Cellulase Production by some Fungi Cultured on Pineapple Waste

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Abstract: Cellulase production from cellulosic pineapple waste using *Trichoderma longibrachiatum, Aspergillus niger* and *Saccharomyces cerevisiae* was assessed. The wastes were dried, pre-treated with alkali and steam, re-dried and then blended. The powdered wastes were then used as substrates in separate shake-flasks which contained mineral salts medium (MSM) and inoculi of *Trichoderma longibrachiatum, Aspergillus niger* and *Saccharomyces cerevisiae.* Fermentations were carried out in flasks containing the MSM, the waste substrate and the inoculum at pH 5.0, 1% substrate concentration, 10% inoculum size and cultured on a rotary shaker at 29±1ºC initially for 5 days to verify cellulase production by the organisms from the waste substrates, then for 7 days or 9 days while varying different fermentation parameters. Cellulase activity and amount of glucose produced by the three test organisms from the waste substrates were determined and compared. The amount of glucose produced was optimized by varying the fermentation parameters: Time, pH, Substrate concentration, Inoculum size and Temperature. The results obtained from the fermentations showed that *Trichoderma longibrachiatum* produced the highest amount of glucose among the cultures tested (0.92mg/0.5ml). This was produced from pineapple pulp at pH 4.5 and temperature of 45ºC on Day7 of fermentation. The highest amount of glucose produced by *Aspergillus niger* was also from pineapple pulp (0.63mg/0.5ml) at pH 3.5 and temperature of 40ºC on Day5 of fermentation. The highest amount of glucose produced by *Saccharomyces cerevisiae* was from pineapple pulp (0.54mg/0.5ml) at pH 4.5 and temperature of 45ºC on Day5 of fermentation. [Nature and Science. 2008;6(2):64-79]. ISSN: 1545-0740.

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INTRODUCTION

Cellulose is the principal constituent of the cell wall of most terrestrial plants. The source of cellulose is in plants and it is found as micro-fibrils (2-20nm in diameter and 100 – 40,000nm long). These form the structurally strong frame work in the cell walls.

Despite a worldwide and enormous utilization of natural cellulosic sources, there are still abundant quantities of cellulose-containing raw materials and waste products that are not exploited or which could be used more efficiently. The problem in this respect is however to develop processes that are economically profitable. Cellulose-containing wastes may be agricultural, urban, or industrial in origin, sewage sludge might also be considered a source of cellulose since its cellulosic content provides the carbon needed for methane production in the anaerobic digestion of sludge. Agricultural wastes include crop residue, animal excreta and crop processing wastes slashing generated in logging, saw dust formed in timber production and wood products in forestry originated activities.

The previous negative attitude in which wastes were viewed self consciously as valueless and even offensive and for disposal only has been replaced in large part by a positive view in which wastes are recognized as raw materials of potential value (Pranner, 1979).

Currently, there are two major ways of converting cellulose to glucose: chemical versus enzymatic. Enzymatic hydrolysis of cellulose is an important reaction in nature for it marks the first step in the decay of cellulose, the most abundantly occurring organic material. In the early 1970s, the oil crisis generated interest in using cellulose as a chemical and energy resource. One promising approach was to hydrolyze the cellulose to glucose with fungal enzymes and then to ferment the glucose to ethanol which could be used as a liquid fuel (Mandels *et al.,* 1974).

The research on both methods has for decades occupied the attention of many investigators world wide because each cellulose molecule is an unbranched polymer of 1000 to 1 million D- glucose units, linked together with β-1, 4 glucosidic bonds. Cellulose from various sources is all the same at the molecular level. However, they differ in the crystalline structures and bindings by other biochemicals (Nam, 1979).

Pineapple fruits are compound, oval fruits, six to eight inches long with spiky robust leaves at the top of the fruit. The tough waxy rind is green, brown and yellowish in colour with a scale-like appearance. The flesh of the pineapple is juicy and yellow to white in colour.

