Screening and Production of β-galactosidase by *Trichoderma* species.

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Abstract: Twelve *Trichoderma* strains (*Trichoderma reesei, Trichoderma harzarium, Trichoderma viride, Trichoderma longibrachiatum, Trichoderma asperellum, Trichoderma arundinaceum, Trichoderma konnigii, Trichoderma pseudokonnigii, Trichoderma ciroviride, Trichoderma fertile, Trichoderma polysporum* and *Trichoderma crassum*) were isolated from soil samples collected from different locations in Ibadan metropolis. Among the strains *Trichoderma crassum* (12.6%) has the highest frequency of occurrence. All the isolates were screened for β–galactosidase production using X-gal (5-bromo-4-chloroindolyl-β-D-galactopyranoside) and IPTG solution as an inducer. Six isolates were selected as the best producer of β–galactosidase. β–galactosidase production ranged from 0.3476 - 2.2369 U/ml in which *Trichoderma crassum* has the highest production. On submerged fermentation, static condition gave a profound increase in β-galactosidase production. The best yield of β-galactosidase production was obtained at 35^{o}C, pH 4, Tween80 at 0.15ml concentration and 6 days of incubation. Among the carbon and nitrogen sources tested manitol and casein supported β–galactosidase production (2.7666 - 6.9888 U/ml) by *Trichoderma crassum*.

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1. Introduction

Trichoderma is a cosmopolitan organism in soils and on decaying wood and vegetable matter. It belongs to the family Hypocreacae. They belong to an aggregate of *Deuteromycetes* species whose teleomorphs belong to the *Ascomycetes*. *Trichoderma* is a fungal genus first proposed by Persoon on the basis of material collected in Germany (Persoon, 1794). Species of *Trichoderma* are fungi that are present in substantial numbers in many regions of the world. They are frequently dominant components of the soil microflora in widely varying habitats. This may be attributable to the diverse metabolic capability of *Trichoderma* species and their aggressively competitive nature (Samuels, 1996). Strains within this genus include a wide spectrum of evolutionary solutions that range from very effective soil colonizers with high biodegradation potential to non-strict plant symbionts that colonize the rhizosphere. Species concepts within *Trichoderma* are very wide, which has resulted in the recognition of many intraspecific groups. Some groups of biotypes within this group are able to antagonize phytopathogenic fungi by using substrate colonization, antibiosis and or mycoparasitism as the main mechanisms. This antagonistic potential is the basis for effective applications of different *Trichoderma* strains as an alternative to chemical control against a wide set of fungal plant pathogens (Harman and Bjorkman, 1998).

Recently, (Szekeres et al., 2004) have reviewed antagonisitc metabolites produced by *Trichoderma* spp. The metabolites are linear, amphipathic polypeptides, namely, peptaibols and peptabiotics. They also discussed the physio-chemical and biological properties of these antibiotics compounds which included the disruption of lipid membranes, anti-microbial activities and induction of plant resistance.

β-galactosidase or β-D-galactoside-galactohydrolase is used industrially to obtain the hydrolyzed lactose from milk and milk whey for utilization in bakery products, ice creams, animal feed and as a sugar source for several fermentation products. Monosaccharides derived from hydrolysis are highly soluble and usually prevent the crystallization of the remaining lactose. Enzymatic hydrolysis of lactose from milk and milk whey is also desirable for lactose-intolerant individuals (Greenberg and Mahoney, 1981).

2. Material and Methods

Collection of Samples

Soil samples were collected aseptically at a depth of 2cm from three different locations in Ibadan: Cocoa Research institute of Nigeria, University of Ibadan Botanical Garden and a decayed wood sample in Microbiology Department, University of Ibadan. The samples were conveyed to the laboratory in sterile polythene bags for further analysis.

Isolation and identification of *Trichoderma* species from collected samples.

