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EXHIBITION OF HIGHER TITRE OF CELLULASE BY STREPTOMYCES AUREOFACIENS OVER STREPTOMYCES ALBIDOFLAVUS ON BANANA WASTE SUBSTRATE

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Abstract

The utilization of residual wastes from banana for the production of higher titre of cellulase enzymes in submerged fermentation was evaluated. *Streptomyces aureofaciens* (MTCC 325) exhibited faster and higher growth in the basal salt medium with banana agro waste compared to *Streptomyces albidoflavus*. The maximum cellulase production (6.641 IU/ml) of *S. aureofaciens* using glucose as a sole carbon source in submerged fermentation was comparatively higher than *S. albidoflavus*. Similarly, while using yeast extract as sole nitrogen source, *S. aureofaciens* has showed maximum cellulase production of 5.74 IU/ml, which was comparatively higher than *S. Albidoflvus (*4.88IU/ml). SDS-PAGE analysis showed that the molecular weight of the partially purified enzyme was 70 kDa and 72 kDa for *Streptomyces aureofaciens* and *Streptomyces albidoflavus* respectively. The optimum culture conditions for cellulase production using the above two novel actinomycetes were also studied. The maximum cellulase activity was observed on the 5th day of incubation at 35-45^{o}C, at the pH range of 6.0 – 7.0 for both the strains.

Keywords: Banana agro waste, cellulase, *S. aureofaciens*, *S.albidoflavus*, submerged fermentation

1. Introduction

Cellulose is one of the most essential sources of carbon on this earth and its annual biosynthesis by both land plants and marine algae occurs at a rate of 0.85×10^{11} tonnes per annum (Nowak *et al.,* 2005). Agricultural, industrial and also municipal cellulosic wastes have been accumulating abundantly in developing countries (Kim et al., 2003). Recently, the utilization of cellulosic biomass receives worldwide interest in the view of its wide ranges including in foods, pharmaceuticals and cosmetics that is, as an anti-cholesterimic, hypolipemic, oil absorber, or moisturing agent (Pradeep and Narasimha, 2011). Cellulases provide a key opportunity for achieving tremendous benefits of biomass utilization and a potential challenging area where it has a central role in the bioconversion of renewable cellulosic biomass to commodity chemicals (Ibrahim and El-Diwany, 2007).

Since the commercial substrates for the production of cellulases are relatively expensive the use of waste agro-biomass as a carbon source for the production of cellulase has been suggested due to its lower cost (Sukumaran *et al.,* 2009). The availability of agricultural waste in India per year is estimated to be 322 million tonne which includes crop residues, processed grains, cotton stalks, maize stalk, banana waste and cobs (Chaturvedi, 2001). Banana is one of the major cash crops of south India generating vast agricultural waste after harvesting including dried leaves, pseudostem and fruit stalk having cellulose content of 23.85% (Baig *et al.,* 2004).

There has been much research aimed at obtaining new microorganisms producing cellulolytic enzymes with higher specific activities and greater efficiency (Johnvesly *et al.,* 2002). But majority of studies on cellulase production have focused on fungi, with relatively lesser emphasis on bacterial sources (Bajaj et al., 2009). Actinomycete is one of the known cellulase-producers, has attracted considerable research interest due to its potential applications in recovery of fermentable sugars from cellulose that can be of benefit for human consumption (Arunachalam *et al.,* 2010). The industrial bioconversion of lignocelluloses requires multifunctional cellulases with broader substrate utilization as well as the application of enzymes that can work efficiently in a wide range of temperatures and pH conditions (Sangkharak *et al.,* 2010). Moreover, the optimization of fermentation conditions is an important problem in the development of economically feasible bioprocesses. Many authors have been reported the influence of various parameters such as pH, Temperature, carbon, nitrogen and substrate concentration sources on cellulase production (El-Sersy *et al.,* 2010).

Since an intensive investigation is required to establish the optimum condition to scale up enzyme production in an individual fermentation process using renewable resources, the present study was aimed to utilize the residual wastes from banana for the production of higher titre of cellulase enzymes and also to optimize the culture conditions for higher cellulase activity using *Streptomyces aureofaciens* and *Streptomyces albidoflavus*.

2. Materials and Methods

2.1 Microorganisms

A total of 10 strains of bacteria including 2 strains of actinomycetes (*Streptomyces aureofaciens* MTCC 325 and *Streptomyces albidoflavus* MTCC 327) were obtained from Institute of Microbial Technology, Chandigarh, India. A preliminary qualitative analysis for cellulolytic activity was conducted by using Congo red dye (Ariffin *et al.,* 2006). The bacteria were inoculated on CMC agar plates, incubated at 37°C for 5 days to allow for the secretion of cellulase. At the end of the incubation, the agar medium was flooded with an aqueous solution of Congo red (1% w/v) for 15 minutes. The Congo red solution was then poured off and the plates were further treated by flooding with 1M NaCl for 15 minutes. The formation of a clear zone of hydrolysis indicated cellulose degradation. Both the prominent strains of actinomycetes *S. aureofaciens* MTCC 325 and *S. albidoflavus* MTCC 327 with highest cellulase activity were sub-cultured, incubated at 30^{o}C for 5 days and subsequently stored at 4^{o}C for inoculums preparation.