Pineapple is a tropical plant and fruit. Its scientific name is *Ananas comosus* and it belongs to the class: Liliopsida and family Bromeliaceae (Morton, 1987). Pineapple is the source of the proteolytic

enzyme bromelain which is used in commercial meat tenderizers and which constitutes practice as a soft tissue anti-inflammatory and for topical debridement.

In addition to its nourishing uses, the pineapple has long served medical purposes in folk medicine. It is used to arouse appetite, effective as diuretic and contraceptive and in the expulsion of internal worms. It has been used to prevent ulcers, enhance fat excretion, among many other uses.

MATERIALS AND METHODS

Microorganisms and Waste substrates

The organisms used for this study were isolated from three sources: *Aspergillus niger* was isolated from rotten wood (RW) picked up on the premises of University of Ilorin campus, *Saccharomyces cerevisiae* was isolated from palm wine (PW) bought from a palm wine tapper at Offa garage area in Ilorin metropolis and *Trichoderma longibrachiatum* was collected from the Faculty of Agriculture, University of Ilorin. The microorganisms were identified in the Microbiology laboratory, University of Ilorin, Nigeria.

The waste substrates used were in this study were pineapple peel (**ppe**) and pineapple pulp (**ppu**). Pineapple fruit was washed and peeled. The pineapple peel was drained to remove excess water. The pineapple pulp was prepared from the after extraction of the juice from the pineapple fruit. The juice was removed with the aid of a juicer in order to separate the juice from the pulp. The pulp was then washed in water to remove excess juice. Both the peel and pulp were dried in the oven at 70ºC for 3 days. After drying, the pineapple wastes were pounded into small pieces using a mortar and pestle. A modified method of Ali *et al*., (1991) which involved alkali and steam treatments was used in the pretreatment of the waste substrates.

The pounded pieces of the samples were autoclaved for one hour at 121ºC with 5% (w/v) NaOH (20ml per gram of substrate) in separate conical flasks for delignification. The autoclaved ppe and ppu waste substrates were filtered through muslin cloth. They were then washed thoroughly with water and neutralized with 1M HCl. The wastes were finally washed with distilled water and dried at 70ºC. After drying, the treated wastes were ground in a blender for 10 minutes each.

Enzyme assays

The three selected test fungi were cultivated using the submerged culture technique and later maintained on PDA slants. A 10ml three to four day old spore suspension was made from PDA slants of

each culture using sterile distilled water. The fermentation media used was Mary Mandels' mineral salts solution and it was used along with different carbon and nitrogen sources. The medium (**M1**) contained the following (per L) Cellulose,10g ; Peptone,1g ; $(NH_4)_2SO_4$, 1.4g ; KH_2PO_4, 2g ; $CaCl_2$, 0.3g ; $MgSO_4.7H_20$, 0.3g ; Urea, 0.3g ; *trace metal solution (2.5g $FeSO_4$; 0.98g $MnSO_4.H_2O$; 1.76g $ZnSO_4.H_2O$; 1.83g $CoCl_2.6H_2O$ dissolved in 495ml of distilled water and 5ml of conc. HCl), 1ml ; pH 4.8 (Jeffries, 1996).

*Trace metal stock solution (1ml is used per L).

Mineral salts glucose medium was prepared and approximately 2.8×10^6 spores/cells of each culture were inoculated into 500 ml flasks containing 100 ml of medium each. The spores/ cells were counted using a Neubauer counting chamber. The flasks were incubated for 24 hours at 29±1ºC on a Gallenkamp (England) rotary shaker at 250rpm to develop the inoculum.

The waste substrates provided the carbon sources in the fermentation media. These were combined with M1 to give Mineral salts glucose medium (MSGM), Mineral salts pineapple peel medium (MSppeM) and Mineral salts pineapple pulp medium (MSppuM).

