

## Improvement of Extracellular $\beta$ -Galactosidase Production by Thermophilic Fungi *Chaetomium thermophile* and *Thermomyces lanuginosus*

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**Abstract:** Two local isolates of thermophilic fungi; *Thermomyces lanuginosus* and *Chaetomium thermophile* were grown on liquid culture containing various carbon sources in addition to lactose. Maximum extracellular  $\beta$ -galactosidase (3.94 and 2.86 U mg<sup>-1</sup>, respectively) was obtained after 7 d at 45°C and initial pH 5.5. Inoculum of 200 CFU was used. The optimal basal solution (OBS) contained (g L<sup>-1</sup>): 15 polygalacturonic acid; 5.2, casein; 1, K<sub>2</sub>HPO<sub>4</sub>; 1, MgSO<sub>4</sub> and 0.01 FeSO<sub>4</sub>. Under solid substrate cultivation (SSC) on wheat bran and lupine - seed powder (LSP) the yield of the enzyme was highly increased. Each Erlenmeyer flask (250 ml) was charged with 10 g LSP thoroughly mixed with 10 ml of OBS (50 % initial moisture content), inoculated with 200 CFU  $\mu$ L<sup>-1</sup> and incubated at 45 °C for 7d. SSC increased the enzyme activity about 10 folds. The hyperproduction of the enzyme by the two local isolates is promising in commercial production of  $\beta$ -galactosidase.

**Key words:** Thermophilic fungi -  $\beta$ - galactosidase

### INTRODUCTION

$\beta$ -Galactosidase (EC 3.2.1,  $\beta$ -Gals), commonly known as lactases, catalyse the hydrolysis of lactose to glucose and galactose. In addition, they were shown to catalyse transgalactosylation reactions of various  $\beta$ -D-galactopyranosides including lactose as well. Both hydrolase and transferase activity of  $\beta$ -Gals can be useful for different applications (Domingues, 2005).

Treatment of milk and other dairy products with  $\beta$ -Gal reduces the content of lactose (Rao *et al.*, 1988). Thus, products free of lactose or with a low lactose content can be used without problems by people suffering lactose - intolerance or galactosemia (Gekas and Lopez-Leiva, 1985; Novelli and Reichardt, 2000 and Campell *et al.*, 2005). Enzymic lactose hydrolysis in whey produce glucose -galactose syrup as a sugar substitute (Budriene *et al.*, 2005). Addition of  $\beta$ -Gal during manufacture of certain dairy products, e.g. yogurt, fresh cheese and ice cream, gives sweeter products and prevents undesired lactose crystallization (Baumgartner and Hinrich, 2000 and Domingues *et al.*, 2005). Galacto-oligosaccharides (GOS) are commercially produced from lactose by transgalactosylation reaction of  $\beta$ -Gal -GOS have many human health benefits by stimulating the growth and/or activity of bifidobacteria in the colon (Boon *et al.*, 2000; Hung and Lee, 2002 and Pruksasri, 2007). Transgalactosylation activities of  $\beta$ -Gal were adopted for manufacture of different therapeutic drugs (Scheckermann *et al.*, 1997 and Nakano *et al.*, 2000).

The occurrence of this enzyme in nature is diverse i.e. in plants, animals and microorganisms (Gekas and Lopez-Leiva, 1985).  $\beta$ -galactosidase from filamentous fungi are generally extracellular and broad stability profiles. Only few reports exist on enzyme from thermophilic fungi (Chung *et al.*, 1985; Fisher *et al.*, 1995; Shaikh *et al.*, 1997 and Nakkat and Haltrich, 2006). Industrial  $\beta$ -Gal production is hampered by the high costs associated with its production and purification (Domingues *et al.*, 2005).

The purpose of this work was to formulate culture media for enhancement of extracellular  $\beta$ -Gal production by *Chaetomium thermophile* and *Thermomyces lanuginosus* locally isolated from compost soil.