Serial dilution of the collected samples was carried out (Olutiola *et al.*2000) and 1ml of the diluents was pour plated on Potato Dextrose agar

(PDA) supplemented with streptomycin. The plates were incubated at 28°C for 3 days. Morphological appearances of the inoculated plates (at room temperature) were observed and distinct colonies were sub-cultured to obtain pure isolates which were then maintained on PDA slants and stored at 4□C for further study. Microscopic observations were made for the pattern of conidiation and hyphal branching of the pure fungi isolates after which identification was done with reference to Barnett's Compendium of Soil Fungi (1980), Rifai (1969) and other relevant electronic documentations on the genus *Trichoderma*.

Screening for β-galactosidase production.

Trichoderma isolates were screened with X-gal.one colony of isolated fungi were grown on MEA agar plates containing 60µl X-gal (5-bromo-4-chloroindolyl-β-D-galactopyranoside, #R0401,fermentas,20mg/ml DMF) and IPTG (iso-propyl-thio-β-D-galactopyranoside ,clioxane free #R0391,fermentas) solution as an inducer. Plates were incubated at 37°C for 3-5 days. Colonies producing β-galactosidase were green (Vinderola and Runheimer, 2003).

Production of β-galactosidase enzyme by submerged fermentation

All the fungi isolates were grown in a basal medium containing Lactose 10g, KH_2PO_4 5g, $(NH_4)_2SO_4$ 1.2g, $MgSO_4$. $7H_2O$ 0.4g and yeast extract 1g in 1 liter of 0.2M potassium phosphate buffer, pH= 5.5. Sterilization of the medium was done at 121°C for 15min, lactose was sterilized by filtration, β-galactosidase was produced in 500ml Erlenmeyer flask with 150ml of culture medium. The culture was incubated in an orbital shaking incubator for 5- 6days at 180rpm and 30°C (Manera et al., 2008).

Dry Cell Weight Determination

The mycelium from each flask was filtered and then washed. The washed mycelium was dried in British-made Gallenkamp oven at 110□C to a constant mass and the mass was determined using an automatic electronic balance.

Statistical analysis

Experiments were performed in triplicate and the results were analyzed statistically. The treatment effects were compared and the significant difference among replicates has been presented as Duncan's multiple range tests in the form of probability values.

3. Results

A total of twelve fungi belonging to the genera *Trichoderma* (*Trichoderma reesei, Trichoderma harzarium, Trichoderma viride, Trichoderma longibrachiatum, Trichoderma asperellum, Trichoderma arundinaceum, Trichoderma konnigii, Trichoderma pseudokonnigii, Trichoderma ciroviride, Trichoderma fertile, Trichoderma polysporum* and *Trichoderma crassum*) were isolated from soil samples collected from University of Ibadan Botanical garden, Cocoa Research Institute of Nigeria and decayed wood in the Department of Microbiology garden. Identification of genus was based on morphological and cultural characteristics compared to fungi compendium (1980), and an illustrated manual on identification of *Trichoderma* species was used. Table 1 shows the cultural, morphological and microscopic characteristics of the *Trichoderma* species.

The frequency of occurrence of the fungal isolates is shown in Figure 1. *Trichoderma asperellum* had the highest frequency of occurrence (16.8%) followed by *Trichoderma crassum* (12.6%) and *Tricoderma arundinaceum* (8.9%). Table 2 shows the isolates that were screened for β-galactosidase using X-gal as an indicator. Six of these isolates (*Trichoderma viride, Trichoderma arundinaceum, Trichoderma fertile, Trichoderma longibrachiatum, Trichoderma crassum,* and *Trichoderma asperellum*) Colonies producing β-galactosidase were green. They were good β-galactosidase producer.

Table 3.1 shows the β-galactosidase production by the selected *Trichoderma* species cultivated in submerged fermentation. It was observed that there was variation in β-galactosidase production by the selected isolates during the fermentation period. Optimum production of β-galactosidase was recorded when the fermentation medium was static. β-galactosidase production ranges from 0.4296 - 4.0926 U/ml in which *Trichoderma viride* had the highest production.