All the media mentioned above were prepared separately and dispensed in conical flasks. They were sterilized in the autoclave at 121ºC for 15 minutes.

The pH of the fermenting media containing the waste substrates at a level of 10g/L was adjusted to 5.0. The suspension of germinated spores was inoculated at a level of 10% (v/v) into the production medium contained in flasks. These were incubated at 29±1ºC on a shaker at 100rpm. Glucose production in the medium was measured on Day 5 of fermentation (Srivastava *et al*. 1987; Jeffries, 1996; Bukoye, 2001).

Cellulase activity was determined colorimetrically by measuring the increase in reducing groups by the hydrolysis of a carboxymethylcellulose (CMC) substrate (Jeffries, 1996). The procedure followed the 0.5ml assay described by Jeffries (1996).

Samples were withdrawn from the culture at 2-day intervals over a period of 7-9 days and the supernatant that resulted following centrifugation at 3000 rpm for 15 minutes to remove solids, were assayed for total reducing sugars using DNSA method of Miller (1959). Enzyme solutions were diluted in 0.05M citrate buffer, pH 4.8. The enzyme diluted in buffer and one percent CMC (0.5ml each) was mixed well and incubated for 30 minutes at 50ºC. Three milliliters of the DNSA was added and the tubes were placed in boiling water bath for five minutes. The tubes were cooled and the reducing sugar, glucose was determined (Jeffries, 1996). The sample, enzyme blank, glucose standard and control were boiled together and absorbance was read at 540nm using a spectrophotometer. A control (substrate and buffer) was used to set the spectrophotometer at zero absorbance. During the course of the experiments, the absorbance of the

sample tube, corrected by subtraction of the enzyme blank was translated into glucose during the reaction using a glucose standard. The linear glucose standard was used to translate the absorbance values of the sample tubes into glucose i.e. mg glucose produced during the reaction. For a 30-minute assay, 1mg of glucose equals 0.185unit $\left(\frac{1}{30 \times 0.18}\right)$.

Determination of Optimal Conditions for Enzyme Production

Effect of varying time: Cellulase activity was measured at regular intervals while fermentation was observed at 29±1ºC for a period of 9 days and the period of maximum enzyme production was determined.

Effect of varying pH: The pH of the fermentation media were adjusted to various values ranging from 2.0-6.0 with 0.1N NaOH or 0.1N HCl. The pH was determined using the pH meter

Effect of varying substrate concentration: Different concentration of the waste substrates ranging from 1.0% to 5.0% were used in the fermentation media.

Effect of varying Temperature: The fermentation was carried out at different temperatures ranging from 30ºC to 45ºC.

Effect of varying inoculum size: Each cellulosic waste was fed with varying sizes of inoculum of the organisms. The inoculum size was varied from 2% to 10%.

Optimization experiments were carried out and each of the organisms were grown on each of the substrates and hydrolyzed using parameters that produced maximal activity of the enzyme from all the earlier experiments. In accord with the International Union of Biochemistry, one enzyme unit equals 1micromole (μ) of substrate hydrolyzed per minute (Ghose, 1987).

RESULTS AND DISCUSSION

Table 1. Fermentation of Pineapple wastes substrates by Test Fungi

WASTE SUBSTRATE	GLUCOSE PRODUCED (mg/0.5ml)		
	T. longibrachiatum	*A. niger*	*S. cerevisiae*
Pineapple peel	0.52	0.41	0.18
Pineapple pulp	0.43	0.49	0.43

Fermentation conditions: Substrate concentration 1%, pH: 5.0, inoculum size: 10%, temp: 29±1ºC, time: 5 days

Table 2: Effect of Substrate Concentration on the Fermentation of Pineapple waste by test fungi

Fermentation conditions: pH: 5.0, inoculum size: 10%, temp: 30ºC, time: 5 days.

