ศักยภาพในการผลิตเอนไซม์กลูโคอะไมเลสและเซลลูเลสจากเชื้อผสม *Aspergillus niger* TISTR 3254 และ *Trichoderma reesei* TISTR 3081

Potential of Glucoamylase and Cellulase Production Using Mixed Culture of *Aspergillus niger* TISTR 3254 and *Trichoderma reesei* TISTR 3081

กรองจันทร์ รัตนประดิษฐ์ (Krongchan Ratanaphadit) [1]*
ขวัญใจ แก้วจันทร์ (Kwanjai Kaewjan)[1]
สมจิตต์ ปาละกาศ (Somchit Palakas)[2,3]

บทคัดย่อ

การศึกษาศักยภาพของการผลิตเอนไซม์กลูโคสอะไมเลสและเซลลูเลสจากการเลี้ยงเชื้อแบบผสมของ *Aspergillus niger* TISTR 3254 และ *Trichoderma reesei* TISTR 3081 โดยเลี้ยงในอาหารประยุกต์สูตร Mandel ที่ใช้กากมันสำปะหลังแห้งร้อยละ 1-3 เป็นแหล่งคาร์บอนทดแทน CMC ร่วมกับการใช้แหล่งไนโตรเจนเดี่ยว ร้อยละ 1 (แอมโมเนียมซัลเฟต ยูเรียหรือแป้งถั่วเหลือง) จากการทดลองทำการเลี้ยงแบบอาหารเหลว บนเครื่องเขย่าที่ความเร็ว 150 รอบต่อนาที ที่อุณหภูมิห้อง เป็นเวลา 5 วัน พบว่า การเจริญของเชื้อผสม *Aspergillus niger* TISTR 3254 และ *Trichoderma reesei* TISTR 3081 ที่เลี้ยงด้วยกากมันสำปะหลังร้อยละ 2 ร่วมกับแอมโนเนียมซัลเฟตร้อยละ 1 ให้ค่ากิจกรรมเอนไซม์สูงที่สุด โดยมีค่ากิจกรรมของเอนไซม์กลูโคสอะไมเลส เท่ากับ 7,734 ± 0.84 ยูนิตต่อมิลลิลิตร และค่ากิจกรรมเอนไซม์ย่อยเซลลูโลสเท่ากับ 5.76 ± 0.09 (FPase) และ 27.96 ± 0.44 (CMCase) ยูนิตต่อมิลลิลิตร ตามลำดับ

Abstract

Production of glucoamylase and cellulase using mixed culture of *Aspergillus niger* TISTR 3254 and *Trichoderma reesei* TISTR 3081 as well as potential of using cassava bagasse as carbon source for enzymes production were investigated. Culture media comprising of cassava bagasse (1-3%) as carbon source instead of CMC supplemented with 1% of selected nitrogen source (ammonium sulphate, urea or soy bean meal) were used. Experiments under submerge fermentation condition were conducted at room temperature and under shaking speed of 150 rpm during 5 days. The results showed that mixed culture of *Aspergillus niger* TISTR 3254 and *Trichoderma reesei* TISTR 3081 grown in culture medium containing of 2%

[1] *Department of Biotechnology, Faculty of Sciences, Burapha University, Chonburi, 20131, Thailand*
[2] *Department of Applied Radiation and Isotopes, Faculty of Science, Kasetsart University, Bangkok, 10900, Thailand*
[3] *Gamma Irradiation Service and Nuclear Technology Research Centre, Kasetsart University, Bangkok, 10900, Thailand*
* *Corresponding author, e-mail: k_ratanapradit@yahoo.com*

cassava bagasse and 1% ammonium sulphate exhibited the highest enzyme activity. Glucoamylase enzyme activity of 7,734 ± 0.84 U ml^{-1} and cellulolytic enzyme activities of 5.76 ± 0.09 (FPase) and 27.96 ± 0.44 (CMCase) U ml^{-1} were obtained.

คำสำคัญ: กากมันสำปะหลัง *Trichoderma reesei* *Aspergillus niger*

Keywords: Cassava bagasse, *Trichoderma reesei*, *Aspergillus niger*

Introduction

Agro-industrial residues such as cassava bagasse, sugar cane bagasse, and pineapple pomace, etc. are alternative low cost raw materials for value added production of microbial derived bio-products such as single cell protein, amino acid, ethanol, organic acid, and some specific enzymes (Pandey et al., 2000). Cassava bagasse is a main residue from tapioca production. It contains 50-70% (dry basis) of starch and 20-30% (dry basis) of fibers (Pandey et al., 2000; Sriroth et al., 2000). The use of cassava bagasse in enzyme production will benefit not only the value adding of agro-industrial waste but also the reduction of water pollution from tapioca factory.