2.2 Preparation of Substrate

The substrate banana agro wastes (Pseudo stem, leaves, fruit stalk) were collected from local agricultural fields, sun dried for a period of three weeks and subsequently oven dried slowly at 50°C for 2 days. The dried substrate was chopped into small bits, pulverized into coarse particle sizes and then washed in several changes of hot water and used for enzyme assay. The protein and the initial cellulose content of the residual waste of banana were determined (Ghose, 1987 & Lowry *et al.,* 1951).

2.3 Cellulase Productivity

The cellulase production was carried out by submerged fermentation process in 250ml conical flasks with 100ml of basal salt medium containing 5% [w/v] banana agro waste as a sole carbon source (Ariffin *et al.,*

2006). The flasks were sterilized by using autoclave at 121°C for 15 min. After cooling at room temperature, 2% inoculum was introduced aseptically and fermentation proceeded at agitation rate of 200 rpm with maintained aeration at 1.0 vvm. The fermentation was maintained at 37°C for 7 days. Samples were withdrawn periodically, centrifuged at 5000 rpm for 15 minutes and the supernatant containing extracellular enzyme was taken for partial purification.

2.4 Partial Purification of Cellulase

Ammonium sulphate precipitation has widely been used for enzyme purification (Arunachalam *et al.*, 2010). The supernatant obtained from the fermentation flask was treated with different saturation levels of solid ammonium sulphate (20, 40, 60 and 80%), with continuous overnight stirring. The precipitated enzyme was collected by centrifugation (10000 rpm for 15 min.) and dissolved in 0.1M citrate buffer (pH 5.0). The enzyme solution was dialyzed against the same buffer for 48hrs with several intermitted buffer changes. The partially purified enzyme obtained was lyophilized and used for further study.

2.5 Enzyme Assay

Filter paper activity (FPase) or (total cellulase activity) was determined according to the standard method (Ghose, 1987). 50 mg of whatman No. 1 filter paper strip were immersed in 1 mL of 0.05M Sodium citrate buffer containing 0.1% of partially purified enzyme (pH 5.0). After incubation at 50 ± 2ºC for 1 hr, the reducing sugar released was estimated by dinitrosalicylic acid (DNS) method (Miller, 1959). One unit of filter paper (FPU) activity was defined as the amount of enzyme releasing 1μmole of reducing sugar from filter paper per ml per min. Endoglucanase activity (CMCase) was measured in the same method using a reaction mixture containing 1mL of 1% carboxy methyl cellulose (CMC) in 0.5M citrate acetate buffer (pH 5.0). One unit (IU) of endoglucanase activity was defined as the amount of enzyme releasing 1 μmole of reducing sugar per min. β-glucosidase activity was assayed by the method of Herr (Herr, 1979) using a reaction mixture containing 0.2 mL of 5 mM ρ – nitro phenyl β - D - glucopyranoside (PNPG), 1.6 mL of 0.05 M sodium citrate buffer (pH 4.8) and 0.2 mL of enzyme solution. After incubation for 30 minutes at 50ºC the reaction was stopped by the addition of 4 mL of 0.05 M NaOH glycine buffer (pH 10.6) and the yellow colored para nitro phenyl was measured at 420 nm in spectrometer. One unit of glucosidase activity is defined as that released mole of PNP from PNPG per minute per mL.

2.6 SDS-PAGE analysis

Sodium dodecyl sulfate–polyacylamide gel electrophoresis (SDS–PAGE) was used to verify the protein purity of the enzyme under denaturing conditions, as described by (Laemmli, 1970). To 100 µL of protein sample, 50 µL of sample buffer (0.05% bromophenol blue, 5% *β*-mercap - toethanol, 10% glycerol, and 1% SDS in 0.25 M Tris– HCl buffer; pH 6.8) was added and kept in boiling water bath for 5 min, cooled at room temperature and loaded onto the gel. Electrophoresis was performed at room temperature for 2.5 hrs with a 120 Volt and then gel was placed in fixing solution for 20 minutes followed by washing with three changes of distal water over 30 minutes time period. The protein bands were visualized by staining with Coomassie Brilliant Blue G (Sigma) and destaining was done again with distilled water and kept for overnight in water at room temperature. The molecular weight of cellulase was determined in comparison to standard marker proteins.