### MATERIALS AND METHODS

#### **Fungi:**

Thermophilic fungi were isolated from compost soil samples (47 samples) collected from decaying manure heaps in "Aiyat" rural area, Giza, Egypt. One g of each soil was blended in 10 ml of sterilized water and

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dilutions from the supernatant liquid were plated in yeast-lactose agar containing chloramphenicol (50 mg L<sup>-1</sup>) before gelling. After incubation at 45 °C for 5 d, pure cultures of well isolated colonies were transferred to yeast - glucose slants. Five isolates of the most frequent fungi were chosen for a preliminary survey of  $\beta$ -galactosidase production. These fungi were : *Aspergillus niger* van Tieghem, *Chaetomium thermophile* La Touche, *Myceliophthora thermophila* (Apinis) van Oorschot, *Talaromyces thermophilus* (Stolk) Stolk & Samson and *Thermomyces lanuginosus* Tsiklinsky. The first strain is thermotolerant and the others are true thermophiles.

#### **Culture Media and Conditions:**

##### **-Liquid Culture:**

Two liquid culture media were used for screening strains for  $\beta$ -galactosidase ( $\beta$ -Gal) activity. These were yeast - lactose (YL) and Czapek's -Lactose (CL). The composition of YL medium was as follows (g L<sup>-1</sup>): Lactose, 20.0 and yeast extract, 10.0; while the composition of CL medium was as follows (g L<sup>-1</sup>): Lactose, 20.0- NaNO<sub>3</sub>, 2.0 - K<sub>2</sub>HPO<sub>4</sub>, 1.0 - KCl, 0.5 - MgSO<sub>4</sub>.7H<sub>2</sub>O, 0.5 - FeSO<sub>4</sub>.5H<sub>2</sub>O, 0.01, with pH adjusted to 5.0 (phosphate buffer, 100 m mol L<sup>-1</sup>). One ml of uniformly prepared funga; suspension (200 CFU  $\mu$ l<sup>-1</sup>) from 7-d-old cultures grown on yeast -glucose agar was used as inoculum.

CL medium was used as basal medium in further experiments for optimization of  $\beta$ -Gal. production by *C. thermophile* and *T. lanuginosus*, terminally selected as potent  $\beta$ -Gal producers. The effect of incubation period up to 10 d on  $\beta$ -Gal production was followed. The effect of incubation temperature (30 - 55 °C) and the initial pH (3.0 - 7.0) on  $\beta$ -Gal production was investigated. Phosphate buffer (100 mmol L<sup>-1</sup>) was used for pH adjustment. The effect of lactose and a variety of related carbon sources (cellobiose, galactan, galactose, maltose, polygalacturonic acid- PGA and sucrose; final concentration 1%) on  $\beta$ -Gal activity was studied. The effect of different concentrations (0.5 - 2.0%) of galactan, lactose and PGA on  $\beta$ -Gal production was also investigated. Expression of  $\beta$ -Gal in the presence of equimolecular amounts of nitrogen from NaNO<sub>3</sub>, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, casein and peptone as well as different concentrations of casein (0.13 - 0.78 %) was studied. At the end of incubation period, the culture filtrate was centrifuged (5.0min and 4000rev.min<sup>-1</sup>). The supernatant cell - free filtrate was used for  $\beta$ -Gal assay and soluble protein determination.

##### **Solid - State Culture:**

In case of solid - state culture (SSC); 250 ml Erlenmeyer flasks were separately charged with 5.0 g of either starch - free wheat bran (WB) or lupine - seed powder (LSP), 400  $\mu$ mesh. Different volumes (2.5, 5.0, 10 and 15 ml) of the optimized basal solution (OBS) minus carbon substrate and containing (gL<sup>-1</sup>): Casein, 5.2 - K<sub>2</sub>HPO<sub>4</sub>, 1.0 - KCl, 0.5 - MgSO<sub>4</sub>.7H<sub>2</sub>O, 0.5 - FeSO<sub>4</sub>.5H<sub>2</sub>O, 0.0; were thoroughly mixed with the carbon substrate to allow initial moisture content 33.3, 50.0, 60.0 and 66.5 % (Carter *et al.*, 2004). pH adjustment, inoculation, incubation period and incubation temperature proceed as mentioned in case of liquid culture. The effect of LSP loadage (5 - 20 g/250 ml flask), 50.0 % initial moisture content) on  $\beta$ -Gal activity was studied. After incubation at 45°C for 7 days; the contents of each flask were pressed through nylon cloth and centrifuged (5min, 4000rev.min<sup>-1</sup>). The supernatant liquid was used for  $\beta$ -Gal assay and protein determination.