Substrate concentration	Glucose produced (mg/0.5ml)					
	T. longibrachiatum		*A. niger*		*S. cerevisiae*	
	ppl	ppu	ppl	Ppu	ppl	ppu
1%	0.28	0.30	0.21	0.25	0.19	0.21
2%	0.29	0.31	0.29	0.27	0.26	0.268
3%	0.34	0.36	0.25	0.28	0.31	0.294
4%	0.31	0.36	0.26	0.26	0.29	0.326
5%	5.87	0.33	0.27	0.27	0.31	0.296

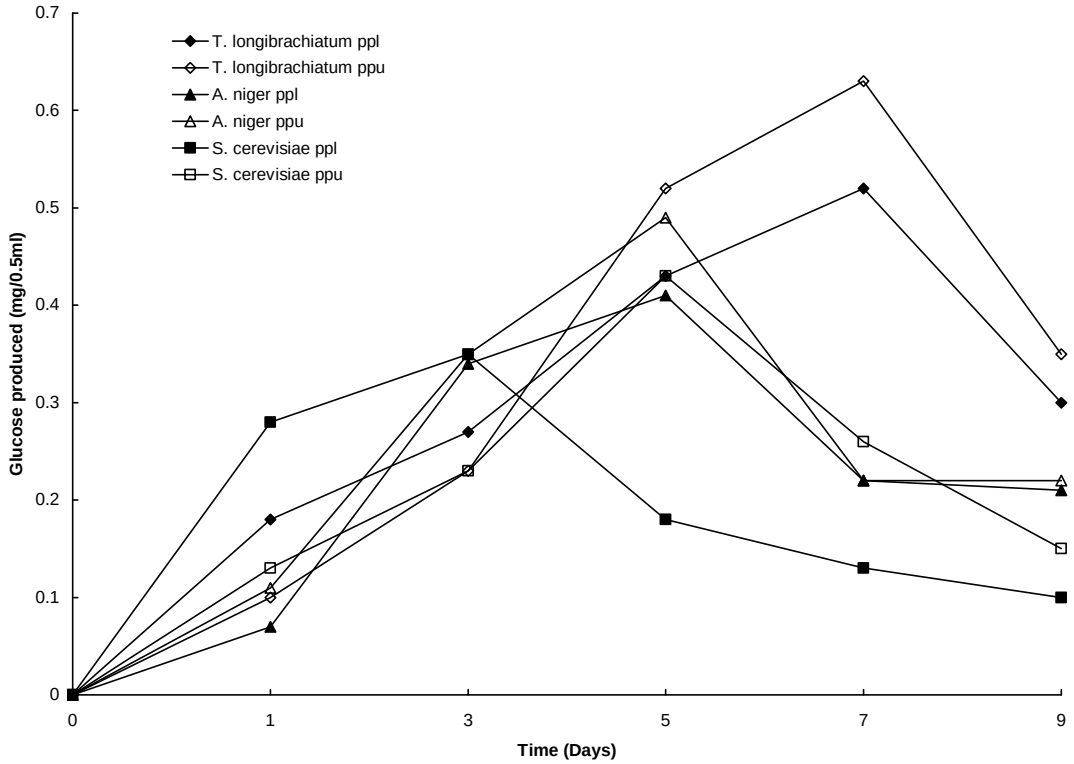

Figure 1. Effect of varying time on the fermentation of Pineapple waste substrates using test fungi.

Table 3. Effect of pH on the Fermentation of Pineapple waste by test fungi

WASTE SUBSTRATE	pH2.0	pH2.5	pH3.0	pH3.5	pH4.0	pH4.5	pH5.0	pH5.5	pH6.0
T. longibrachiatum									
Pineapple peel	0.200_e	0.178_e	0.208_d	0.200_e	0.242_c	0.312_f	0.296_f	0.210_e	0.212_e
Pineapple pulp	0.152_b	0.192_c	0.262_c	0.250_c	0.338_f	0.366_f	0.280_c	0.292_f	0.234_c
Aspergillus niger									
Pineapple peel	0.162_b	0.164_b	0.218_b	0.290	0.240	0.232_b	0.208_b	0.252	0.236_c
Pineapple pulp	0.184_b	0.170b	0.240	0.316	0.292	0.300	0.246	0.294	0.264
Saccharomyces cerevisiae									
Pineapple peel	0.146	0.146	0.150	0.198	0.158	0.198	0.188	0.142a	0.154
Pineapple pulp	0.146	0.152	0.168	0.216	0.188	0.248_b	0.210	0.214	0.234_b