The effect of temperature on the production of β-galactosidase was shown in Table 3. 2, it ranged from 1.0422 - 4.3796 U/ml and the highest production (4.37962 U/ml) was attained at 35°C by *T. crassum*. Figure3.10a shows the effect of incubation time on β-galactosidase production by the selected isolates and at 6days optimum production (7.8171 U/ml) of β-galactosidase was attained by *T. crassum*. Table 3.3 shows the highest production (2.2369U/ml) of β-galactosidase at pH 4 by *T. crassum*. β-galactosidase production ranged from 0.3476 - 2.2369 U/ml. Table 3.4 shows the effect Tween80 which is a supplement. β-galactosidase production ranged from 0.2353 - 3.8999 U/ml in which *Trichoderma viride* had the highest production at 0.15% concentration.

Effect of different carbon sources on β-galactosidase production is shown in Table 3.5. It was observed that manitol induced the higher level of β-galactosidase production and it ranged from 2.7666 - 6.9888 U/ml in which *Trichoderma crassum* had the

highest production. Table 3.6 shows the effect of different nitrogen sources on β-galactosidase production by the selected isolates. Casein supported the optimal production of β-galactosidase enzyme; it ranges from 2.6773 - 7.4853 U/ml in which *Trichoderma crassum* had the highest production.

Table 1: Cultural and Morphological Characteristic of *Trichoderma* species obtained from soil samples.

Isolate code	Appearance on Agar	Growth pattern	Microscopic spore shape	Probable Identity
ASB1	Dark green granular colony	Rapid with coconut odour	Phialides typically crowded arising from broad cells, Conidiophore with branches	*Trichoderma viride*
AS2	Yellow green conidia formed densely over the center and in undulating concentric rings	Fairly rapid	Globose, intercalary hyphae and Terminal phialides	*Trichoderma harzianum*
AW3	Dark green, mottled with white flecks	Fairly rapid	Phialides mainly arising singly, in divergent whorls and typically cylindrical	*Trichoderma longibrachiatum*
AW14	White with a diffusing yellow pigment	Fairly rapid	Phialides in whorls at the tip of fertile branches	*Trichoderma polysporum*
AW5	Wooly green rings	Fairy rapid	Paired lateral branches	*Trichoderma pseudokoningii*
AW16	Yellowish green	Rapid	Phialides held in whorls	*Trichoderma arundinaceum*
AS7	Greenish mycelium	Fairly rapid	Phialides supported by a base cell typically terminating cells of branches in pairs	*Trichoderma citrinoviride*
AW8	Dark green, dense wooly colony	Rapid	Phialides formed on conidiophores within pustules	*Trichoderma asperellum*
AS9	Yellowish brown granules	Rapid	Intercalary within hyphae	*Trichoderma crassum*
AW10	Diffusing yellow pigment conidiation	Fairly rapid	Phialides held in whorls	*Trichoderma konnigii*
NUB5	Greenish, uniformly dispersed colonies	Fairly rapid	Long straight phialides, typically flask-shaped and enlarged in the middle	*Trichoderma reesei*
NUB8	Conidia formed densely in a central disk and concentric rings of conidial production. No pigment in the agar	Fairly rapid	Basal phialides tending to be held in more or less divergent whorl while terminal phialides slightly hooked	*Trichoderma fertile*