In enzyme industry, *Trichoderma reesei* and *Aspergillus niger*, are the high potential funguses that have been in use to produce many extracellular enzymes. *Trichoderma reesei* can produce cellulolytic enzymes comprising of endoglucanase and exoglucanase as well as other enzymes including lignolytic enzyme, pectinolytic enzyme and hemicellulase. *Aspergillus niger* is particularly used not only in cellulolytic enzyme production, endoglucanase, but also in the production of amylolytic enzyme such as amylase and glucoamylase as well as pectinolytic enzyme.

Several groups have reported the use of these funguses in degradation of raw material containing cellulose, starch, hemicellulose, pectin and lignocellulose both in form of microbial degradation and utilization of enzyme produced from both funguses (Gutierrez-Correa et al., 1999; Pandey et al., 2000; Howard et al., 2003; Olsson et al., 2003; Liming and Xueliang, 2004; Juhasz et al., 2005). However, capability of microorganisms in production of specific enzyme is different due to various factors especially carbon and nitrogen sources that can affect the production of different enzymes (Chávez et al., 2004)

The current work aims to investigate the ability of *Aspergillus niger* TISTR 3254 and *Trichoderma reesei* TISTR 3081 in production of glucoamylase and cellulolytic enzymes under submerged mixed culture condition. In order to minimize problem associated with disposal of an agro-industrial residue, cassava bagasse from tapioca industry was used as carbon source in modified Mandel culture medium instead of carboxymethyl cellulose (CMC).

Materials and methods

Microorganism strains

Aspergillus niger TISTR 3254 and *Trichoderma reesei* TISTR 3081 were obtained from Thailand Institute of Scientific and Technological Research (TISTR), Pratumthanee province, Thailand. The organisms were maintained on potato dextrose agar at 4 °C and were sub-cultured monthly.

Substrate

Dry Cassava bagasse was a generous gift from Cho-Chaiwat Industry Co. Ltd., Chonburi province, Thailand. CMC was purchased from Fluka (Finland).

Culture medium and cultivation

Mandel culture medium (Mandel & Weber, 1969) containing 7.5 g/l of CMC, urea (0.3 g/l), $(NH_4)_2SO_4$ (1.4 g/l), peptone (0.75 g/l), yeast extract (0.25 g/l), KH_2PO_4 (2.0 g/l), $CaCl_2.2H_2O$ (0.4 g/l), $MgSO_4.7H_2O$ (0.3 g/l), $FeSO_4.7H_2O$ (5 mg/l) $MnSO_4.4H_2O$ (1.6 mg/l), $ZnSO_4.7H_2O$ (1.4 mg/l) and $CoCl_2.6H_2O$ (20 mg/l) was used as control medium.

Preliminary experiments were carried out in order to evaluate the capability of both funguses to use cassava bagasse as carbon source for production of glucoamylase and cellulolytic enzymes. For each fungal strain, stock culture was prepared on potato dextrose agar at room temperature during 7 days. Afterwards, spores were harvested and suspended in sterile solution of 0.9% NaCl (w/v). Subsequently, 107 spores/ml were inoculated to 500 mL flask containing 150 mL of either Mandel culture medium or modified Mandel culture medium. In Mandel medium, 7.5 g/l of CMC was used as carbon source meanwhile the same amount of cassava bagasse was used in modified medium. The inoculated flasks were then incubated on rotary shaker at room temperature with the shaking speed of 150 rpm during 5 days.

In optimization experiments, carbon and nitrogen sources in the modified Mandel culture medium were considered. Cassava bagasse with different concentrations (1-3%) was used as carbon source instead of 7.5 g/l of CMC. Conventional mixed nitrogen sources in Mandel medium comprising of urea (0.3 g/l), $(NH_4)2SO_4$ (1.4 g/l), peptone (0.75 g/l) and yeast extract (0.25 g/l) were replaced by 1% (w/v) of selected single nitrogen source, namely, ammonium sulphate, urea or soybean meal.

