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Effects of glucose as carbon catabolite repressor on alpha-amylase and glucoamylase production in Indonesian indigenous fungi

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ABSTRACT

Aims: The study aimed to investigate the effect of glucose on alpha-amylase and glucoamylase production in some Indonesian indigenous fungi.
Methodology and results: Fungi were screened for their ability to produce alpha-amylase and glucoamylase in the presence of glucose. The strains were grown in a medium containing starch and glucose as carbon sources with glucose concentrations varying from 0 to 5% for four days, and the alpha-amylase and glucoamylase were analyzed at the end of the growth period. Most strains showed repression on the amylases production when glucose was added to the medium. However, some strains showed no repression on amylases production when glucose was supplemented to the medium. The addition of glucose repressed glucoamylase production, but no repression on alpha-amylase was noted for strain KKB4, vice versa, there was repression on alpha-amylase production but no repression on glucoamylase production for strain FIG1. Strains FNCC 6151 and MLT1J1 showed no repression on both alpha-amylase and glucoamylase production when glucose was added to the medium up to 5%. The occurrence of repression in the production of alpha-amylase and glucoamylase was strain-specific.
Conclusion, significance and impact of study: Out of the nine indigenous fungi strains examined, strains FNCC 6151 and MLT1J1 showed no repression on both alpha-amylase and glucoamylase production when glucose was added to the medium up to 5%. Those two strains have the potential to be improved further to produce both alpha-amylase and glucoamylase.

Keywords: Alpha-amylase, glucoamylase, indigenous fungi, glucose, repression

INTRODUCTION

Amylases consist of 3 enzymes, namely alpha-amylase, beta-amylase and glucoamylase, which cleave alpha-1,4 glycosidic bonds of starch. Alpha-amylase is an enzyme that hydrolyses starch randomly at the middle starch molecule and produces at least maltotriose. Beta-amylase is an enzyme that hydrolyses starch from the non-reducing end of every two glucose monomers to produce maltose. Glucoamylase is an enzyme that hydrolyses starch from non-reducing ends to produce glucose (Sundarram and Murthy, 2014). Amylases are important industrial enzymes accounting for 25% of the industrial enzyme market (Sindhu *et al.*, 2017). Amylase as a catalyst has a vital role in various food industries such as corn, maltose and glucose syrups, juice clarification, alcohol fermentation, bread making and as a food additive (Mojsov, 2014).

Bacteria and fungi produce amylases and the enzyme produced by fungi due to hyphae's morphology can grow well, tolerate low water activity (a_w), high osmotic pressure and has the most efficient substrate bioconversion (Singh *et al.*, 2014). Fungi are naturally found in many sources with uncomplicated nutritional requirements (Saranraj and Stella, 2013). The fungi that produce many amylases are *Aspergillus* (Veerapagu *et al.*, 2016), *Aspergillus oryzae* (Avwioroko *et al.*, 2018), *A. niger* (Obafemi *et al.*, 2017), *Rhizopus oryzae* (Ferreira *et al.*, 2015), *Trichoderma harzianum* (Mohamed *et al.*, 2011), *A. nidulans* and *Penicillium* (Saleem and Ebrahim, 2014).

In many cases, carbon catabolite repression (CCR) affects enzymes production (Magasanik, 1961), i.e., glucose as the end product of starch hydrolysis will repress the amylases production. CCR is part of a series of carbon metabolism regulations responsible for

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adaptation to environmental and physiological changes for energy efficiency (Adnan *et al.*, 2018). Previous studies reported that the effect of glucose acting as a carbon catabolite repressor was significant (New *et al.*, 2014; Kayikci and Nielsen, 2015; Adnan *et al.*, 2018). The effect of glucose on amylase production has also been widely reported (Jensen *et al.*, 2002; de Oliveira Santos and Martins, 2003; Varalakshmi *et al.*, 2009; Kayikci and Nielsen, 2015). However, fermentation with solid-state cultivation has a different effect; the addition of glucose can induce the production of alpha-amylase and glucoamylase (Carrillo-Sancen *et al.*, 2016).