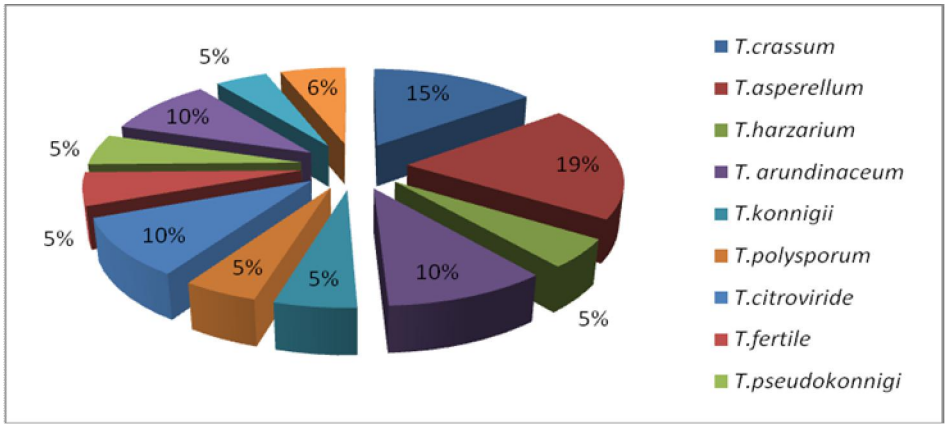

Figure 1 Percentage frequency of occurrence of microorganisms isolated from the soil samples

Table 2: Screening of the *Trichoderma* species for β-galactosidase production on solid agar.

Probable Isolates	Mycelium colour Day 4	Mycelium colour Day 5	Mycelium colour Day 6
Trichoderma viride	White	Light green	Dark green
Trichoderma harzianum	Cream	Cream	Light green
Trichoderma longibrachiatum	White	Light green	Dark green
Trichoderma polysporum	Cream	Light green	Light green
Trichoderma pseudokoningii	White	Light green	Dark green
Trichoderma arundinaceum	Cream	Green	Dark green
Trichoderma citrinoviride	Cream	Cream	Light green
Trichoderma asperellum	Light green	Dark green	Dark green
Trichoderma crassum	Light green	Dark green	Dark green
Trichoderma konnigii	White	Light green	Light green
Trichoderma reesei	Cream	Light green	Light green
Trichoderma fertile	Cream	Green	Dark green

Table 3. 1: β-galactosidase production (U/ml) by *Trichoderma* species under static and agitation condition.

Isolate code	β-galactosidase production (U/ml)	
	Static	Agitation
Trichoderma viride	4.0926^{a}	1.1398^{f}
T. polysporum	2.6379^{f}	1.1808^{e}
T. pseudokonnigii	1.0376^{k}	0.779^{k}
T. crassum	3.9216^{b}	1.9352^{a}
T. konnigii	2.0737^{j}	0.8446^{i}
T. arundinaceum	0.4296^{l}	0.8036^{j}
T. reesei	2.4747^{g}	0.8938^{h}
T. harzarium	3.1069^{c}	1.3612^{c}
T. asperellum	2.7244^{e}	0.9026^{g}
T. fertile	2.9984^{d}	0.7544^{l}
T. longibrachiatum	2.3038^{h}	1.4186^{b}
T. citroviride	2.2898^{i}	1.2218^{d}

Values are means of three triplicates. Mean values in columns with different superscript letters are significantly different (P≥0.05), NG- NO GROWTH

Table 3. 2: Effect of temperature on β-galactosidase production (U/ml) by *Trichoderma* species

Isolate code	β-galactosidase production (units/ml) $x10^{-7}$				
	Temperature (^{o}C)				
	25	30	35	40	45
T. reesei	3.1776^{b}	3.6457^{a}	2.3862^{c}	1.6990^{d}	1.5965^{e}
T. viride	1.7400^{b}	2.6801^{a}	1.4296^{c}	1.0455^{e}	1.4612^{d}
T. harzarium	1.8113^{c}	2.4509^{a}	1.7999^{d}	2.3837^{b}	1.5186^{e}
T. longibrachiatum	2.1303^{b}	2.4194^{a}	1.5953^{d}	1.1512^{e}	1.6211^{c}
T .crassum	2.4591^{b}	4.3796^{a}	2.4173^{c}	2.7125^{d}	1.6375^{e}
T.asperellum	2.0508^{c}	2.7566^{a}	2.5731^{b}	2.1598^{d}	1.0422^{e}