(Fermentation conditions: Substrate concentration: 1 %, Temp: 29±1ºC, inoculum size: 10%)

Values are presented as mean.

All groups are compared to each other at $p<\alpha=0.05$.

Values with different subscripts are statistically different.

TABLE 4. Effect of Substrate Concentration on the Fermentation of Pineapple waste by test fungi

n	1%	2%	3%	4%	5%
SUBSTRATE					
Pineapple peel	0.280	0.288	0.338	0.314	5.874a
Pineapple pulp	0.296	0.314	0.360	0.360	0.334
Aspergillus niger					
Pineapple peel	0.208	0.288	0.250	0.256	0.268
Pineapple pulp	0.246	0.266	0.278	0.264	0.274
Saccharomyces cerevisiae					
Pineapple peel	0.188	0.262	0.308	0.286	0.314
Pineapple pulp	0.210	0.268	0.294	0.326	0.296

(Fermentation conditions: pH :5.0, Temperature : 29±1ºC

Values are presented as mean.

All groups are compared to each other at $p<\alpha=0.05$.

Values with different subscripts are statistically different.

TABLE 5. Effect of Inoculum size on the Fermentation of Pineapple waste by test fungi

WASTE SUBSTRATE	2%	4%	6%	8%	10%
Trichoderma longibrachiatum					
Pineapple peel	0.144	9.200_e	0.134	0.198	0.280
Pineapple pulp	0.136	0.140	0.192	0.166	0.296_a
Aspergillus niger					
Pineapple peel	0.124	0.216	0.302_a	0.288_a	0.208
Pineapple pulp	0.122	0.230	0.286_a	0.280	0.234
Saccharomyces cerevisiae					
Pineapple peel	0.232	0.228	0.284_a	0.264	0.188
Pineapple pulp	0.264	0.268	0.292_a	0.272	0.210

(Fermentation conditions: Temperature: 29±1ºC, pH: 5.0, Substrate concentration: 1%)

Values are presented as mean.

All groups are compared to each other at $p<\alpha=0.05$.

Values with different subscripts are statistically different.

TABLE 6**.** Effect of Temperature on the Fermentation of Pineapple waste by test fungi

WASTE SUBSTRATE	30ºC	35ºC	40ºC	45ºC
Trichoderma longibrachiatum				
Pineapple peel	0.280^{b}	0.242^{b}	0.300^{b}	0.368^{c}
Pineapple pulp	0.296^{b}	0.252^{b}	0.302^{b}	0.360^{c}
Aspergillus niger				
Pineapple peel	0.208^{b}	0.200^{a}	0.282^{b}	0.304^{c}
Pineapple pulp	0.246^{b}	0.206^{a}	0.328^{c}	0.324^{c}
Saccharomyces cerevisiae				
Pineapple peel	0.188	0.192	0.258^{a}	0.276^{a}
Pineapple pulp	0.210	0.214	0.270^{a}	0.296^{c}

Values are presented as mean.

All groups are compared to each other at $p<\alpha=0.05$.

Values with different subscripts are statistically different.

Table 7. Fermentation of Pineapple wastes using optimized fermentation parameters using the test fungi

WASTE SUBSTRATE	*T.longibrachiatum*	*A. niger*	*S. cerevisiae*
Pineapple peel	0.453_c	0.375_b	0.297_a
Pineapple pulp	0.565_d	0.375_b	0.323_a

Values are presented as mean.