CCR presents a disadvantage during the production of amylases. This CCR should be eliminated in the strain used in commercial amylases production. Therefore, the presence of CCR in the production strains should be determined first and destroyed if necessary. The effort to eliminate the effects of repression by glucose has been carried out through physical and chemical mutations (Haq *et al.*, 2014; Wang *et al.*, 2016; Aleem *et al.*, 2018) and genetic engineering (Todd *et al.*, 2000; Hunter *et al.*, 2013; Ichinose *et al.*, 2014). As a tropical country with abundant biodiversity, Indonesia has 64,000 fungal isolates, of which 881 have been studied (Elizabeth *et al.*, 2014). In this study, several Indonesian indigenous fungi were examined for the presence of CCR in amylase production. Therefore, this study aimed to investigate the effect of glucose on alpha-amylase and glucoamylase activity in some Indonesian indigenous fungi.

MATERIALS AND METHODS

Indigenous fungi

Indigenous fungi used in this study were isolated from various sources (Table 1).

Effect of glucose on the production of amylase

The effect of glucose on the production of amylase was studied in a plate and liquid media. The strains were grown in an agar plate containing Mandel's mineral salt solution (Mandels *et al.*, 1974), 10 g/L starch, 0.1% (v/v) Triton X-100 and 17.5 g/L agar. The medium was supplemented with 0%, 1%, 3% and 5% glucose as a repressor. The pH was adjusted to 4.8 and the medium was sterilized at 121 °C for 15 min. The strains were inoculated on the agar plates and incubated at 28 °C for three days. The plates were further incubated at 50 °C for 18 h to accelerate the activities of the amylases produced and hence to make the clear zones more obvious (Sadhasivam *et al.*, 2018; Fadhil *et al.*, 2020). The plates were stained using 1% iodine solution. Then, the diameters of the entire clear zone and the fungal colony were measured. The clear zone was a ratio between the diameter of the entire clear area and the diameter of the fungal colony.

The preparation of a liquid medium was similar to the solid one, except there was no addition of agar and Triton X-100. The liquid medium was supplemented with 0%, 1%, 3% and 5% glucose. The medium was inoculated with the strain at 1×10^6 spores/mL and incubated at 30 °C with shaking for four days. The culture was centrifuged at 21,000× *g*, 4 °C for 10 min and the supernatant was analyzed for alpha-amylase and glucoamylase activities (Chand *et al.*, 2005). The pellet was washed with distilled water and dried at 105 °C for 24 h to obtain the dry biomass weight produced during fungal growth (Zangirolami *et al.*, 2002).

Enzymatic assay

The alpha-amylase activity was analyzed using an alpha-amylase assay kit (K-CERA) from Megazyme International Ireland Ltd, Ireland. The substrate used was *p*-nitrophenyl maltoheptaoside, where its non-reducing end was blocked. The action of alpha-amylase on the substrate was followed by the cleavage by glucoamylase and alpha-glucosidase, which was an integral part of the substrate mixture to release glucose and *p*-nitrophenol. The free *p*-nitrophenol was measured by a spectrophotometer at 400 nm. One unit of alpha-amylase is defined as the amount of enzyme required to release one micromole of *p*-nitrophenol per minute.

Glucoamylase activity was analyzed using amyloglucosidase assay reagent (R-AMGR3) from Megazyme International Ireland Ltd, Ireland. The reagent contains *p*-nitrophenyl, β-maltoside and β-glucosidase. The action of glucoamylase on the substrate was followed by the cleavage of β-glucosidase to release glucose and *p*-nitrophenol. The free *p*-nitrophenol was measured by a spectrophotometer at 400 nm. One unit of glucoamylase is defined as the amount of enzyme required to release one micromole of *p*-nitrophenol per minute.

