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RESEARCH ARTICLE

Enhancement of FPase and β-Glucosidase Production by *Trichoderma harzianum* CKP01 in Carbon and Nitrogen Sources

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ABSTRACT

Wastes are produced virtually by all types of industries. The drift in the world today is to convert waste into useful products through application of microorganisms. The current study aimed at the production of FPase and β-glucosidase enzyme by the filamentous fungi, *Trichoderma harzianum* CKP 01 in different carbon and nitrogen sources. A number of indigenously isolated fungi were screened for cellulolytic potential and *Trichoderma harzianum* CKP 01 was selected since, it showed maximum hydrolytic zone. Identifiation of the fungal isolate was done based on biochemical and molecular characterization by sequencing the 18S rRNA coding gene. Of the two enzymes FPase and β- glucosidase showed maximum activity and was in 2% dextrose (9.18 and 7.13 I Umg^{-1}) at intracellular level and 2% sucrose (9.76 and 6.45 Umg^{-1}) at extracellular level. Among different nitrogen sources, the highest activity was recorded in FPase (9.84 Umg^{-1}) followed by *β*-glucosidase (7.72Umg^{-1}) in 3.0% potassium nitrate when compared to control (0.71 I Umg^{-1}) at an intracellular level. Potassium nitrate and peptone were the best nitrogen sources for the production of cellulase enzyme with FPase activity of 9.49Umg^{-1} and *β*-glucosidase of 6.62Umg^{-1} at an extracellular level by *Trichoderma harzianum* CKP 01 on 9th day of incubation.

INTRODUCTION

Advances in industrial biotechnology offer potential economic utilization of agro-industrial residues, particularly those originating from tropical regions. In recent years, there has been an increasing trend towards more efficient utilization of agro-industrial residues such as sugarcane bagasse, sugar beet pulp, coffee pulp/husk, apple pomace etc. Several processes have been developed that utilize these raw materials for the production of bulk chemicals and value-added fine products such as ethanol, Single cell protein, mushrooms, enzymes, organic acids, amino acids and biologically active secondary metabolites etc. (Pothiraj and Eyini, 2007). Sugarcane is one of the important cash crops in India and plays pivotal role in both agricultural and industrial economy of the country. India is one of the largest producers of sugar and is in close competition with Brazil for the top position. In India, sugarcane is cultivated over an area of 4 million hectares and the production is estimated to be about 325 million tonnes with productivity of 70 tonnes per hectares. In Tamil Nadu, (India), sugarcane is cultivated in an area of 3.22 lakh hectares with an average productivity of 101.8 tonnes (Mohan and Ponnusamy, 2011). Enormous amount of agricultural lignocellulosic wastes such as bagasse is constantly piled up therefore, these materials are particularly attractive in the bioconversion process because of their low cost and plentiful supply (Verma *et al.,* 2011).

Cellulases are industrially important hydrolytic enzymes of great significance in present day biotechnology. These enzymes are produced by numerous microorganisms such as *Aspergillus, Cladosporium, Fusarium, Geotrichum, Myrothecium, Paecilomyces, Penicillium and Trichoderma species* (Azzaz, 2009).

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Many research being achieved in cellulases production and their characterization during recent years (Murad and Azzaz, 2010., Ong *et al.*, 2010; Roslan *et al*., 2011). Since the production of cellulase enzyme is a major process and economically viable, major attention has been given to use lignocellulosics as substrate for cellulase production. The level of cellulase activity and its application depend on the microbial producing strain, the media composition and process control (Ghose, 1987; Kheng *et al.*, 2006). In this work, the hydrolytic potential of total cellulase (FPase) and β- glucosidase enzyme production by *Trichoderma harzianum* CKP 01 showing maximum cellulolytic activity isolated from sugar cane bagasse disposal area soil samples were compared.

MATERIALS AND METHODS

Potato Dextrose Agar Medium for fungal isolation

From peeled Potato (250 g), PDA medium was prepared by adding dextrose (20 g) and agar (15 g). The volume was made upto 1000ml.

Isolation of Native Mycoflora

Native mycoflora from sugar cane bagasse disposal area soil was screened by cellulose enriched PDA medium. The fungal isolates were identified based on their morphology, mycelial structure and spore formation.