All groups are compared to each other at $p<\alpha=0.05$.

Values with different subscripts are statistically different.

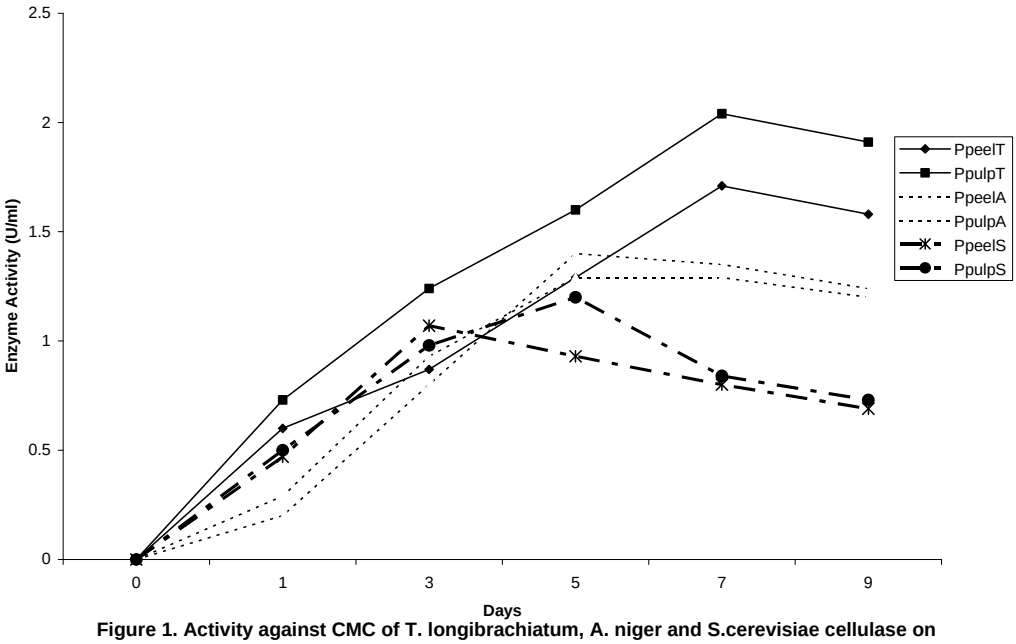

Figure 1. Activity against CMC of T. longibrachiatum, A. niger and S.cerevisiae cellulase on Pineapple Wastes

Figure 2.

The three test organisms are capable of producing cellulase as evidenced by the production of glucose (Table 1). Optimal glucose production from *T. longibrachiatum* was observed on Day 7 of fermentation, Day 5 for *A. niger* and Day 3 for pineapple peel and Day 5 for pineapple pulp for *S. cerevisiae*. Pineapple pulp released higher amount of glucose from the hydrolysis than pineapple peel (Table 2). Hydrolysis rates decline with time due to depletion of the more amorphous substrates, product inhibition and enzyme inactivation (Ghose, 1987). Caritas and Humphrey (2006) and Narasimha *et al*. (2006) also gave similar time course reports of maximum glucose yield on 5th day of fermentation using *A. niger*. Effect of pH on glucose production from the two waste substrates by the three microorganisms was shown on Table 3. This supports the findings of Lee *et al*. (2002) who reported that CMCase, Avicelase and FPase activities exhibit a pH optimum of approximately 4, while the pH optimum of β- glucosidase was between pH 5 and 6.

The effect of substrate concentration was shown on Table 4. Further increase in cellulose concentration beyond the level that gave the optimum glucose did not result in proportionate increase in glucose yield. Haapela *et al*., (1995) and Jeffries, (1996) reported that maximum endoglucanase activity was recovered on the medium with cellulose at 10g/l. Mandels and Reese (1959) also reported that maximal yields of cellulase were obtained on one percent substrate (cellulose, lactose, cellobiose and glucose) using *T. viride* and *Myrothecium verrucia.* These reports support the findings of this study as substrate concentration of 10g/L gave the highest amount of glucose from *T. longibrachiatum* on pineapple pulp.