Statistical analysis

The experiment was statistically analyzed through the One-way ANOVA method by Duncan Multiple Range tests (DMRT) at 99% of the significance level. This analysis was performed using SPSS v.25 (SPSS Inc., USA).

RESULTS AND DISCUSSION

Effect of glucose on amylase production in plate medium

When the fungal strain grew in plate medium and produced amylases, the starch as the sole carbon source was hydrolyzed to oligosaccharides or simple sugars and gave no colour when stained with iodine solution. It appeared as a clear zone surrounding the colony. The starch hydrolysis was due to the action of amylases that might be produced by the strain, i.e., alpha-amylase, beta-amylase and glucoamylase. All colonies of the strains showed clear zones on medium without glucose supplementation (Figure 1). However, when they were grown on a medium supplemented with glucose, most of the clear zones formed, expressed as the ratio between

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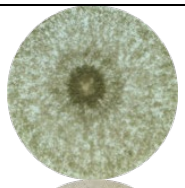
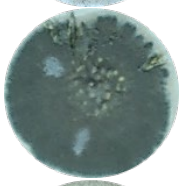
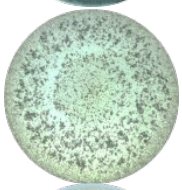
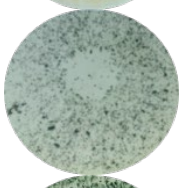
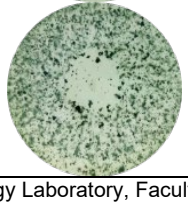
Table 1: List of Indonesian indigenous fungi.

No.	Indigenous fungi		Origin	Coordinate
1	*Aspergillus tamarii* FNCC 6151		Koji, Bantul, Yogyakarta	-7°53'17.02" S 110°19'44.00" E
2	*Aspergillus oryzae* KKB4*		Koji, Kebumen	7°40'34.284" S 109°39'49.316" E
3	*Aspergillus aculeatus* FIG1*		Cacao, Gunungkidul	-7°58'59.99" S 110°37'0.01" E
4	*Rhizopus oryzae* GAP1*		Fermented cassava, Sleman	-7°42'56.02" S 110°21'20.02" E
5	*Penicillium citrinum* G2J2*		Oil palm empty fruit bunches, Garut	7°13'40.4616" S 107°54'31.3164" E
6	*Trichoderma asperellum* PK1J2*		Oil palm empty fruit bunches, Pekanbaru	0°30'37.5840" N 101°26'17.9124" E
7	*Trichoderma asperellum* MLT1J1*		Rotten Coconut Husk, Central Maluku	-3°17'30.01" S 128°58'3.00" E
8	*Trichoderma asperellum* MLT3J2*		Rotten Coconut Petioles, Central Maluku	-3°17'30.01" S 128°58'3.00" E
9	*Trichoderma asperellum* MLT5J1*		Soil in Wotay, Central Maluku	-3°17'30.01" S 128°58'3.00" E

*Strains were a collection of Biotechnology Laboratory, Faculty of Agricultural Technology, Gadjah Mada University.