##### **$\beta$ -Galactosidase Assay:**

$\beta$ -Galactosidase activity was assayed at 45 °C using the chromogenic substrate o-nitrophenyl  $\beta$ -Galactopyranoside (oNPG) supplied by Sigma chemical Co. Assay mixtures containing 480  $\mu$ L oNPG (10 m mol L<sup>-1</sup>) and phosphate buffer (100 m mol L<sup>-1</sup>, pH 5.5). The reaction started by addition of cell-free culture filtrate 20  $\mu$ L and allowed to proceed for 5 min. The reaction was stopped by addition of 750  $\mu$ L of 10 m mol L<sup>-1</sup> NaNO<sub>3</sub> and the o-nitrophenol (oNP) released from the substrate oNPG was measured at 405 nm using Carl Zeiss, Speakol 11 spectrophotometer. oNPG unit of enzyme activity was defined as the amount of enzyme necessary to catalyze the formation of 1  $\mu$  mol of oNP per minute under stated conditions.

##### **Determination of Proteins:**

The total amount of soluble proteins in the cell-free culture filtrates was determined according to the method of Lowery *et al.* (1951), using bovine serum albumin as standard. Throughout this paper  $\beta$ -galactosidase activity was expressed in terms of specific activity (oNPG units/mg total soluble protein). The experiments were carried out in duplicate and the results presented are average value, with less than 10 % error.

## RESULTS AND DISCUSSION

Twenty two thermophilous fungal strains were isolated from compost soil samples in Aiyat, Egypt. Thermophilic fungi are the chief component of microbiota of habitats rich in organic materials (Emerson, 1968; Tansey and Brock, 1978; Mouchacca, 1997 and Maheshwari *et al.*, 2000). Five strains namely *Aspergillus niger*, *Chaetomium thermophile*, *Myceliophthora thermophila*, *Talaromyces thermophilus* and *Thermomyces lanuginosus* were subjected for a preliminary survey. Utilization of thermophilic fungi for production of extracellular  $\beta$ -Gal suitable for commercial applications is intended for the following reasons: 1) Ease of isolation and maintenance of cultures. 2) Reduced risk of contamination. 3) Simple nutrition and rapid growth. 4) Extracellular enzymes are secreted in the growth media and culture filtrate can be obtained in substantial quantities. 5) Purification protocols of secretory enzymes are cost-effective than cell associated (intracellular) enzymes. 6) Desired properties of the enzymes.

The five fungal strains grown on either YL or CL medium showed a considerable  $\beta$ -Gal activity (Table 1); higher activity was noticed in CL medium. *T. lanuginosus* and *C. thermophile* were the most potent producing strains exhibiting the higher specific activity (0.813 and 0.807 oNPG U mg<sup>-1</sup>). Few reports exist on production of  $\beta$ -Gal from thermophilic fungi (Chung *et al.*, 1985; Fisher *et al.*, 1995; Shaikh *et al.*, 1997 and Nakkat and Haltrich, 2006). A lactose-inducible intracellular  $\beta$ -Gal of the thermophilic fungus *T.lanuginosus* ATCC 16455 was detected (2.2 nkat pNPG U mg<sup>-1</sup>) in submerged culture. The medium contained (gL<sup>-1</sup>) 20, soluble starch; 10, yeast extract; 6, lactose, 1, MgSO<sub>4</sub> AND 1, K<sub>2</sub>HPO<sub>4</sub>; pH 6.8 and incubated at 50 °C and 100 rpm for 24 h (Fisher *et al.*, 1995). Different isolates "strains" from different geographical localities produce the same enzymes with intraspecies differences in enzyme properties. Concerning *C. thermophile*; the best of our knowledge; there are no available reports about  $\beta$ -Gal production.