Enzyme assays

Endoglucanase activity was determined in the form of carboxymethyl cellulase activity (CMCase activity) as described by Mandel (1975). Exoglucanase was determined in the form of filter paper activity (FPase activity) as described by Ghose (1987). One unit of CMCase and FPase was defined as the amount of enzyme that released 1 μmol of glucose equivalent per minute under assay condition.

Glucoamylase activity was determined by dinitrosalicylic acid method of Miller (1959). One unit of glucoamylase was defined as the amount of enzyme necessary to produce reducing sugars equivalent to 1 μmol of reducing sugar as glucose per minute at 40 °C.

Results and Discussion

The mono-culture under submerged fermentation condition was carried out in order to examine the feasibility of using cassava bagasse as carbon source for production of glucoamylase and cellulolytic enzymes. Experiments were performed using Mandel medium in control experiments comparing with the using of modified medium containing 7.5 g/l of cassava bagasses as carbon source instead of CMC. It was found that in the presence of cassava bagasse, *Aspergillus niger* TISTR 3254 produced 11.60±1.14, 2.65±0.09 and 0.26±0.01 U ml^{-1} of glucoamylase, CMCase and FPase, respectively, while the control experiment gave lower enzyme production with the respective values of 7.84±0.12, 1.04±0.04 and 0.19±0.08 U ml^{-1}. For *Trichoderma reesei* TISTR 3081, the culture in the presence of cassava bagasse produced lower glucoamylase and CMCase than *Aspergillus niger*

TISTR 3254 (5.42±1.12 and 0.94±0.15 U ml^{-1}) with a similar value of exoglucanase (0.32±0.06 U ml^{-1}), while those of control were 0.89±0.33, 1.26±0.20 and 0.43±0.08 U ml^{-1}. This result indicated the ability of both funguses to use cassava bagasse as carbon source for production of glucoamylase and cellulolytic enzymes even with slightly production rate. However, the preliminary experiments were further carried out using 1:1 mixed culture of *Aspergillus niger* TISTR 3254 and *Trichoderma reesei* TISTR 3081.

During 96 hours of culture, when cassava bagasse was used as carbon source, both funguses produced high glucoamylase activities with a value of 981.87 ± 0.17 U ml^{-1} or about 4.4-fold of control as shown in Table 1. Regarding cellulases production, using cassava bagasse as carbon source gave lower yield for both endoglucanase in form of CMCase and exoglucanase in form of FPase than those of using carboxymethyl cellulose with approximately 8 times and 21 times, respectively. The results showed that mixed culture of both funguses in different carbon source yielded higher endoglucanase activities than exoglucanase activities. When cassava bagasse was used as carbon source, the high yielding of glucoamylase and low yielding of cellulases were observed. This might due to the fact that cassava bagasse contains high starch content of about 50-70% dry weight with lower cellulose content of about 20-30% dry weight (Panday et al., 2000). In fact, low cellulose content can limit the production of cellulases.

Table 1. Glucoamylase and cellulase enzyme activities produced by mixed fungi culture of *Aspergillus niger* TISTR 3254 and *Trichoderma reesei* TISTR 3081

Carbon source	**Enzyme Activity (U ml^{-1})**		
	Glucoamylase	**CMCase**	**FPase**
Cassava bagasses	981.87±0.17	0.19±0.06	0.03±0.01
Caboxymethyl cellulose	225.36±0.05	1.58±0.04	0.63±0.1

After preliminary study of using cassava bagasse as carbon source for production of glucoamylase and cellulase, its optimum concentration in modified Mandel culture medium has been determined using 1-3% cassava bagasse instead of 7.5 g/l as usual. Figure 1 illustrates the time behaviours of glucoamylase, endoglucanase and exoglucanase activities. The results manifest that mixed culture of *Aspergillus niger* TISTR 3254 and *Trichoderma reesei* TISTR 3081 in modified Mandel medium containing 2% (w/v) of cassava bagasse yielded highest glucoamylase activities of 5,029.22 ± 0.74 U ml^{-1} at 72 hours while similar values of 3,146.46 ± 4.39 U ml^{-1} at 72 hours and 3,063.43 ± 0.06 U ml^{-1} at 48 hours were observed when 1% and 3% of cassava bagasse were used, respectively (Figure 1A). For cellulolytic enzymes, mixed fungi culture of *Aspergillus*