Values are means of three triplicates. Mean values in columns with different superscript letters are significantly different (P≥0.05), NG- NO GROWTH

Table 3. 3: Effect of incubation time on β-galactosidase production (U/ml) by *Trichoderma* species

Isolate code	β-galactosidase production (units/ml) x10^{-7}			
	Incubation Time (Days)			
	3	6	9	12
T. reesei	3.6547^{d}	6.1672^{a}	4.9905^{c}	5.3250^{b}
T. viride	3.2914^{b}	7.2348^{a}	2.9151^{c}	2.8691^{d}
T. harzarium	2.5551^{d}	7.4726^{a}	4.2123^{b}	4.0721^{c}
T.longibrachiatum	3.2857^{d}	7.1594^{a}	5.0938^{b}	3.3005^{c}
T. crassum	5.2168^{b}	7.8170^{a}	5.1217^{c}	3.6432^{d}
T. asperellum	3.7006^{c}	6.1934^{a}	3.4144^{d}	4.4132^{b}

Values are means of three triplicates. Mean values in columns with different superscript letters are significantly different (P≥0.05), NG- NO GROWTH

Table 3. 4: Effect of Tween80 on β-galactosidase production (U/ml) by *Trichoderma* species

Isolate code	β-galactosidase production (units/ml) x10^{-7}				
	Tween80 concentration (ml/l)				
	0.05	0.1	0.15	0.25	0.3
T. reesei	1.5366^{b}	1.3439^{c}	3.8843^{a}	0.2353^{e}	1.0061^{d}
T. viride	1.7351^{c}	1.1931^{e}	3.8999^{a}	1.5809^{d}	1.8163^{b}
T. harzarium	3.7236^{b}	1.6711^{c}	3.2463^{a}	0.1123^{e}	0.6789^{d}
T.longibrachiatum	1.7761^{c}	2.0836^{b}	3.1004^{a}	0.5231^{e}	1.4046^{d}
T. crassum	1.1504^{c}	1.3185^{b}	1.4128^{a}	1.0061^{d}	0.8265^{e}
T. asperellum	0.8569^{e}	0.9700^{d}	2.1090^{a}	0.9831^{c}	1.8409^{b}

Values are means of three triplicates. Mean values in columns with different superscript letters are significantly different (P≥0.05), NG- NO GROWTH

Table 3. 5: Effect of pH on β-galactosidase production (U/ml) by *Trichoderma* species

Isolate code	β-galactosidase production (units/ml) 10^{-7}				
	pH				
	2	4	7	9	12
T.reesei	0.6904^{e}	2.2369^{a}	1.6941^{d}	2.2882^{b}	2.1525^{c}
T.viride	0.5149^{d}	1.5088^{a}	1.3300^{b}	0.9733^{c}	0.0967^{e}
T.harzarium	0.7740^{c}	1.3267^{a}	1.7179^{b}	0.6068^{d}	0.1541^{e}
T.longibrachiatum	0.8085^{c}	2.2935^{a}	1.9007^{b}	0.7978^{d}	0.5526^{e}
T.crassum	0.6789^{b}	2.6264a	0.4780^{e}	0.6371^{c}	0.4805^{d}
T.asperellum	0.6207^{b}	1.2939^{a}	0.3886^{d}	0.4780^{c}	0.3476^{e}

Values are means of three triplicates. Mean values in columns with different superscript letters are significantly different (P≥0.05), NG- NO GROWTH

Table 3 .6: Effect of carbon sources on β-galactosidase production (U/ml) by *Trichoderma* species

