pan *et al.,* 2006]. In the present study, only the native lignocellulosic substrates without pretreatment were used. Use of the pretreated lignocelluloses may result in a higher increased yield of the cellulolytic enzymes by microorganisms subjected to the submerged fermentation. Further studies are necessary for confirmation.

Results of the present study showed that cellulolytic enzymes are produced from cellulolytic bacteria on different substrates as the sole carbon source. To the best of our knowledge, this is the first report on the high level production of the cellulolytic enzymes within 8 days of incubation in submerged fermentation without optimization of the fermentation conditions. Further investigations are required to enhance the enzyme activity using carbon and nitrogen sources etc., and for large scale production of cellulolytic enzymes from submerged fermentation.

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