Primary Screening of Cellulolytic Fungi

The identified fungal strains like *Rhizopus stolonifer*, *Aspergillus niger*, *A. flavus, A. fumigatus, Geotrichum sp., Verticillium sp., Penicillium sp., Cladosporium sp., Trichoderma harzianum, Alternaria* sp. and *Dreschslera* sp. were grown individually on one per cent carboxymethyl cellulose agar plates and incubated at 28°C.

After 5 days of growth, 0.1 per cent Congo red solution was added and counter stained with 1M NaCl for 15-30 min. A clear zone of cellulose hydrolysis gave an indication of cellulase producing organism. The diameter of the clear zone was measured to provide a quantitative comparison of cellulolytic activity. The fungal colony showing largest zone of decolorization was selected for enzyme study.

Identification of fungi by DNA sequencing method (Liu *et al*., 2000)

Fungi werecultured in PDA or SDA plates. DNA was extracted from the fresh mycelia taken from the surface of the plate. The DNA extracts were amplified using ITS1, 5.8S, ITS2 and D1/D2/D3 region by using the specific primer.The PCR amplification product was subsequently analysed by Agarose gel electrophoresis.After analysis, the PCR product was eluted and sequenced by using Sanger's sequencing method with forward and reverse primers. The forward and reverse sequences were aligned into a single sequence for further analysis. Finally, the obtained DNA sequence was compared with reference database of verified fungal sequences by Basic Local Alignment Search Tool (BLAST) analysis for calculating sequence similarity.

Preparation of Culture Medium for enzyme study

Fifty ml of Mandels and Reese liquid medium was dispensed in 250 ml Erlenmeyer flasks and sterilized at 1 atm for 15 minutes. After cooling, one ml of chloramphenicol (10,000 ppm) was added. The pH of the medium was maintained at 4.8, after sterilization. The medium was inoculated with 5 g (disc) of the fungus, *Trichoderma harzianum* CKP 01 and the enzyme activity was analyzed on the 7th, 9th, 11th days of incubation because fermentation period was an important parameter for enzyme production.

Preparation of Culture Filtrate as Enzyme Source (Extracellular)

The mycelium was filtered through Whatman No. 40 filter paper using a Buchner funnel under suction and the clear filtrate was used as a source of an extracellular enzyme.

Preparation of Cell Free Enzyme Source from Fungal Mycelium (Intracellular)

The fungal mycelium was washed with distilled water twice. A quantity of 0.5 g of the washed mycelial mat was macerated in five ml of sodium citrate buffer, pH 4.8 in a pre-chilled mortar and pestle with a pinch of acid washed sand. The homogenate was centrifuged in a refrigerated centrifuge at 10,000 g for 15 minutes. The supernatant served as an intracellular enzyme source (Ghose, 1987).

Optimization of culture conditions for enzyme production

Effect of Carbon and Nitrogen Sources

To assess the enhancement of enzyme production by *Trichoderma harzianum* CKP 01*,* carbon sources like dextrose, sucrose and starch and nitrogen sources like peptone, potassium nitrate and ammonium nitrate at different concentrations like 1 per cent, 2 per cent and 3 per cent were supplemented in the fermentation medium.

Statistical Analysis

Standard errors of means of all the replicates of each variable were computed using computer software, The results were statistically analyzed using 3 way analysis of variance (ANOVA) followed by LSD method to delineate mean differences (Panse, and Sukhatme., 1978.)

RESULTS AND DISCUSSION

Screening of fungi for cellulolytic activity (Hydrolyzing Zone)

Among the number of mycoflora like *Rhizopus stolonifer, Aspergillus niger, A. flavus, A. fumigatus, Geotrichum* sp., *Verticillium* sp., *Penicillium* sp., *Cladosporium* sp., *Trichoderma viride*, *Alternaria* sp. and *Drechslera* sp. isolated from the sugar cane bagasse waste disposal area soil samples, only 4 fungal strains (*Aspergillus flavus, Penicillium chrysogenum, Fusarium oxysporum* and *Trichoderma*) showed maximum hydrolyzing zone.