Isolate code	β-galactosidase production (units/ml) x10^{-7}					
	Carbon sources (g/l)					
	Sucrose	Glucose	Fructose	Lactose	Maltose	Manitol
T.reesei	4.5173^{f}	5.4456^{e}	5.8097^{d}	6.4025^{b}	5.9310^{c}	6.8765^{a}
T.viride	4.8101^{f}	4.9118^{e}	5.4456^{d}	5.4653^{c}	5.5899^{b}	5.7777^{a}
T.harzarium	5.0225^{e}	3.6440^{f}	5.5259^{c}	5.2627^{d}	5.6079^{b}	6.0876^{a}
T.longibrachiatum	5.3209^{d}	3.8982^{e}	5.7629^{c}	6.1672^{b}	2.7666^{f}	6.9601^{a}
T.crassum	3.5936^{f}	4.1073^{e}	5.9716^{b}	5.3890^{c}	4.7330^{d}	6.9888^{a}
T.asperellum	5.1914^{f}	5.8129^{c}	5.6407^{d}	5.8384^{b}	5.2152^{e}	6.1499^{a}

Values are means of three triplicates. Mean values in columns with different superscript letters are significantly different (P≥0.05), NG- NO GROWTH

Table 3. 7: Effect of nitrogen sources on β-galactosidase production (U/ml) by *Trichoderma* species

Isolate code	β-galactosidase production (units/ml) $x10^{-7}$					
	Nitrogen sources (g/l)					
	Casein	Yeast	$NaNo_3$	Urea	NH_4No_3	$(NH_4)_2SO_4$
T. reesei	5.9882^{a}	5.8441^{c}	5.9794^{b}	4.2123^{f}	5.6563^{d}	5.3652^{e}
T. viride	6.8769^{a}	6.0794^{c}	5.7875^{d}	4.1385^{f}	6.1885^{b}	5.0380^{e}
T. harzarium	6.6041^{a}	6.4566^{b}	5.9917^{d}	6.3443^{c}	5.0184^{e}	5.0036^{f}
T.longibrachiatum	5.9498^{a}	4.9003^{e}	5.2734^{d}	5.8016^{c}	5.9376^{b}	2.6773^{f}
T. crassum	7.4853^{a}	6.4710^{b}	4.7633^{f}	5.6998^{d}	5.1118^{e}	6.1204^{c}
T. asperellum	6.6186^{a}	5.1065^{e}	5.8539^{b}	5.1118^{d}	4.5091^{f}	5.3341^{c}

Values are means of three triplicates. Mean values in columns with different superscript letters are significantly different (P≥0.05), NG- NO GROWTH

4. Discussions

Lactose, the main sugar in milk and whey and its corresponding hydrolase, β-galactosidase, have been the subject of extensive research during the past decade. Partly, this is because of the interesting possibilities of using low lactose or lactose free products (Kara, 2004). β-galactosidase optimal production was attained at 30°C and this is in agreement with the work of Kara, (2004). As the temperature increased, there was a gradual increase in the production of the enzyme from 25°C - 40°C. At 6 days incubation time there was optimal production of β-galactosidase which means that the organisms are at their lag phase in which metabolic activities are very high. The best yield of β-galactosidase at pH 4 in this study might be due to the organism's requirement of acidic pH for enzyme production (Puntambekar, 1995). Optimal production of β-galactosidase by *Trichoderma* spp. was attained at pH 4. This report does not concur with Saad (2004).Which said optimal production is attained at pH 5.2. Different concentrations (0.05%, 0.10%, 0.15%, 0.25% and 0.30%) of Tween80 were studied for the effect on enzyme production by the selected *Trichoderma* species. A profound increase of β-galactosidase production was attained at 0.3% of Tween80 by the isolates. The work of El-Halwary and Mostafa, (2001) reported that supplement like Tween80 enhances enzymes activities by increasing availability of nutrients. The mechanism of enhancement by Tween80 at low concentration increases the permeability of the cell membrane allowing for more rapid secretion of the enzyme which in turn leads to greater enzyme synthesis which is in agreement with the work of Kishen *et al*. (1981).

The different carbon sources used supported the production but it was only manitol that gave highest production of β-galactosidase and this result is not in agreement with the work of Dabhole and Joishy, (1998). Among the organic and inorganic nitrogen sources used in this study, casein gave a profound increase in β-galactosidase production.

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