2.7 Optimization of Culture Conditions for Enzyme Production

The various process parameters that influence the enzyme production during submerged fermentation were optimised over a wide range (Jaradat *et al.*, 2008). Process parameters thus standardized included pH (3-9) adjusted with 1 N NaOH or 1N HCL, incubation temperature (30-70ºC) and incubation period (3–7 days). Moreover, supplementation of additional nutrients (0.1%) asparagine, ammonium sulphate, Urea, yeast extract as nitrogen sources and glucose, cellulose, lactose, sucrose as carbon source were also optimised for maximal enzyme production.

2.9 Statistical Analysis

Data presented on the average of three replicates (± SE) are obtained from three independent experiments.

3. Results and Discussion

Low cost production of cellulases from different wastes had been studied by many workers. Agricultural residues such as corn stover (Yao *et al.*, 2010), wheat straw, rice straw (Sun *et al.*, 2008), bagasse, and lignocelluloses (Hendriks *et al.*, 2009), have been used for cellulase production. Shabeb et al. (Shabeb *et al.*, 2010) studied the low cost production and the economic value of cellulase from molasses by *B.subtilis* KO. The biochemical analysis of banana agro waste showed that it contained principally 24.7% cellulose, 0.03%

reducing sugar and 0.01% protein. Similar results had earlier reported that banana agro waste contained 23.85% cellulose and 0.01% of reducing sugar (Crawford and Chamberlain, 2000 & Odeniyi *et al.*, 2009).

3.1 Cellulase productivity

Cellulase, is one of the most important hydrolytic enzymes, produced by most of the actinomycetes isolates (94%) that are identified from soils of Southern- West Ghats (Sangkharak *et al.*, 2010). Several studies have been carried out to produce cellulolytic enzymes in biowaste degradation process by several bacteria (Shankar and Isaiarasu, 2011). The specific cellulolytic activity shown by the bacterial species was reported to depend on the source of occurrence. Table 1 showed a faster utilization of cellulose by both *S.aureofaciens* and *S.albidoflavus* up to 4th day of fermentation beyond that the degradation of cellulose was moderate. On the 5th day of submerged fermentation, 71% of cellulose was utilized by *S.aureofaciens* while *S.albidoflavus* utilized only 67% which clearly indicated that *S. aureofaciens* have higher and faster utilization potential than *S. albidoflavus*. Potential of these organisms can be correlated with the activities of cellulose saccharifying enzymes such as CMCase, filter paper activity and β-glucosidase activity (Ojumu *et al*., 2003). *S.aureofaciens* has showed maximum cellulase activity (CMCase – 2.11; glucosidase – 1.86; FPase – 1.52 IU/ml) on the 5th day of fermentation which was comparatively higher than *S.albidoflavus* (CMCase – 1.92; glucosidase – 1.34; FPase – 1.78 IU/ml) (Table 2). Similar results on earlier studies showed that *Streptomyces* were the best choice organism for the utilization of cellulosic substrate (Alam *et al.*, 2004 & Pandey *et al.*, 2000).

3.2 Enzyme purity

Cellulases obtained from many bacterial strains by fermentation, even without purification was used for commercial application (Bindu *et al.*, 2007); however, purification would enhance the extent of its efficacy as a biobleaching agent and hence purity is warranted. In earlier studies, ammonium sulphate at saturation level of 40 – 60% has been used for cellulases purification from different microbial sources (Hendriks and Zeeman, 2009; Lynd *et al*., 2002). In the present study, a maximum of 86% and 89% of crude cellulase were recovered on partial purification using ammonium sulphate saturation for *S. albidoflavus* and *S. aureofaciens* respectively. A similar earlier study reported a recovery rate of 78.9% xylanase from a fungus *Paecilomyces themophila* upon partial purification using 20-50% ammonium sulphate saturation (Li *et al.*, 2006). The partially purified

cellulase was further resolved on a SDS-PAGE found to be a homogenous monomeric protein as evident by a single band corresponding to 70 kDa and 72 kDa for *S. aureofaciens* and *S. albidoflavus* respectively (Figure 1). The results regarding the molecular weight of the enzyme are close to the findings of Qin *et al*., 2008 and Li *et al.,* 2006, who isolated a CMCase with 54 kDa and 61 – 78 kDa from *Bacillus* sp. Moreover, the zymogram revealed the presence of a zone of hydrolysis that corresponded with Commassie stained band of cellulase on native PAGE, confirming the partially purified protein as cellulase Niture *et al*., 2001 and Mohamed *et al.*, 2006 have reported low and medium ranged molecular weight microbial cellulases which are comparable with the present results.