Table 1. The diameter of colony and hydrolytic zone

S.No.	Fungal strains	Colony diameter (mm)	Hydrolytic zone (mm)
1.	*Fusarium oxysporum* CKP 02	60	30
2.	*Trichoderma harzianum* CKP 01	65	40
3.	*Aspergillus flavus* CKP 03	35	16
4.	*Penicillium chrysogenum* CKP 04	40	20

A significantly highest hydrolyzing zone (clearance zone) of 40 mm (out of colony diameter of 65 mm) was shown by *Trichoderma harzianum* CKP 01 followed by *Fusarium oxysporum* CKP 02,30 mm (out of colony diameter of 60 mm). Since, *T. harzianum* CKP 01 showed remarkably prominent clearance zone, it was selected as the test fungus for further studies related to cultural and physiological parameters for optimization of enzyme activity. Similar result of exhibiting highest zone of clearance around the colony was reported by Gautam, *et al.* (2011). Similar observations were expressed by Charithadevi and Sunilkumar (2012). They have obtained highest hydrolyzing zone of 13 mm out of colony diameter of 17 mm by *Aspergillus niger* isolate JSMW-1 and 12 mm out of colony diameter of 19 mm in *A. niger* isolate PIW-1. The present result coincides with findings of Chandel *et al.* (2013). They also reported that *Trichoderma, Aspergillus, Rhizopus* and *Penicillium* species showed highest clearance zone. Chandel *et al.,* (2013) observed that after screening cellulase producing fungi a total of 45 fungal isolates were isolated from compost samples, out of which 36 fungal isolates were purified and 23 isolates showed the cellulase activity based on the clearance zone. The isolates were identified as *Trichoderma, Aspergillus, Rhizopus* and *Penicillium* species.

Identification of fungi by DNA sequencing method

The identification is achieved by comparing the sequence of a genomic region found in the target sample with a comprehensive reference database and it was identified as *Trichoderma harzianum* CKP01.The ITS1 and ITS2, 5.8S rDNA region of given sample sequence showed 100% sequence coverage and 100% homology with *Hypocrea lixii* voucher HR1889.1 (ACCESSION: JN704349), *Trichoderma harzianum* strain CON4029 (ACCESSION: JX969619) and *Trichoderma harzianum* ATCC58673 (ACCESSION: AF057583) etc. in similarity search with BLAST programme. Also, the LSU (D1/D2/D3 region) of the sequence showed 92% sequence coverage and 100% homology with *Hypocrea lixii* strain CBS 226.95 (ACCESSION No: HM466680) and 82-85% sequence coverage and 100% homology with *Trichoderma* cf. *harzianum* strains (ACCESSION No(s): JN939821, JN939819 etc.) Therefore, this isolate may be *Trichoderma harzianum* (*Hypocrea lixii*).

Optimization of culture conditions for enzyme production

Effect of carbon sources on enzyme production

The effect of carbon sources such as sucrose, dextrose and starch ranging from 1.0 to 3.0% on the production of cellulase enzyme by *Trichoderma harzianum* CKP 01 was investigated.T he extra and intra cellular cellulase enzyme activity showed an increasing trend in carbon sources up to 9 days of incubation and after that it declined

Table 2. β-glucosidase Activity (IUmg-^{1} protein) of *Trichoderma harzianum* CKP01 on carbon sources

	Carbon Source	CONTROL			1% con.			2% con.			3 % con.		
		7 day	9 day	11 day	7day	9 day	11 day	7day	9day	11day	7 day	9 day	11 day
Intracellular	Sucrose	0.23	0.57	0.22	2.90	4.89	1.21	3.86	4.67	2.26	1.76	2.86	2.80
	Dextrose				1.73	3.69	2.70	2.23	7.13	1.14	2.22	4.29	1.82
	Starch				3.16	4.28	2.54	2.83	4.50	1.26	1.72	3.79	1.64
Extracellular	Sucrose	0.25	0.81	0.45	2.80	4.87	1.53	3.30	5.52	1.41	3.27	6.45	2.30
	Dextrose				1.63	2.26	1.57	1.83	4.60	1.67	4.59	5.35	4.80
	Starch				4.80	6.09	4.51	2.63	4.90	1.29	3.81	5.67	1.80
			Intracellular		Extracellular								
	C.D ($P < 0.05$)		0.10		0.11								
	S.E.D		0.05		0.06								