niger TISTR 3254 and *Trichoderma reesei* TISTR 3081 in culture medium containing 2% of cassava bagasse exhibited highest endoglucanase (CMCase) of 63.42 ± 0.90 U ml^{-1} at 120 hours (Figure 1B), meanwhile, highest exoglucanase (FPase) of 6.23 ± 0.61 U ml^{-1} was observed at 96 hours when medium with 3% cassava bagasse was used (Figure 1C). It should be noticed that when concentration of cassava bagasse in culture medium was increased from 7.5 g/l (0.75%) to 2% (w/v), production of enzymes by mixed fungi culture were significantly increased not only for glucoamylase (more than 5 times) but also for endoglucanase (more than 300 times) and exoglucanase (more than 200 times). This result elicits again an important role of starch and cellulose as substrates for enzyme production (Panday et al., 2000). Lower production of all considering enzymes observed in the presence of 1% cassava bagasse may directly relate to inadequate carbon source for fungal growth and enzyme production in comparing with higher cassava bagasse content. While lower production of glucoamylase and endoglucanase found in the presence of 3% cassava bagasse may relate to medium texture that can cause the growth and enzyme production potential of funguses. In the presence of 3% cassava bagasse, culture medium was sticky and viscous, moreover, the majority partition of starch and small solid cellulosic residues were found at the bottom and the top of medium, respectively. The sticky and viscous of medium may reduce oxygen transfer rate in submerge fermentation and lead to reduction of fungal growth and enzyme production as ever reported by Jin et al. (1999). In contrast with glucoamylase and endoglucanase, the highest exoglucanase production was observed in the presence of 3% cassava bagasse. This latter result may relate to the increasing of substrate that can promote enzyme production (Reczey et al., 1996). Moreover, in the presence of 3% cassava bagasse, the solid cellulosic particles floated on the top of medium. This localization of solid cellulosic particles in medium can lead to the reduction of oxygen transfer rate but it is not significantly influence exoglucanase production.