**Table 1:**  $\beta$ -Galactosidase production of fungal strains grown on either YL or CL media.

Fungi	Soluble Protein (mg ml <sup>-1</sup> )		$\beta$ -Galactosidase activity (oNPG units)			
	YL	CL	U ml <sup>-1</sup>		U mg <sup>-1</sup>	
			YL	CL	YL	CL
<i>Aspergillus niger</i> *	1.88	1.09	1.10	0.84	0.585	0.771
<i>Chaetomium thermophile</i>	2.10	0.83	1.83	0.67	0.651	0.807
<i>Myceliophthora thermophila</i>	2.34	1.78	1.17	0.97	0.500	0.545
<i>Talaromyces thermophilus</i>	1.85	2.90	1.09	0.63	0.589	0.217
<i>Thermomyces lanuginosus</i>	1.54	0.80	1.19	0.65	0.773	0.813*
Thermotolerant strain						

Under surface culture conditions on CL medium the two experimental fungi exhibited a considerable  $\beta$ -Gal activity at a wide pH range (3.0 - 7.0) with maximal activity at pH 5.5 (Fig.1) and wide range of incubation temperature (30 - 55°C) with highest activity at 45°C (Fig. 2). Maximal  $\beta$ -Gal activity was obtained after 7 d incubation at 45°C (1.145 and 1.259 U mg<sup>-1</sup> for *C. thermophile* and *T. lanuginosus*, respectively (Fig. 3). Chung *et al.* (1985) reported that the maximum yield of extracellular  $\beta$ -Gal produced by *Aspergillus fumigatus* SJ-1 (0.13 oNPG U ml<sup>-1</sup>) was obtained in the medium potato lactose broth containing 1% lactose (pH 3.5). Cultivation was carried out on a reciprocal shaker (120 spm, 5 cm) at 50°C for 4 d. Skaikh *et al.* (1997) reported that maximum extracellular  $\beta$ -Gal activity (0.21 oNPG U mg<sup>-1</sup>) after 4 days under submerged fermentation condition. Solid state fermentation increased the enzyme activity 9-folds (2.04 U mg<sup>-1</sup>). The temperature range for production of the enzyme was 38 - 55 °C with maximum activity at 45 °C. The thermophilic fungus submerged culture of *Talaromyces thermophilus* CBS 236.58, grown in a medium containing (gL<sup>-1</sup>): 20, lactose; 10, yeast extract; 1, MgSO<sub>4</sub> and 1, K<sub>2</sub>HPO<sub>4</sub> (pH 5.0 - 5.5, 110 rpm, 40°C) and incubated for 5 d showed 3.8 (oNPG U ml<sup>-1</sup>) of extracellular  $\beta$ -Gal activity (Nakkharat and Haltrich, 2006).

Concerning the effect of a variety of carbon substrates (1%) on  $\beta$ -Gal production by *C. thermophile* and *T. lanuginosus*; results (Fig. 4) show that regardless of the carbon source nature,  $\beta$ -Gal activity was detected in a considerable amounts. Maximum yield was detected in case of PGA followed by lactose and galactan. Increase of galactan or lactose concentration up to 1% and PGA up to 1.5 % increased the  $\beta$ -Gal production by the two experimental fungi (Table 2). Similar results were obtained by Comp and Lester (1971) and Montero *et al.* (1989). *Ophiostoma ulmi* secreted a considerable amount of  $\beta$ -Gal (1.45 nkat ml<sup>-1</sup>) when grown on galacturonic acid or sodium pectate. Rhamnose and pectin also stimulated the secretion of the enzyme to a lesser extent (Binz *et al.*, 1997). McKay (1991) stated that the  $\beta$ -Gal enzymes of *Aspergillus oryzae* and *Scopulariopsis* sp. are secreted during growth of the fungi on PGA, but not on a wide range of other carbon substrates, including lactose. Production of  $\beta$ -Gal by the two experimental fungi regardless the carbon source,