(IUmg-^{1} protein) = μ mol p-nitrophenyl-β- D-glucopyranoside (p-NPG) released min^{-1} mg^{-1} protein

Table 3. β-glucosidase Activity (IUmg-^{1} protein) of *Trichoderma harzianum* CKP01 on nitrogen sources

	Nitrogen Source	CONTROL			1% con.			2% con.			3 % con.		
		7 day	9day	11 day	7day	9 day	11 day	7day	9day	11day	7 day	9 day	11 day
Intracellular	Ammonium nitrate	0.23	0.57	0.22	2.81	7.72	1.65	3.65	4.84	1.36	1.34	5.82	1.94
	Potassium nitrate				3.79	4.40	2.63	3.86	4.78	3.88	3.79	4.80	2.88
	Peptone				3.46	5.55	4.31	4.86	5.50	4.64	3.91	5.62	4.87
Extracellular	Ammonium nitrate	0.25	0.81	0.45	2.45	6.31	3.20	1.40	5.86	3.28	1.16	4.84	3.43
	Potassium nitrate				3.28	6.33	1.65	1.06	4.70	1.33	1.43	4.89	1.79
	Peptone				3.80	4.37	1.16	4.86	6.62	1.82	1.44	6.48	1.84
			Intracellular			Extracellular							
	C.D ($P < 0.05$)		0.12			0.11							
	S.E.D		0.06			0.05							

(IUmg-^{1} protein) = μ mol p-nitrophenyl-β- D-glucopyranoside (p-NPG) released min^{-1} mg^{-1} protein

Table 4. FPase activity (IUmg-^{1} protein) of *Trichoderma* harzianum CKP01 on carbon sources

	Carbon Sources	CONTROL			1% con.			2% con.			3 % con.		
		7 day	9 day	11 day	7day	9 day	11 day	7day	9day	11day	7 day	9 day	11 day
Intracellular	Sucrose	0.29	0.76	0.32	4.09	8.12	1.28	2.14	9.03	1.35	1.16	1.65	1.42
	Dextrose				4.15	9.18	6.42	1.66	4.39	1.79	2.39	6.65	2.16
	Starch				3.10	7.25	1.26	2.62	9.64	6.11	1.30	4.44	2.75
Extracellular	Sucrose	0.33	0.84	0.28	1.16	8.61	0.41	1.87	9.76	5.11	1.88	8.13	2.36
	Dextrose				1.60	5.27	2.16	3.20	2.10	4.16	2.88	5.11	2.37
	Starch				3.61	7.22	4.24	4.45	6.82	4.16	4.27	4.51	4.24
	Intracellular		Extracellular										
	C.D ($P < 0.05$)		0.26		0.13								
	S.E.D		0.13		0.07								

(IUmg-^{1} protein) = μ mol glucose released min^{-1} mg^{-1} protein

Table 5. FPase activity (IUmg-^{1} protein) of *Trichoderma harzianum* CKP01 on nitrogen sources

	Nitrogen Source	CONTROL			1% con.			2% con.			3 % con.		
		7 day	9 day	11day	7day	9 day	11 day	7day	9day	11day	7 day	9 day	11 day
Intracellular	Ammonium nitrate	0.29	0.76	0.32	4.39	6.49	3.37	6.88	8.82	4.30	4.30	7.87	2.55
	Potassium nitrate				4.53	8.42	1.41	4.80	8.56	1.68	4.39	9.84	1.14
	Peptone				1.12	7.35	2.39	4.22	6.36	4.18	4.21	7.70	4.30
Extracellular	Ammonium nitrate	0.33	0.84	0.28	1.53	6.84	5.30	5.22	8.85	2.61	3.71	8.71	1.65
	Potassium nitrate				3.19	6.63	4.09	4.33	9.49	6.09	1.52	8.82	4.26
	Peptone				4.44	7.66	2.54	3.28	5.37	2.51	3.40	4.09	1.53
			Intracellular			Extracellular							
	C.D ($P < 0.05$)		0.16			0.12							
	S.E.D		0.08			0.06							