3.3 Optimization of Culture Conditions

3.3.1 pH

The enzyme activity gradually increased when increasing the pH up to the optimum followed by a gradual fall in activity. Results of enzyme assay showed that the cellulase enzymes was completely active in a large pH range (5-8) and presented an optimum cellulase activity of 2.27 U/mL and 1.91 U/mL at a pH of 7 by *S. aurofaciens* and *S. albidoflavus* respectively (Fig. 1 & 2) which was little higher than *Streptomyces lividans* and higher than those from *Mucor circinelloides*, 4.0 - 6.6 (Niture *et al*., 2001) and *Bacillus circulans*, 4.5 - 6.8 (Sukumaran *et al.,* 2009). Whereas, any further increase in pH from optimum value, cellulase showed decreasing trends in its activity. Effect of pH on cellulase production by these fungi supports the findings of (Ojumu *et al*., 2003) who reported that CMCase, Avicelase, and FPase activities exhibit a pH optimum of approximately 4, while the pH optimumof *β*-glucosidase was between pH 5-6 showed that the optimum pH for endoglucanase from a strain of was 5.5.

3.3.2 Temperature

The selected strains were found to show heavy growth and liquefaction and maximum enzyme activity after 4 days of incubation period. Temperature and pH-values were found to be important parameters that influenced enzyme activities and production (Odeniyi *et al*., 2009). The maximum cellulase productivity was observed at 40°C in the fermentation medium inoculated with both *S. aurofaciens* and *S. albidoflavus* with the optimum range of 35 - 45°C and the enzyme activity was decreased when the temperature increased above 45ºC (Fig. 3 & 4). These results are similar to that reported by Alam *et al.,* 2004 who studied the heavy growth and

high cellulose activity by *Streptomyces omiyaensis* at 35 and 40°C. Furthermore, Arunachalum *et al.,* 2010 have reported an optimal temperature for cellulase activity in the range of 40 - 55°C for several *Streptomyces* species including *Streptomyces lividans*, *Streptomyces flavogrisus*, and *Streptomyces nitrosporus*. Jaradat *et al.,* 2008 found that the maximum CMCase activity of *Streptomyces sp.* (isolate J2) was recorded at 60°C with no significant difference ($p < 0.05$) between 50 and 60°C. Many workers have reported different temperatures for maximum cellulase production either in flask or in fermentor studies using *Aspergillus* sp. and *Trichoderma* sp. suggesting that the optimal temperature for cellulase production also depends on the strain variation of the microorganism (Ojumu *et al.,* 2003; Pandey *et al*., 2000).

3.3.3 Nutrient supplements

The results showed that among other sources of carbon, a concentration of 1.0% glucose had a maximum production of cellulase enzyme. The highest cellulase production by *S. aurofaciens* of exoglucanase (2.61 U/mL), endoglucanase (1.62U/mL), and *β*-glucosidase (2.41U/mL) was observed with the addition of 1.0% glucose (Table 3). These results have also shown that banana waste itself can act as a source of carbon, nitrogen and minerals as well as growth factors. Cellulase biosynthesis seems to require residual inducer in the culture medium for a period of approximately 30-50 hr when cellulose, lactose or glucose is the growth substrate. However addition of these sugars at concentrations above 1% level led to a significant reduction in enzyme synthesis. It was inferred that supplementing with glucose at reduced concentration was sufficient for enhancing enzyme production (Jaradat *et al*., 2008).

The effect of different nitrogen sources (1.0%) on the production of cellulase enzyme by *S. aurofaciens* and *S. albidoflavus* was investigated. The overall rate of cellulase production was increased with the addition of various nitrogen sources.The results showed that among other sources of nitrogen, a concentration of 1.0% yeast extract led to a maximum production of cellulase enzyme. The highest cellulase production by *S. aurofaciens* of exoglucanase (2.38 U/mL), endoglucanase (1.42U/mL), and *β*-glucosidase (2.24U/mL) was observed with the addition of 1.0% yeast extract (Table 4). Additional supply of nitrogen sources influenced with the CMCase activity to a certain extent, where as the influence on FPase and β-glucosidase activity was comparatively lower.

4. Conclusion

The potential application of agricultural waste residues would not only provide cheap, over all alternative substrates for the production of bulk chemicals and value-added products, avoiding the moral conflict and economic competition from using food crops, but it also would help in solving pollution problems. The present work, therefore, shows that, at least at a laboratory scale, simple and low cost saccharification of agricultural residues can be performed under mild conditions and the quantity of saccharifying enzymes obtained are useful for bioconversion to value added products. However, it remains to be established if this is suitable for industrial scale processes in future.