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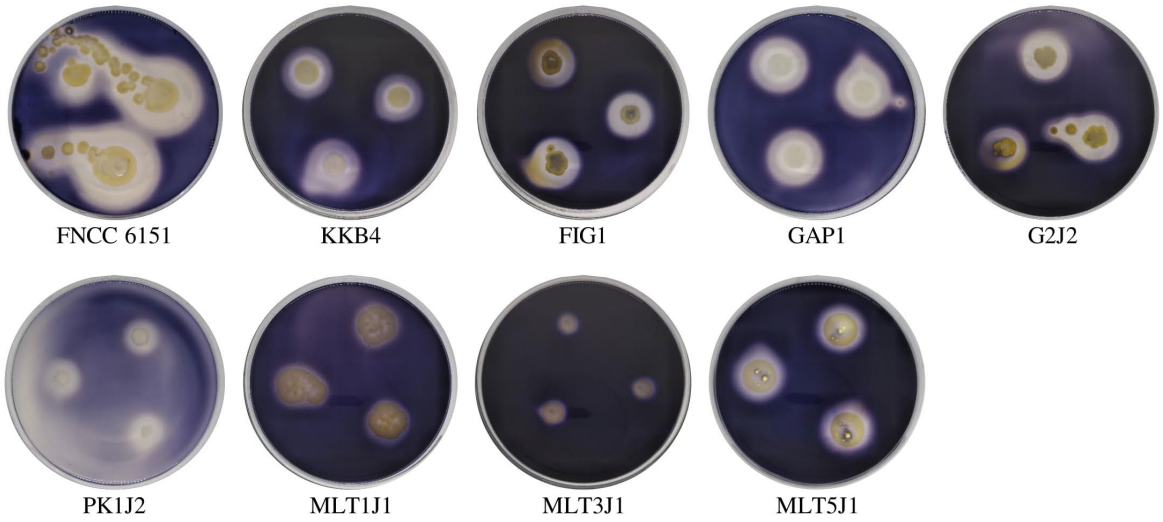

Figure 1: Clear zone appearance of nine indigenous fungi without glucose supplementation.

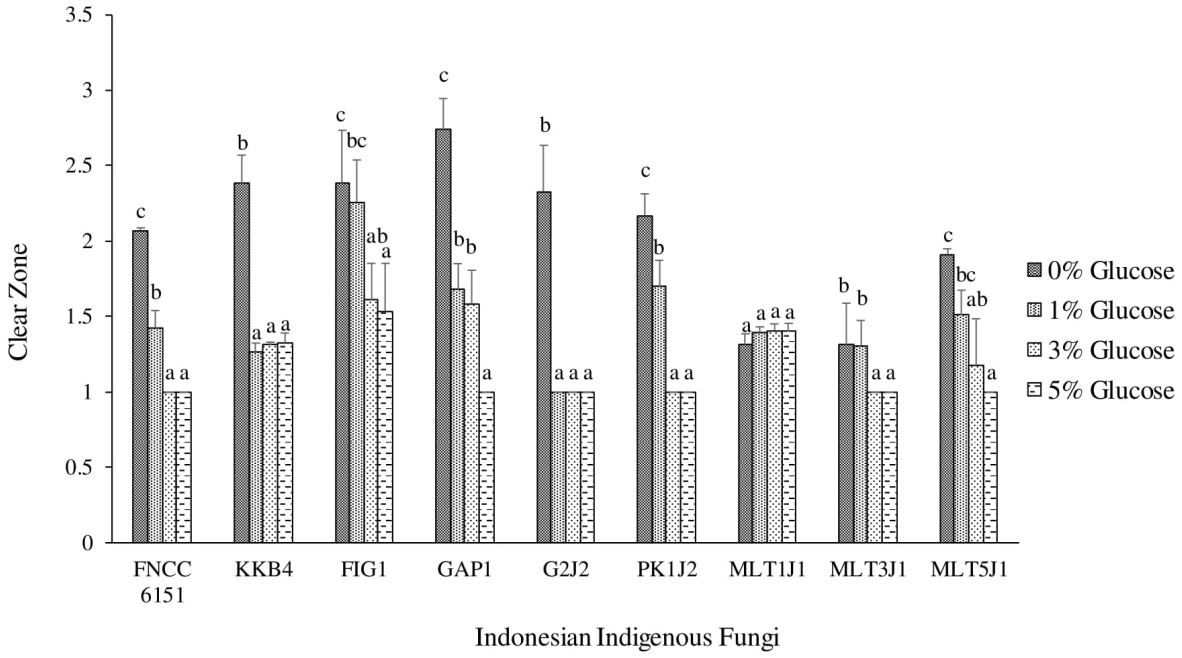

Figure 2: Clear zone of nine indigenous fungi with 0%, 1%, 3%, 5% glucose as carbon catabolite repressor. MLT1J1 showed no repression by glucose. The clear zone is a ratio between clear zone diameter and colony diameter; a clear zone equal to 1 means no amylase activity. The different letters outside the end of the bar indicated a significant difference between glucose concentration (p<0.01) for the same strain according to the Duncan test.