(IUmg-^{1} protein) = μ mol glucose released min^{-1} mg^{-1} protein

gradually (Table -2, 3). Among the carbon sources(sucrose, dextrose and starch) examined, 2% starch was found to be the best inducer for cellulase enzyme production with maximum FPase activity of 9.64 I Umg^{-1} followed by β-glucosidase activity of 7.13 I Umg^{-1} (2% dextrose) when compared to control (0.22 I Umg^{-1}) at intracellular level. At an extracellular level, 2% and 3% sucrose were found to be the best carbon source for enzyme by *Trichoderma harzianum* CKP 01 showing maximum production of FP ase activity of 9.76 I Umg^{-1} and 6.45 Umg^{-1} β-glucosidase activity of 6.45 Umg^{-1} protein than the control. This study substantiates the work of Kathiresan and Manivannan (2006) and Devanathan *et al.,* 2007) who demonstrated lactose as best inducer of *Aspergillus* sp*.,* Nochure

*et al., (*1993) identified fructose as the best inducer of cellulase in *Clostridium thermocellum*. In another study dextrin was found to enhance the production of cellulase by *Trichoderma* sp. in SSF (Pang *et al.,* 2006).

Effect of nitrogen sources on enzyme production

The extra and intra cellular cellulase enzyme activity showed an increasing trend in nitrogen sources up to 9 days of incubation and after that it declined gradually (Table-4, 5). The nitrogen sources tested ranged from 1 to 3.0% were peptone, potassium nitrate, and ammonium nitrate. Among different nitrogen sources, the highest activity was recorded in FPase (9.84 Umg^{-1}) on 3.0% potassium nitrate as nitrogen sources followed by *β*-glucosidase (7.72Umg^{-1}) in 1% ammonium nitrate when compared to control (0.22 I Umg^{-1}) at intracellular level. Results of the present study indicated that the sources of nitrogen greatly influenced the production of cellulase enzyme. Potassium nitrate and peptone were the best nitrogen sources for the production of cellulase enzyme with (FPase activity of 9.49 Umg^{-1} and *β*-glucosidase of 6.62Umg^{-1} at extracellular level by *Trichoderma harzianum* CKP 01 on 9th day of incubation. Yousoff *et al.* (2000) during their study on sugar cane bagasse degradation by mixed culture of *Trichoderma viride* and *Aspergillus terreus* obtained highest β-glucosidase activity of 0.07 U ml^{-1} and FPase activity of 1.4 Uml^{-1}. Baig (2005) found that the nitrogen source peptone enhanced FPase production of 0.20 Uml^{-1} and CMCase activity of 0.041 Uml^{-1} by *Trichoderma lignorum.* Ali and El-Dieu (2008) reported highest β-glucosidase activity in sodium nitrate among the other two nitrogen sources (Ammonium nitrate and peptone). Similar finding was expressed by Gautam *et al.* (2010). They have obtained maximum FPase activity of 2.21 Uml^{-1} and β-glucosidase activity of 1.94 Uml^{-1} by *Trichoderma viride* in 1 per cent peptone as nitrogen source among the different concentrations of different nitrogen sources (yeast extract, ammonium nitrate, sodium nitrate, beef extract. Effect of carbon sources on enzyme production after (Pang *et al.,* 2006). Andrade *et al*. (2011) also reported similar finding of highest FPase activity of 1.20 Uml^{-1} in wheat bran as carbon source. Similar finding was observed by Shanmugapriya *et al.* (2012). They also found that maximum maximum cellulase production of 10.96 U/mg with glucose as source cellulase producing bacteria. Chandel *et al*. (2013) registered that maximum production of cellulase (13.32 U/ml) was obtained by *Trichoderma* spp. with 1.0% sucrose as the carbon source.

Conclusion

Enzymes have attracted attention of researchers all over the world because of wide range of physiological, analytical and industrial application, especially, the production from microorganisms because of their broad biochemical diversity, feasibility of mass culture and ease of genetic manipulation. Thus, it can be deduced from the present investigation, that the candidate *Trichoderma harzianum* CKP1, a cellulolytic fungus can be effectively harnessed for the maximum production cellulase enzyme with different carbon and nitrogen sources.

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