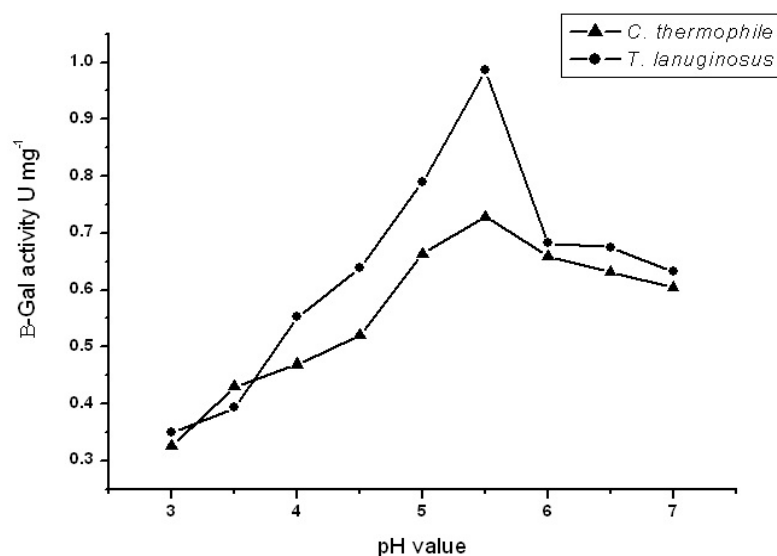


Fig. 1: Effect of CL medium on β-Galactosidase production by *C. thermophile* and *T. lanuginosus*.

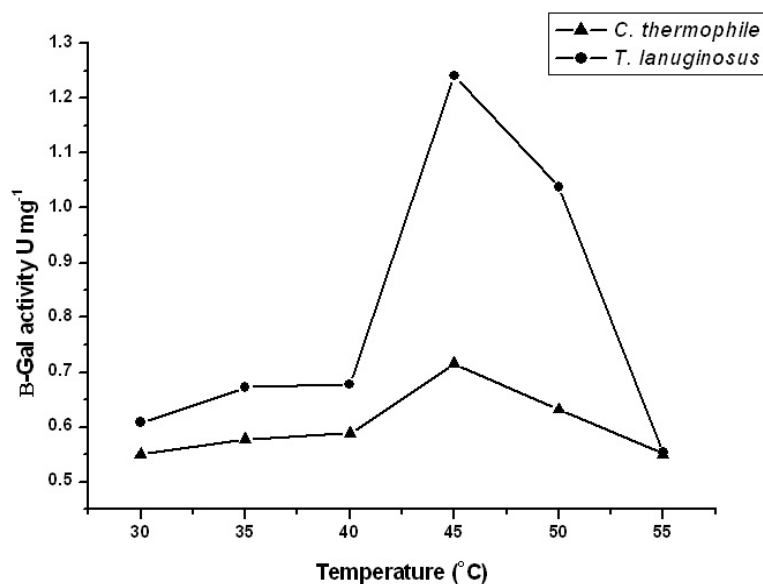


Fig. 2: Effect of temperature of incubation on β-Galactosidase production by *C. thermophile* and *T. lanuginosus*.

Table 2: Effect of varying concentration (w/v) of the best carbon sources on β-Galactosidase production by *C. thermophile* and *T. lanuginosus*.

Carbon source	Conc. (% w/v)	β- Galactosidase activity (U mg <sup>-1</sup> )	
		<i>C. thermophile</i>	<i>T. lanuginosus</i>
Galactan	0.5	0.533	0.710
	1.0	1.627	1.919
	1.5	0.480	0.667
	2.0	0.438	0.583
Lactose	0.5	0.402	0.639
	1.0	1.339	1.466
	1.5	0.667	0.778
	2.0	0.401	0.419
PGA	0.5	1.317	1.338
	1.0	1.906	1.924
	1.5	2.665	2.980
	2.0	2.255	2.556