the diameter of the entire clear zone and the diameter of the fungal colony, differed from those grown on medium without glucose (Figure 2). The decrease of the clear zone was due to the presence of glucose in the medium. It might indicate that the production of amylases was repressed by glucose on a solid medium. The production of amylases by strain MLT1J1 was not repressed by glucose up to 5%. However, the production of amylases by strain G2J2 was repressed by glucose at a concentration as low as 1%. The degree of repression on amylases production in the other strains was varied. The amylases production by strain F1G1 and MLT3J1 was slightly repressed by 1% glucose, but it was considerably repressed by glucose at higher concentrations. The other strains showed considerable repression on amylases production when the strains were grown on a medium containing glucose at a concentration between 1 to 5%. It required 3% glucose to repress the amylases production

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in strains FNCC 6151, PK1J2 and MLT3J1 and it required 5% glucose for strains GAP1 and MLT5J1 to repress the production of amylases on a solid medium.

There were some works on the assessment of amylase production using clear zones surrounding colonies, such as in *Aspergillus niger* (Saleem and Ebrahim, 2014), *A. fumigatus* (Singh *et al.*, 2014), *A. luchuensis* bs1 (Sadhasivam *et al.*, 2018) and *Penicillium expansum* (Fifendy *et al.*, 2020). However, only the work of Hiramoto *et al.* (2015) presented data on the repression of amylase production in solid media. The growth of *A. oryzae* in plate medium supplemented with 0.5% glucose showed a decrease in clear zone ratio compared to media without glucose (Hiramoto *et al.*, 2015).

Effect of glucose on alpha-amylase and glucoamylase production in liquid medium

Production of amylase in a liquid medium allows us to examine each amylase, namely alpha-amylase and glucoamylase. The addition of glucose to the liquid growth medium brought above repression of alpha-amylase and glucoamylase in the same strain but no repression in other strains. Alpha-amylase production by strains FNCC 6151, KKB4 and MLT1J1 did not decrease considerably by adding glucose up to 5% to the growth medium. However, the addition of 1% glucose to the growth medium decreased the alpha-amylase production in other strains (Figure 3).

Although glucose repressed the production of alpha-amylase in one strain, it did not repress the production of glucoamylase or vice versa. The production of alpha-amylase in FIG1 was repressed by 1% glucose. However, the production of glucoamylase was not repressed by glucose up to 5% glucose (Figure 4). Strain KKB4 showed no repression of alpha-amylase production, but it showed repression of glucoamylase production by 3% glucose (Figure 4). Strains FNCC 6151 and MLT1J1 showed no repression to both alpha-amylase and glucoamylase by glucose up to 5%. The rest of the strains showed repression of glucoamylase production by 1% glucose (Figure 4).

Several studies reported that the addition of 0.5% glucose could repress alpha-amylase activity in bacteria such as *Bacillus* sp. (de Oliveira Santos and Martins, 2003) and *B. licheniformis* (Samanta *et al.*, 2017). Meanwhile, 1% glucose could repress amylase activity in *Thermomyces lanuginosus* (Kunamneni *et al.*, 2005). Glucose repression in yeast and fungi had also been investigated, especially in filamentous fungi (Adnan *et al.*, 2018) such as *A. oryzae* (Ichinose *et al.*, 2018), *A. nidulans* (Shroff *et al.*, 1996), *T. reseei* (Portnoy *et al.*, 2011) and *Aspergillus* (Lubertozzi and Keasling, 2009).