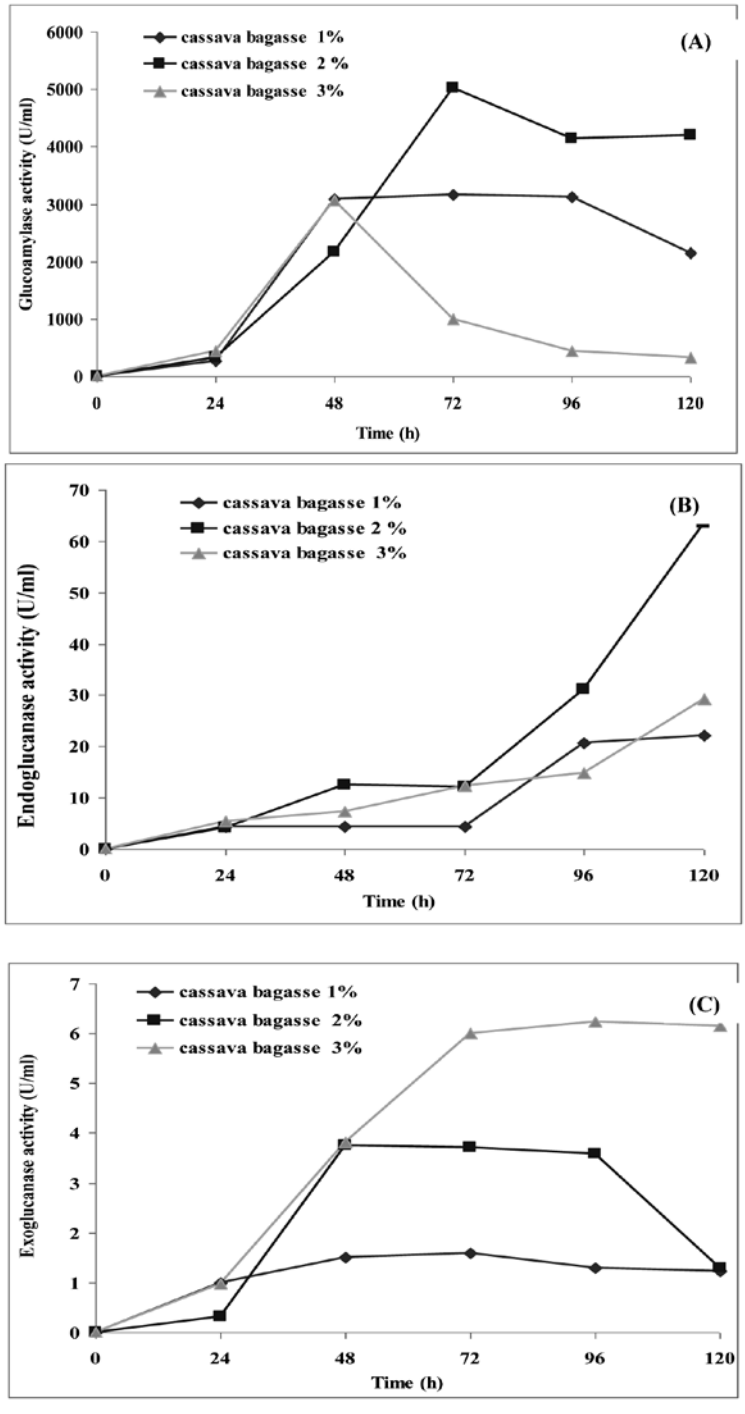

Figure 1. Effect of different cassava bagasse concentration on production of glucoamylase (A) endoglucanase (B) and exoglucanase (C) by mixed fungi culture of *Aspergillus niger* TISTR 3254 and *Trichoderma reesei* TISTR 3081 under submerged fermentation in modified Mandel medium.

Besides carbon source, nitrogen source used in culture medium take also an important role in growth of microbial cell mass and production of microbial metabolite products including various enzymes. In this study, optimization of nitrogen sources for mixed culture of both funguses was conducted using modified Mandel medium containing 2% (w/v) cassava bagasse as carbon source in stead of 7.5 g/l of CMC and 1% (w/v) of selected single nitrogen source, ammonium sulphate, urea or soy bean meal was used to replace mixed nitrogen source normally used in Mandel medium.

Effect of different nitrogen source on glucoamylase, endoglucanase and exoglucanase activities are illustrated in Figure 2. When ammonium sulphate was used as nitrogen source, the organisms produced highest glucoamylase activities of 7,734.21 ± 0.84 U ml^{-1} at 96 hours followed by the using of soy bean meal (5,613.01 ± 1.06 U ml^{-1} at 96 hours) and urea (2,701.57 ± 3.15 U ml^{-1} at 48 hours), respectively. Similarly, culture in the presence of ammonium sulphate gave highest endoglucanase and exoglucanase with respective values of 27.96 ± 0.44 U ml^{-1} at 96 hours and 5.76 ± 0.09 U ml^{-1} at 72 hours. The less appropriated nitrogen source for endoglucanase production were soy bean meal and urea, respectively, meanwhile, those for exoglucanase production were urea and soy bean meal, respectively. The lowest exoglucanase activities in the presence of soy bean meal as nitrogen source observed in this study is incoherent with the works of Gutierrez-Correa et al. (1999) which production of cellulase by mixed culture of *Aspergillus niger* ATCC 10864 with either wild type or mutant of *Trichoderma reesei* LM-UCA4 using sugar cane bagasse as carbon source and soy bean meal as nitrogen source exhibit higher enzyme activities in comparing with using of urea and ammonium sulphate as nitrogen source. However, the same manner that observed both in this work and that of Gutierrez-Correa et al. (1999) is, culture of mixed funguses in the presence of organic nitrogen source like soy bean meal could reduced time for maximum enzyme production in comparing with the culture in the presence of inorganic nitrogen sources as urea and ammonium sulphate.

The results found in this work underlines the fact that cassava bagasse is a potent carbon source for microbial production of bio-products (Panday et.al., 2000), moreover, both organisms used in this work are able to produce cellulase and glucoamylase (Rattanachomsri et al., 2009; Sriroth et al., 2000).