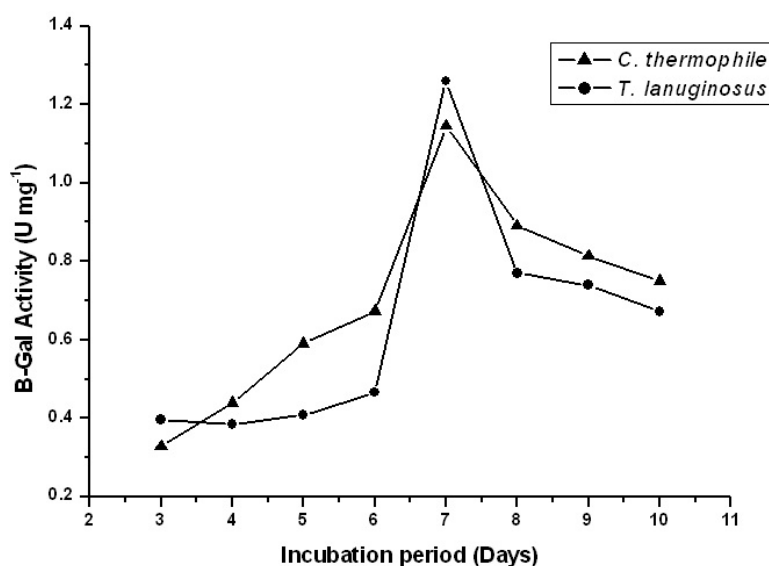


Fig. 3: Effect of incubation period on β-Galactosidase production by *C. thermophile* and *T. lanuginosus*.

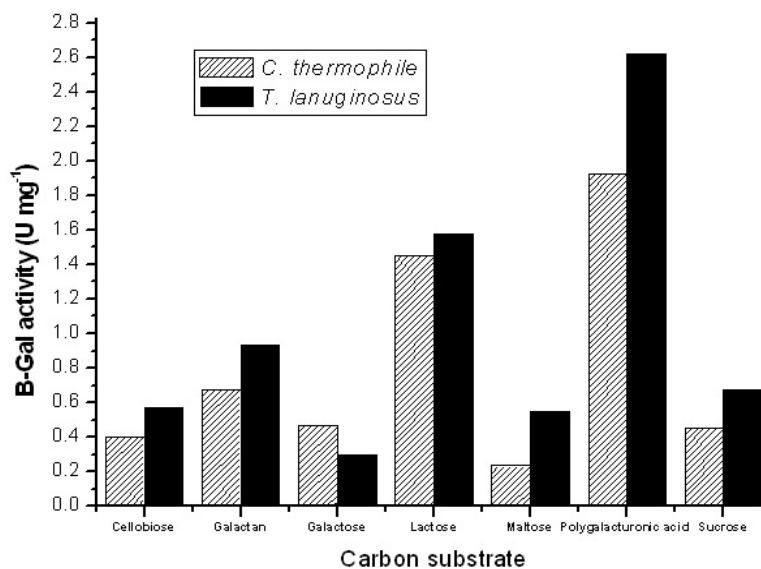


Fig. 4: Effect of Carbon substrates (1%, w/v) on β-Galactosidase production by *C. thermophile* and *T. lanuginosus*.

show that it is apparently not under catabolic repression control. Galactose also supported the secretion of β-Gal to a lesser extent; (Fig. 4); indicating that the released galactose does not inhibit β-Gal production i.e. galactose has no catabolic repression effect on β-Gal production process by the two experimental fungi. PGA is a complex polysaccharide with structure based on a poly (1-4) linked D-galacturonan. L-Rhamnose residues may be interspersed between the galacturonic acid residues of the central polymer chain. Side chains of neutral sugar residues such as galactose, arabinose or xylose are attached to galacturonan backbone of the molecule (Aspinall, 1980). β-Gal enzyme is involved in the hydrolysis of 4-linked galactose from PGA. Galactans especially 4-linked β-D-galactan chains are subject of hydrolysis by the action of β-Gal enzyme to release galactose. Utilization of PGA or Galactan in β-Gal production may be expensive, vegetable wastes (pectic substrates) from food processing industries are optional substrate for β-Gal production.