The strains FNCC 6151, KKB4 and MLT1J1 are suitable to produce both alpha-amylase and glucoamylase production since the production of both enzymes in those strains was not repressed by glucose up to 1%. These findings indicated that strains of FNCC 6151, KKB4 and MLT1J1 could be used to produce a mixture of alpha-amylase and glucoamylase. In industrial applications, the mixture of alpha-amylase and glucoamylase is widely used in glucose syrup production.

None of the strains examined is suitable for the production of alpha-amylase because alpha-amylase production in all strains was subject to repression by 1% glucose. The repression of alpha-amylase production in those strains should be disturbed before the strains are used for alpha-amylase production.

The strains of FIG1 are more suitable for glucoamylase production since the enzyme production in this strain was not repressed by glucose. However, the production of alpha-amylase was repressed by glucose. Glucoamylase is widely used in the starch saccharification process in juice and beer products (de Souza and de Oliveira Magalhães, 2010), glucose production as a sweetener and glucose syrup (Kumar and Satyanarayana, 2009). Glucoamylase plays a role in producing glucose to increase the Maillard reaction, responsible for the crust's browning process, leading to the unique bread flavour (Kumar and Satyanarayana, 2009; Saini *et al.*, 2017).

Effect of glucose in fungal biomass

Figure 5 showed that the fungal biomass produced generally increased as the initial glucose concentration in the medium increased regardless of the presence of glucose repression on amylases production. Although there were repressions on alpha-amylase and glucoamylase productions in strains GAP1, G2J2, PK1J2, MLT3J1 and MLT5J1 but the biomass produced increased as the initial glucose concentration in the medium increased. The biomass produced by strains FNCC 6151, KKB4, FIG1 and MLT1J1, which possessed no repression on either one or both alpha-amylase or glucoamylase, increased as the initial glucose concentration in the medium increased.

Glucose present in the growth medium was metabolized by the cells and the energy produced as ATP was used to synthesize the cell components. Most heterotrophic microorganisms prefer glucose as the carbon and energy source; they will use other carbon sources, such as other simple sugars or starch, when glucose is depleted in the medium (Hobbie and Hobbie, 2013; Morales-Sánchez *et al.*, 2013). The strain which possessed glucose repression on amylases production did not produce amylases when the glucose concentration in the medium was relatively high and used existing glucose for growth. When the initial glucose in the medium diminished, the strain started to produce amylases and hydrolyze starch in the medium into glucose. Strain with no glucose repression on amylases production kept producing amylases as long as starch as an inducer was present in the medium and produced glucose. Growth medium with higher initial glucose had a higher total carbon source which led to higher biomass to be produced. Similar results were shown by works of

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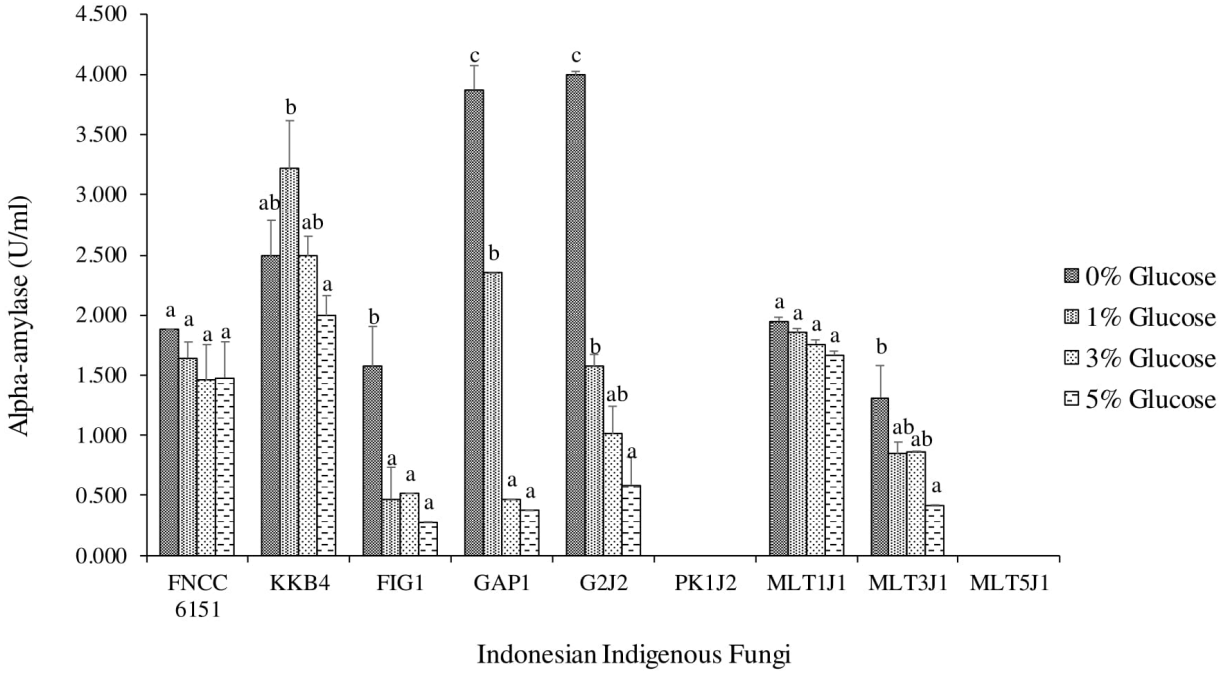