Plate 1. SDS – PAGE molecular weight determination of the partially purified enzyme [A] Standard Molecular markers [B] *S. aurofaciens* [C] *S. albidoflavus*

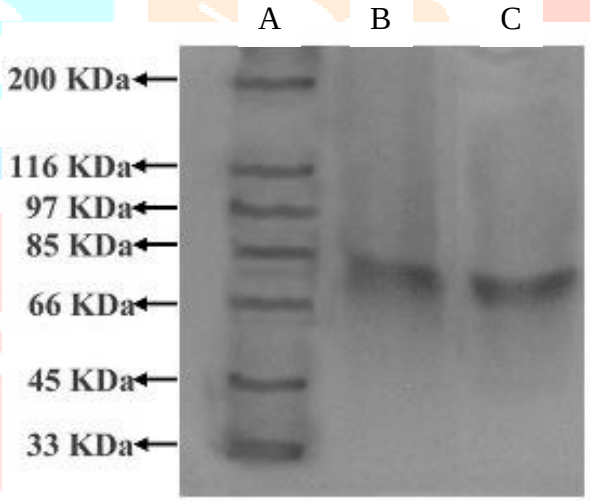


Table 1: Cellulose content [mg/ml] of the substrate on microbial titres in a submerged fermentation system

Results are mean ± SE of three replicates.

S.No.	Name of the organisms	Period of fermentation [Days]							
		0	1	2	3	4	5	6	7
1.	*Streptomyces aureofaciens*	247.5±10.9	213.3±11.2	172.6±10.9	138.1±10.8	105.4±3.7	71.2±4.2	71.0±4.4	70.2±3.3
2.	*Streptomyces albidoflavus*	247.6±10.9	223.5±11.1	185.3±10.9	144.2±9.7	116±6.7	82.3±4.3	80.4±4.3	80.0±3.1

Table 2 : Cellulase enzyme activities [IU/ml] of the substrate on microbial titres in a submerged fermentation system

Results are mean ± SE of three replicates.

S. No.	Name of the organisms	Enzyme activities [IU/ml]	Period of fermentation [Days]						
			1	2	3	4	5	6	7
1.	S. aureofaciens	*CMCase*	0.51±0.001	1.13±0.002	1.48±0.002	1.72±0.02	2.11±0.08	1.91±0.01	1.27±0.09
		FPase	0.23±0.001	0.47±0.001	0.77±0.002	1.31±0.004	1.52±0.02	1.48±0.02	1.44±0.04
		β-glucosidase	0.32±0.002	0.72±0.005	1.29±0.005	1.72±0.005	1.86±0.02	1.81±0.04	1.72±0.03
2.	*S. albidoflavus*	*CMCase*	0.4±0.001	0.92±0.001	1.31±0.004	1.62±0.002	1.92±0.05	1.81±0.06	1.72±0.06
		FPase	0.17±0.005	0.38±0.005	0.62±0.008	1.12±0.005	1.34±0.02	1.36±0.02	1.28±0.02
		β-glucosidase	0.28±0.001	0.58±0.001	1.18±0.002	1.52±0.005	1.78±0.03	1.74±0.05	1.68±0.04

Table: 3 Effect of carbon sources on the production of extra cellular protein, reducing sugar level, % saccharification and cellulase activity of the *S. aureofaciens* and *S. albidoflavus* on the 5th day of fermentation.

Name of the organisms	Sources of Carbon	Cellulase Activity [IU/ml]			Extracellular protein [mg/ml]	Reducing Sugar [mg/ml]	% Saccharification
		CMCase	FPase	β-glucosidase			
Streptomyces aureofaciens	Glucose	2.61 ±0.04	1.62 ±0.01	2.41 ±0.06	0.41	4.26	8.52
	Lactose	2.44 ±0.04	1.57 ±0.02	2.28 ±0.05	0.36	4.18	8.36
	Sucrose	2.31 ±0.02	1.39 ±0.04	2.09 ±0.04	0.32	4.14	8.28
	Cellulose	2.26 ±0.03	1.46 ±0.02	2.35 ±0.06	0.39	4.21	8.42
S. *albidoflavus*	Glucose	2.44 ±0.03	1.54 ±0.02	2.31 ±0.04	0.37	4.08	8.16
	Lactose	2.34 ±0.02	1.47 ±0.04	2.17 ±0.03	0.31	3.89	7.78
	Sucrose	2.31 ±0.02	1.31 ±0.03	2.11 ±0.04	0.36	3.81	7.62
	Cellulose	2.27 ±0.01	1.50 ±0.02	2.28 ±0.03	0.36	3.92	7.84

Table: 4 Effect of Nitrogen sources on the production of extra cellular protein, reducing sugar level, % saccharification and cellulase activity of the *S. aureofaciens* and *S. albidoflavus* on the 5th day of fermentation.