The effect of equimolecular amounts of certain nitrogen sources (Fig. 5) on β-Gal production by the two experimental fungi was investigated. Results show that maximum yield of β-Gal was obtained on medium containing casein followed by peptone. Increase of casein concentration up to 5.2 % increased β-Gal yield (3.942 and 2.860 U mg<sup>-1</sup>) in case of *T. lanuginosus* and *C. thermophile*, respectively (Fig. 6).

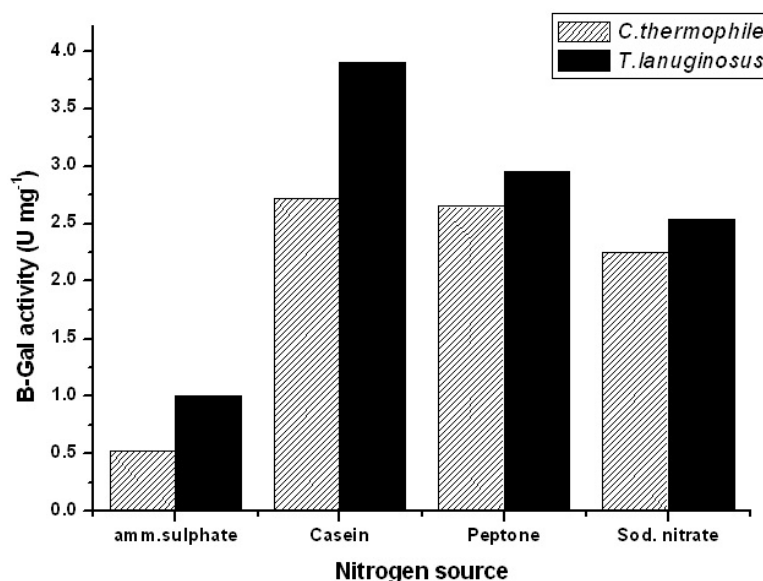


Fig. 5: Effect of different nitrogen sources on  $\beta$ -Galactosidase production by *C. thermophile* and *T. lanuginosus*.

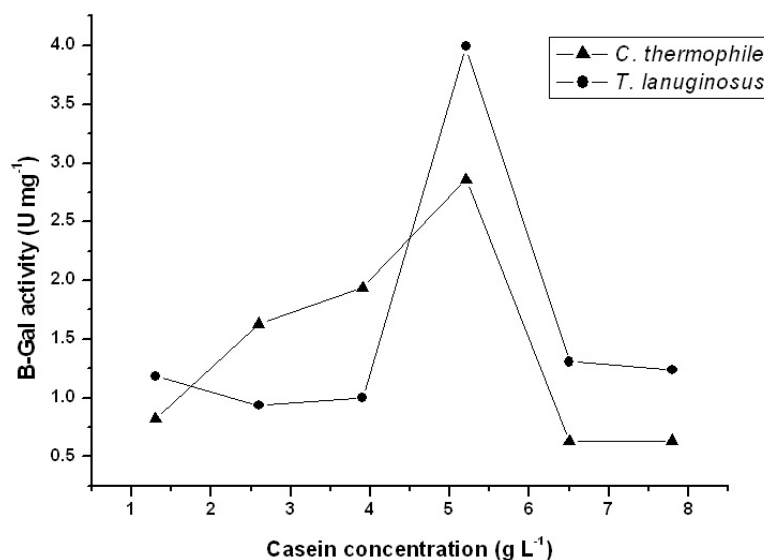


Fig. 6: Effect of casein concentration on  $\beta$ -Galactosidase production by *C. thermophile* and *T. lanuginosus*

Organic nitrogen sources are hydrolysed by the action of proteolytic enzymes of the experimental fungi to produce a variety of amino acids, from which  $\beta$ -Gal enzyme is synthesized through transamination process.