Since the substrates contain different minerals apart from carbon which may serve as nutrient supplements, increase in substrate concentration leads to increase in these nutrients which may adversely affect the cell concentration. The increase in glucose production until the optimum that was obtained was due to the availability of cellulose in the medium; while a decrease in production beyond optimum concentration is explained to be as a result of an inhibitory effect of accumulated cellobiose and cellodextrins of low degree of polymerization to the growth medium. It might also be due to the specific binding of the enzymes with the substrates (Gilkes, *et al*., 1984). Low glucose production after optimum very probably highlights sugar depletion from the substrates into the medium (Brien and Craig, 1996).

Effect of inoculum size was shown on Table 5. Decrease in amounts of glucose production resulted at inoculum sizes above 6% and 8% for fermentations using *A. niger*. This decrease in glucose production with further increase in inoculum might be due to clumping of cells which could have reduced sugar and oxygen uptake rate and also, enzyme release (Srivastava, *et al*., 1987). Effect of Temperature was shown on Table 6. The optimum temperature for the synthesis of enzymes for saccharification of agrowaste in all cases to enzymatic hydrolysis can be attributed to lignin content of the material. Pretreatment of lignocellulosic material enhances enzyme activity and maximum saccharification was achieved within the range 30-45^{o}C coinciding with the characteristics of mesophiles (Baig *et al*., 2004).

Optimum glucose from the waste substrates using *T. longibrachiatum,*was produced at 10% inoculum size at 45ºC on Day7 but at pH 5.0 and 3% substrate concentration for pineapple peel and pH 4.5 and 1% substrate concentration for pineapple pulp.

Optimum glucose from the waste substrates using *A. niger* was produced at pH 3.5, 2% substrate concentration, 6% inoculum size on Day5 but at 45ºC for pineapple peel and 40ºC for pineapple pulp.

Optimum glucose from the waste substrates using *S. cerevisiae* was produced at pH 3.5, 3% substrate concentration, 6% inoculum size, 45ºC on Day3 for pineapple peel and pH 4.5, 4% substrate concentration, 2% inoculum size and 45ºC on Day5 for pineapple pulp.

These optimal conditions were combined in single fermentations for each organism and cellulase activity was measured (figure 1). Cellulase activity from pineapple peel was 1.71U/ml when hydrolyzed by *T. longibrachiatum*, 1.29 U/ml when hydrolyzed by *A. niger* and 1.07U/ml when hydrolyzed by *S. cerevisiae.* Cellulase activity from pineapple pulp was 2.04U/ml when hydrolyzed by *T. longibrachiatum*, 1.40U/ml when hydrolyzed by *A. niger* and 1.20U/ml when hydrolyzed by *S. cerevisiae*. Cellulase activity of *S. cerevisiae* was lowest. The exo-β-1, 3-glucanases produced by *S. cerevisiae* yield glucose as the end product, whereas endo-β-1, 3-glucanase releases a mixture of oligosaccharides with glucose as the minor product. Because β-1, 3-glucan is the main structural polysaccharide responsible for the strength and rigidity of the yeast cell wall, β-1, 3-glucanases have been suggested to play a role in important morphogenetic processes involving the controlled autolysis of β-1, 3 glucan. During vegetative growth, several endo- and exo-1, 3- β- glucanases are synthesized, some of which are secreted only to remain entrapped in the cell wall whereas others are released to the surrounding medium (Lee *et al*., 2002).

In conclusion, this study revealed that pineapple peel and pulp, which are examples of domestic and industrial agro-wastes, produce large amounts of cellulase enzymes when hydrolyzed by cellulolytic microorganisms and instead of being left behind for natural degradation can be utilized effectively under these conditions, to produce cellulase.

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