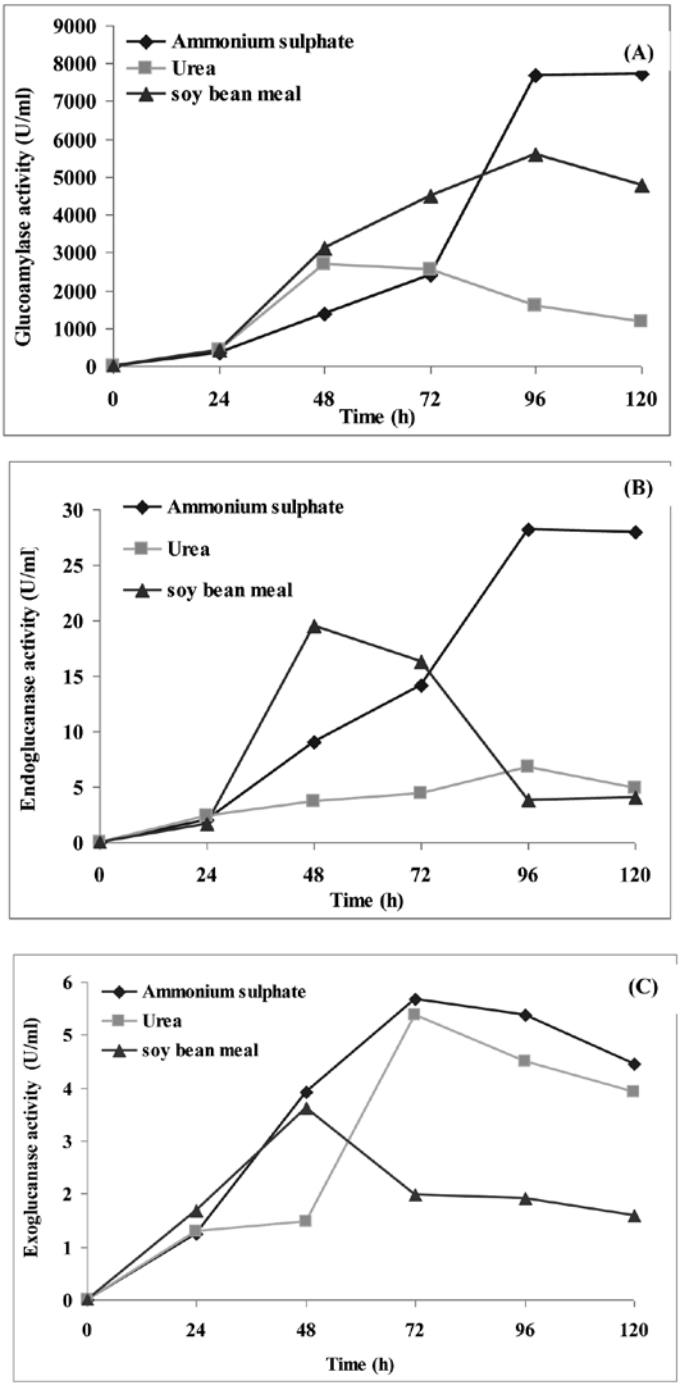

Figure 2. Effect of different nitrogen source on production of glucoamylase (A) endoglucanase (B) and exoglucanase (C) by mixed culture of *Aspergillus niger* TISTR 3254 and *Trichoderma reesei* TISTR 3081 under submerged fermentation in modified Mandel medium with 2 % cassava bagasse supplemented with selected nitrogen source.

In addition, the results indicate the simultaneous production of all considering enzymes, as already observed for several enzyme production both with single culture (Kovács et al., 2008; Omojasola and Jilani, 2008; Rattanachomsri et al., 2009; Sohail et al., 2009) and mixed culture of fungi (Wen, Liao and Chen, 2005).

Furthermore, when time of enzyme production is considered, it should be remarked that, in submerge mixed culture in the presence of cassava bagasse as carbon source, *Aspergillus niger* TISTR 3254 and *Trichoderma reesei* TISTR 3081 have to take a relatively long time for production of glucoamylase (72 to 96 hours), endoglucanase (120 hours) and exoglucanase (72 hours). These results agree with several works on microbial production of α-amylase and glucoamylase (Chávez et al., 2004), exoglucanase in FPase form and β-glucosidase (Kovács el al., 2008) as well as endoglucanase (Chandra et al., 2009).

Conclusion

In this research, microbial hydrolysis of cassava bagasse for production of glucoamylase and cellulase by mixed fungi culture has been investigated. Submerged mixed culture of *Aspergillus niger* TISTR 3254 and *Trichoderma reesei* TISTR 3081 in modified Mandel medium containing 2% of cassava bagasse as carbon source supplemented with 1% of ammonium sulphate as nitrogen source exhibited the highest glucoamylase enzyme activity of 7,734 ± 0.84 U ml-1 including cellulytic enzyme activities of 5.76 ± 0.09 (FPase) and 27.96 ± 0.44 (CMCase) U ml-1. The results convince that cassava bagasse is a suitable carbon source for production of glucoamylase and cellulases by submerged fermentation by mixed fungi culture of *Aspergillus niger* TISTR 3254 and *Trichoderma reesei* TISTR 3081, however, in order to improve enzyme production and feasibility of industrial application, optimum conditions of temperature, pH and even the use of raw cassava bagasse are needed to further investigation.

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