Production of  $\beta$ -Gal by *C. thermophile* and *T. lanuginosus*, under solid-state cultivation (SSC) conditions, was investigated on two substrates namely WB and LSP. WB is a popular substrate for commercial production of fungal enzymes. Both of the substrates contain pectic substances. LSP contains also galactan in addition to pectic substances. The two substrates increased  $\beta$ -Gal production of the investigated fungi (Table 3). *T. lanuginosus* grown on LSP or WB exhibited 38.51 and 37.594 U mg<sup>-1</sup> of  $\beta$ -Gal activity, respectively. *C. thermophile* produced 27.07 and 24.89 U mg<sup>-1</sup> on LSP and WB, respectively.

The maximum activities were obtained at initial moisture content 50.0 % where the ratio of substrate: OBS was 1:1, w/v (5 g substrate: 5 ml OBS). Increase in the initial moisture content decrease the  $\beta$ -Gal production by the two fungi. The moisture content is known to affect the production of hydrolytic enzymes in solid substrate cultivation (Battalino *et al.*, 1991). Lower moisture level leads to sub-optimal fungal growth, low degree of substrate swelling and high surface tension; whereas a high moisture level decrease the medium

porosity which initiate anaerobic condition, stickness of substrate particles and lower oxygen transfer (Lonsane and Ghildyal, 1992). Increase of LSP loadage up to 10g / flask increased  $\beta$ -Gal activity to 40.5 and 28.6 U  $\text{mg}^{-1}$  in case *T. lanuginosus* and *C. thermophile*, respectively (Table 4). Decrease in  $\beta$ -Gal production was observed with increase of substrate loadage. The high substrate loadage reduce heat transfer, while lower loadage makes heat dissipation easy. It is necessary to maintain an appropriate loadage (Wang *et al.*, 2004). Under SSC condition  $\beta$ -Gal production increased about 10-fold (from 3.9 to 40.5 and from 2.71 to 28.6 U  $\text{mg}^{-1}$  in case of *T. lanuginosus* and *C. thermophile*, respectively. Commercial production of fungal enzymes under SSC conditions has many advantages: 1) Cost-effective in equipment and operation, 2) Lower risk of contamination, 3) More concentrated yield of enzymes. 4) Mimicking, to some extent, the natural habitat of enzyme producing fungi, 5) Lower input and output of water and 6) Utilization of certain agro-industrial wastes as substrates for enzyme production, which increase their added value and clean-up the environment.

**Table 3:** Effect of initial moisture content of either wheat bran (WB) or lupine seed powder (LSP) on  $\beta$ -Galactosidase production by *C. thermophile* and *T. lanuginosus* under solid-state culture (SSC) conditions.

Moisture content (% w/v)	Substrate / nutrient solution ratio	$\beta$ -Galactosidase activity (U $\text{mg}^{-1}$ )			
		<i>C. thermophile</i>		<i>T. lanuginosus</i>	
		WB	LSP	WB	LSP
33.3	1 : 0.5	18.03	25.18	33.000	36.10
50.0	1 : 1	24.89	27.07	37.594	38.51
60.0	1 : 2	21.48	20.53	30.650	32.40
66.5	1 : 3	19.93	17.53	20.170	25.03

**Table 4:** Effect of LSP loadage on the production of  $\beta$ -Galactosidase by *C. thermophile* and *T. lanuginosus*.

LSP loadage (g / 250 flask)	$\beta$ -Galactosidase activity (U $\text{mg}^{-1}$ )	
	<i>C. thermophile</i>	<i>T. lanuginosus</i>
	5	26.9
10	28.6	40.5
15	25.2	32.6
20	20.6	30.8

### Conclusion:

Hyper-production of extracellular  $\beta$ -galactosidase by the two locally isolated thermophilic fungi namely: *T. lanuginosus* and *C. thermophile*, grown on LSP under SSC was reported (40.5 and 28.6 oNPG U  $\text{mg}^{-1}$  total soluble proteins, respectively). The two fungal strains may provide alternative sources for commercial production of the enzyme. The work will continue for purification and characterization of the enzyme.

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