Figure 3: Alpha-amylase activity of nine indigenous fungi with 0%, 1%, 3%, 5% glucose as carbon catabolite repressor. FNCC 6151, KKB4 and MLT1J1 showed more resistance to glucose than others. The different letters outside the end of the bar indicated a significant difference between glucose concentration (p<0.01) for the same strain according to the Duncan test.

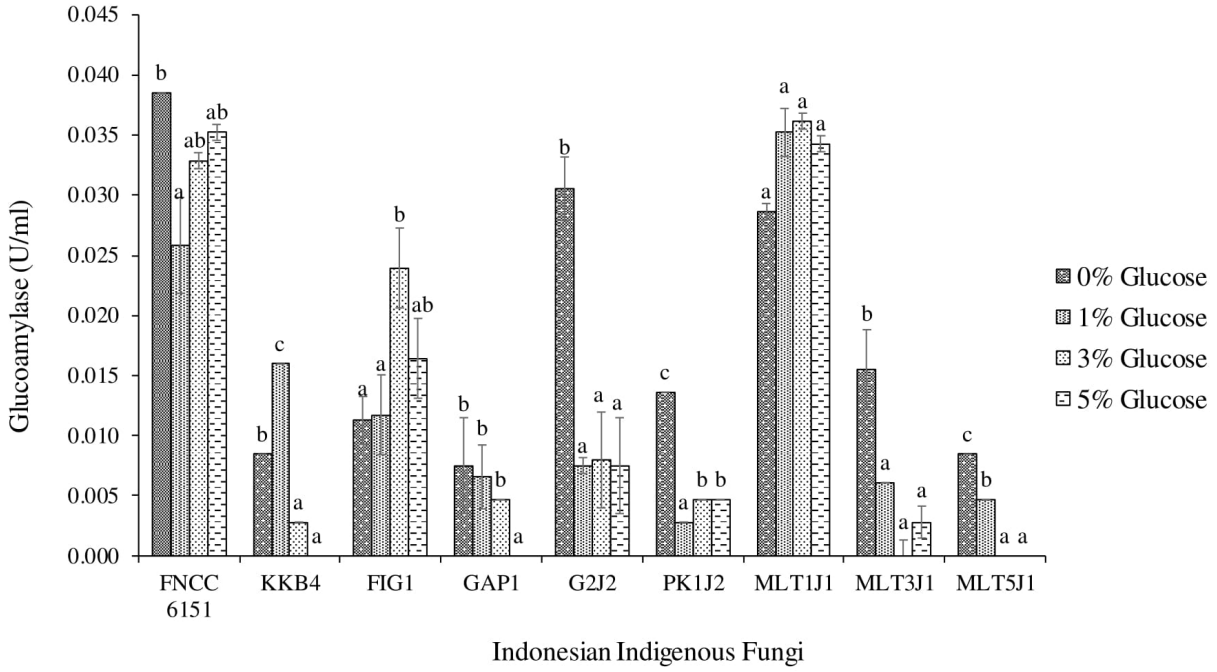

Figure 4: Glucoamylase activity of nine indigenous fungi with 0%, 1%, 3%, 5% glucose as carbon catabolite repressor. FNCC 6151, FIG1 and MLT1J1 showed more resistance to glucose than others. The different letters outside the end of the bar indicated a significant difference between glucose concentration (p<0.01) for the same strain according to the Duncan test.

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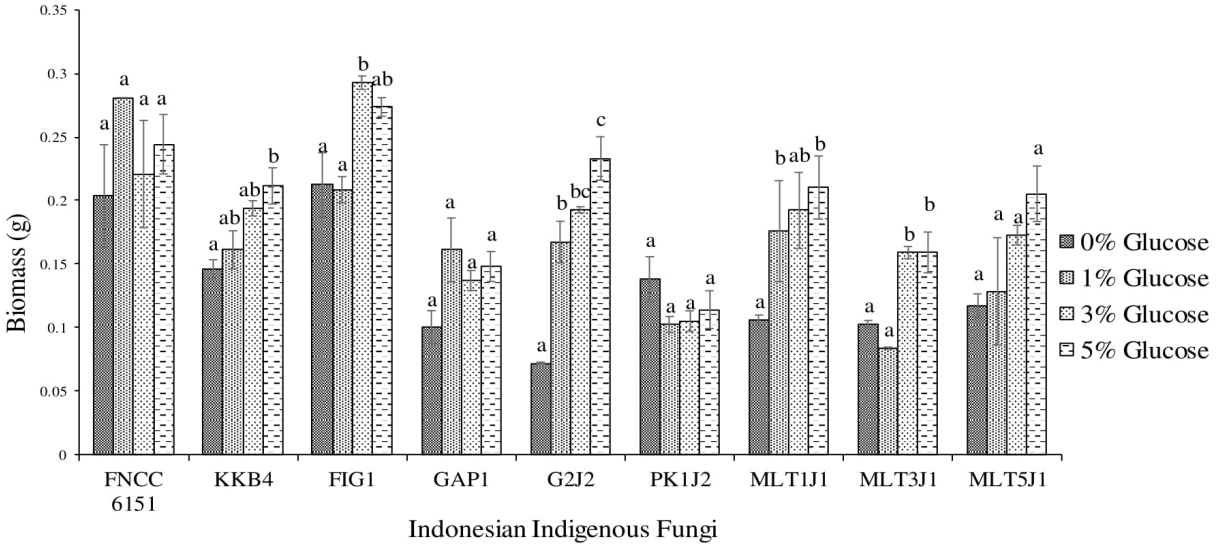

Figure 5: Biomass of nine indigenous fungi with 0%, 1%, 3%, 5% glucose as carbon catabolite repressor. Overall, glucose increases the fungal biomass. The different letters outside the end of the bar indicated a significant difference between glucose concentration (p<0.01) for the same strain according to the Duncan test.

Carlsen *et al.* (1996), Zangirolami *et al.* (2002), New *et al.* (2014) and Hamad *et al.* (2015) that biomass produced during growth was higher when the initial amount of carbon source in the medium was higher.

CONCLUSION

Out of the nine indigenous fungi strains examined, strains FNCC 6151 and MLT1J1 showed no repression on alpha-amylase and glucoamylase production when glucose was added to the medium up to 5%. Strain KKB4 showed no repression on alpha-amylase production, but there was repression on glucoamylase production, whereas strain FIG1 showed repression on alpha-amylase production, but there was no repression on glucoamylase production.

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