Name of the organisms	Sources of Nitrogen	Cellulase Activity [IU/ml]			Extracellular protein [mg/ml]	Reducing Sugar [mg/ml]	% Saccharification
		CMCase	FPase	β-glucosidase			
Streptomyces aureofaciens	Asparagine	2.21 ±0.02	1.38 ±0.03	2.15 ±0.01	0.34	4.04	8.08
	Yeast extract	2.38 ±0.02	1.42 ±0.04	2.24 ±0.02	0.41	4.18	8.36
	$[NH_4]_2 SO_4$	2.17 ±0.02	1.33 ±0.03	2.23 ±0.02	0.36	3.91	7.82
	Urea	2.13 ±0.02	1.27 ±0.01	2.19 ±0.02	0.31	3.83	7.66
Streptomyces albidoflavus	Asparagine	1.83 ±0.01	1.31 ±0.01	1.74 ±0.02	0.31	3.81	7.62
	Yeast extract	1.98 ±0.01	1.39 ±0.01	1.81 ±0.02	0.36	3.91	7.82
	$[NH_4]_2 SO_4$	1.87 ±0.02	1.28 ±0.02	1.71 ±0.03	0.30	3.79	7.58
	Urea	1.81 ±0.02	1.21 ±0.02	1.62 ±0.01	0.28	3.63	7.26

Fig. 1 : Effect of pH on the CMCase, FPase and Beta glucosidase activity of the *Streptomyces aureofaciens* on the 5th day of fermentation

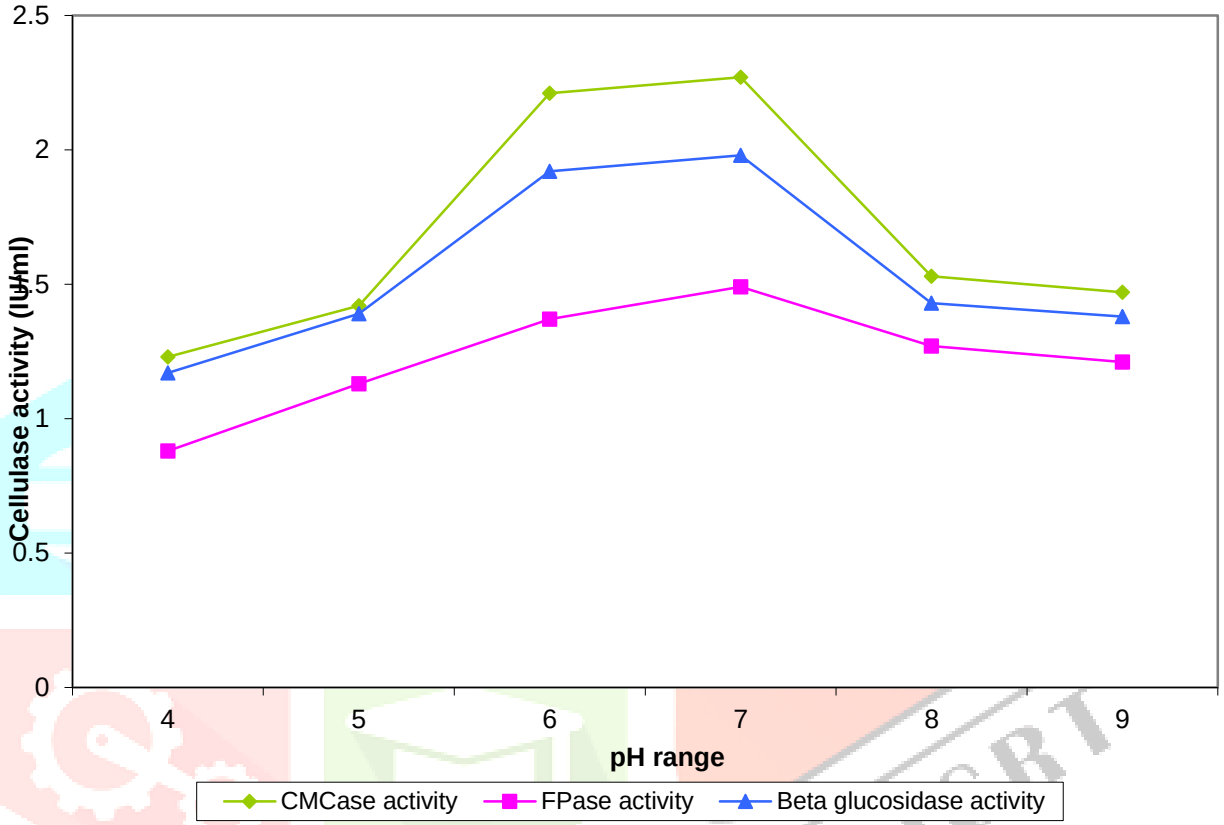


Fig. 2 : Effect of pH on the CMCase, FPase and b glucosidase activity of the *Streptomyces albidoflavus* on the 5th day of fermentation

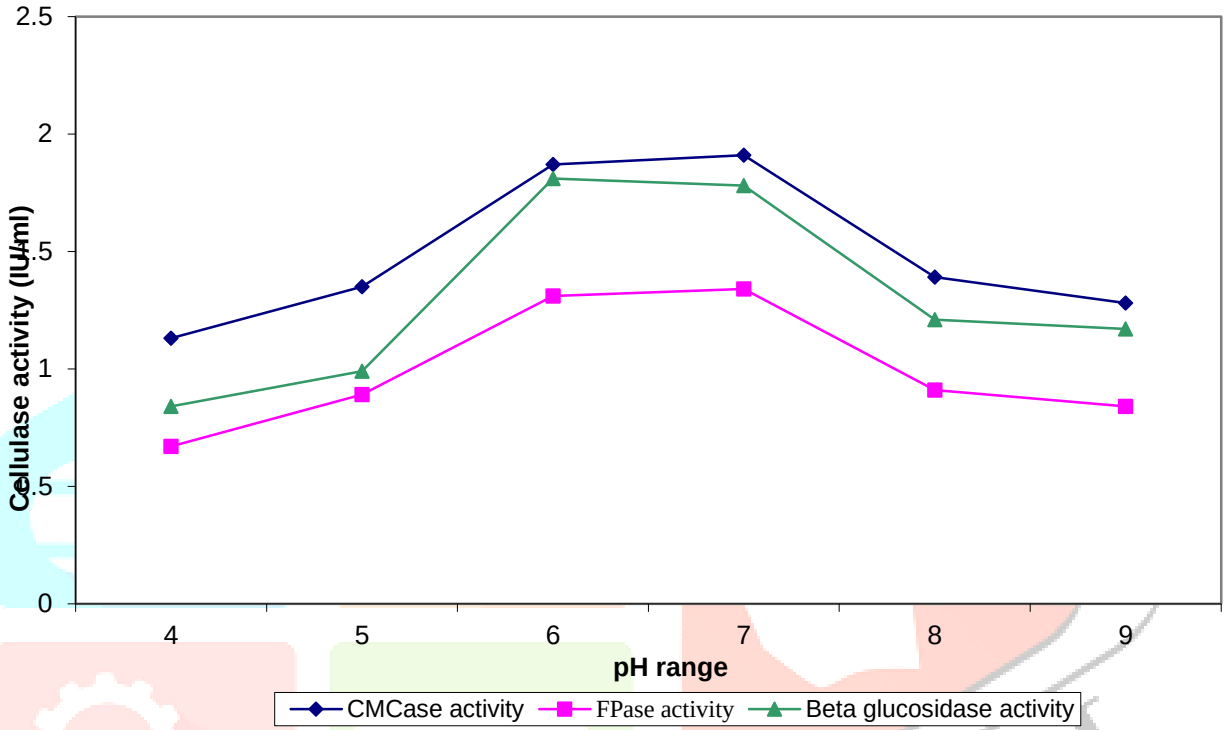


Fig. 3 : Effect of temperature on the CMCase, FPase and β glucosidase activity of the *Streptomyces aureofaciens* during enzyme substrate reaction on the 5th day of fermentation

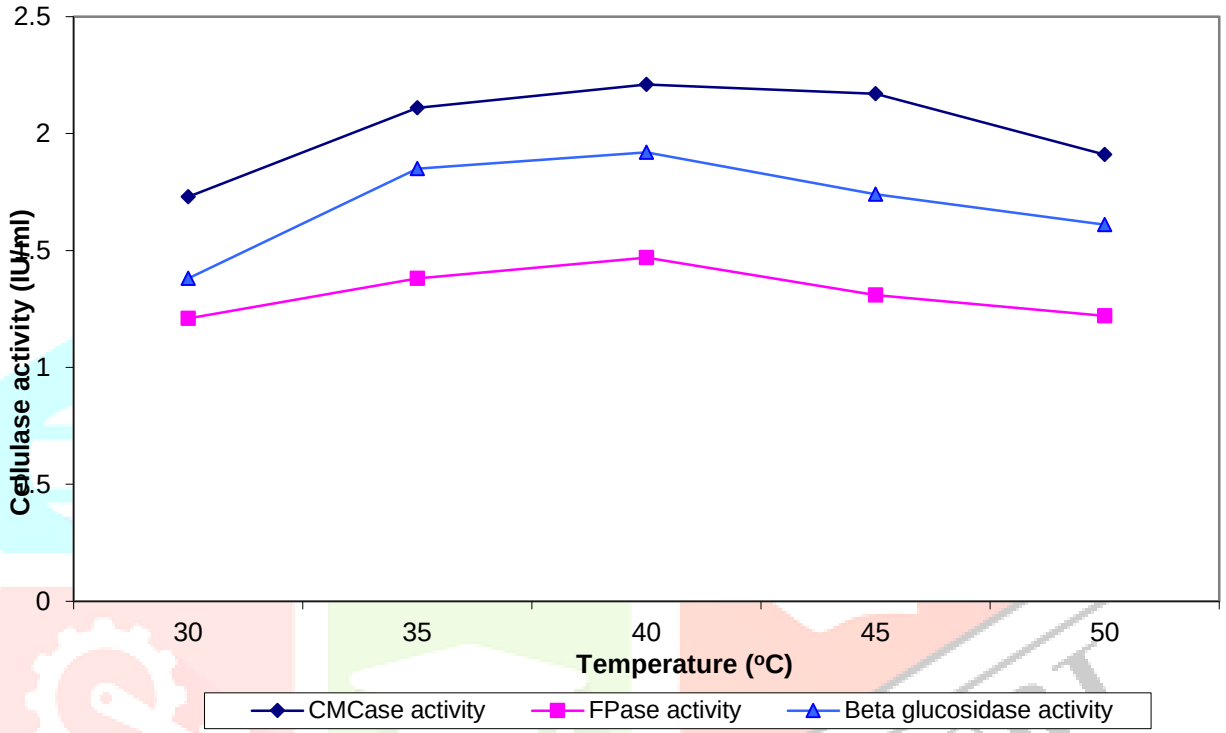
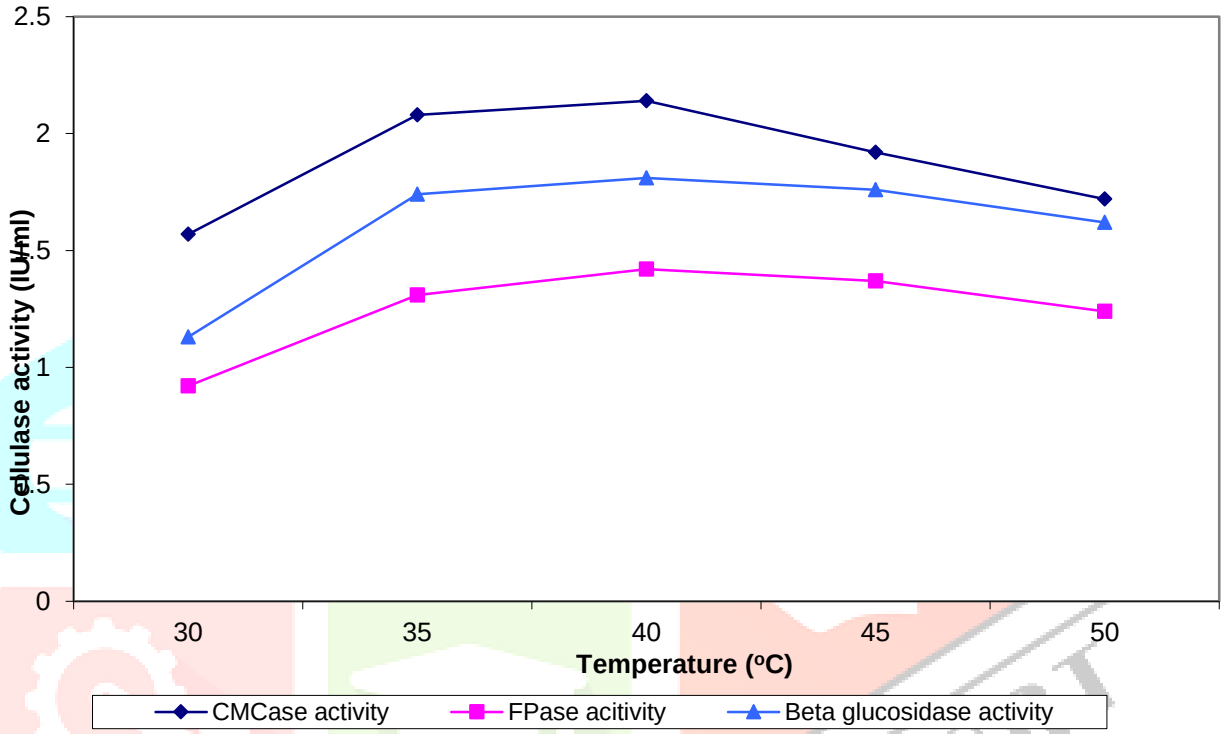


Fig. 4 : Effect of temperature on the CMCase, FPase and β glucosidase activity of the *Streptomyces albidoflavus* during enzyme substrate reaction on the 5th day of fermentation



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