Molecular Expression of a Recombinant Thermostable Bacterial Amylase from *Geobacillus stearothermophilus* SR74 Using Methanol-free *Meyerozyma guilliermondii* Strain SO Yeast System

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α-Amylase, which was isolated from *Geobacillus stearothermophilus* SR74, has shown its potential to be used in industrial applications. However, its expression in the *Pichia pastoris* expression system with the alcohol oxidase 1 promoter (P_{AOX1}) requires high methanol consumption and is time-consuming. This study aimed to express SR74 α-amylase in an alternative yeast system, using *Meyerozyma guilliermondii* strain SO, which was isolated from a spoiled orange (SO) under the regulation of a formaldehyde dehydrogenase promoter (P_{FLD}). Qualitative screening showed that strain SO possessed a native amylase grown on YPD-starch plate at 30 °C. The recombinant SR74 α-amylase was further quantified and validated using the Western blot test. It was confirmed that SR74 α-amylase was expressed by strain SO extracellularly with a size of 59 kDa. Optimization in a shake flask showed that the recombinant SR74 α-amylase, which was regulated by P_{FLD}, was successfully produced (26 U/mL) without any external inducer in the YPT medium after 24 h of cultivation. In conclusion, strain SO was able to produce SR74 amylase without methanol in one-fifth the fermentation time of *P. pastoris*. Further optimization of the expression may be done to improve the yield, as this methanol-free host is still underexplored.

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INTRODUCTION

α-Amylase is one of the most used enzymes in the detergent, food, pharmaceutical, and industries, alongside lipase and protease. It belongs to the glycosyl hydrolase family that randomly cuts polysaccharides, such as starch or glycogen, by cleaving the internal *α*-1,4-glycosidic bond to release simple sugar molecules such as maltoses or glucoses (Metin *et al.* 2010). In industrial practice, enzymes are used in extreme conditions particularly with respect to temperature (Prakash and Jaiswal 2010). Therefore, thermostability is one of main concerns for industrial amylase. Thermophilic microorganisms are preferred for the

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production of thermostable amylases (Kavanagh 2017). The properties offered by microbial thermostable α-amylases have high commercial value, where the optimum temperature and pH range from 50 °C to 100 °C and 5.0 to 10.5, respectively (Lim *et al*. 2020).

The *α*-amylase from a thermophile *Geobacillus stearothermophilus* SR74 has potential to be commercialized for industrial applications due to its thermostability (Gandhi *et al.* 2015). Hence, SR74 is required to be produced in large quantity. However, its production in wild-type *G. stearothermophilus* SR74 is too low (Kassaye 2019). Therefore, the *Pichia pastoris* expression system under an alcohol oxidase promoter (P_{AOX1}) regulation was used to maximize its production (Gandhi *et al.* 2015). Nonetheless, its production in this system required high methanol consumption and long fermentation time (Gandhi *et al.* 2015). Hence, the production cost of SR74 *α*-amylase is expected to be expensive and uneconomical for industrial use.

P. pastoris (methylotrophic yeast) expression system has gained worldwide attention due to the presence of strong promoters, especially P_{AOX1}, leading to high foreign protein production (Hartner and Glieder 2006). However, it also comes with a bottleneck. The P_{AOX1} promoter dependency on methanol is challenging in certain aspects. Methanol is a volatile chemical that easily evaporates. Hence, it is hard to monitor and maintain the methanol concentration, even at room temperature. Promoters, such as formaldehyde dehydrogenase (P_{FLD}), that does not rely on methanol is considered as an alternative (Shen *et al*. 1998). The P_{FLD} was reported to produce foreign protein in comparable yield as the P_{AOX1} in *P. pastoris* (Shen *et al*. 1998). It is a promising alternative as an P_{AOX1} (Cos *et al*. 2005).

Moreover, a newly developed expression host, *M. guillermondii* strain SO, has the potential to produce foreign protein at a faster rate than *P. pastoris* with minimal methanol induction (Oslan *et al*. 2015). *P. pastoris* requires 60 h, whereas *M. guillermondii* strain SO requires 30 h to produce foreign protein at an optimum level (Oslan *et al*. 2015). To date, only P_{AOX1} and P_{FLD} were found in this host. The P_{AOX1} is dependent on the methanol for induction purpose, whereas P_{FLD} is able to respond to an inducer other than methanol, which is methylamine (Shen *et al*. 1998).

The objective of this study was to express SR74 *α*-amylase in *M. guilliermondii* strain SO under the regulation of the P_{FLD}.

EXPERIMENTAL

Materials
Strains and plasmids

Wild-type *M. guilliermondii* strain SO and *Escherichia coli* DH*α*5/pJET/SR74 amylase harboring gene of interest were obtained from previous studies (Kassaye 2009; Oslan *et al.* 2012). *P. pastoris* KM71H, *E. coli* TOP10, and FLD promoter (P_{FLD}) expression kit were purchased from Invitrogen (Carlsbad, CA, USA). *M. guilliermondii* strain SO and *P. pastoris* KM71H were maintained in YPD (yeast extract, peptone, and dextrose) broth, whereas *E. coli* TOP10 was maintained in LB (Luria-Bertani) broth. All of them were kept in glycerol stock at -80 °C in a freezer.

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Methods

Screening of native amylase in M. guilliermondii strain SO

Preliminary screening of α-amylase was completed using a method from a previous study (Effio *et al.* 2000). Wild-type *M. guilliermondii* strain SO was cultured on YPD agar containing 1% (w/v) starch for 2 to 3 days. Lugol solution (0.1% (w/v) iodine and 1% (w/v) potassium iodide) was flooded onto the plate for 5 to 10 min. The protocol was completed at 30 °C without any induction.

Cloning of SR74 α-amylase into P_{FLD}

SR74 α-amylase gene was amplified using the primers flanked with *Pml*I and *Apa*I restriction sites (5'-3') (Fwd SR74 *Pml*I: AT<u>CACGTG</u>TGCCGCACCGTTTAAC) and (Rvs SR74 *Apa*I: AT<u>GGGCCC</u>AGGCCATGCCACCAAC). Standard PCR amplification procedure was used with the annealing temperature 58 °C. Next, the amplicon and FLDα plasmid were digested with *Pml*I and *Apa*I (ThermoFisher Scientific, USA) prior ligation. Then, the ligation mixture was transformed into *E. coli* TOP10 for propagation purposes according to protocols suggested by the EasySelect™ *Pichia* Expression Kit manual, Invitrogen (USA). Finally, it was transformed into *M. guilliermondii* strain SO. The cloning process was completed according to FLDα manual (Invitrogen 2012). The transformant mixture was spread onto YPDS (w/v) (1% yeast extract, 2% peptone, 2% dextrose, 2% agar, and 1 M sorbitol) agar plates containing 100 µg/mL Zeocin™.

Quantitative screening expression of recombinant SR74 α-amylase

Fourteen positive colonies from 60 colonies containing the SR74 α-amylase gene were picked and subjected to expression screening. Minimum glycerol with ammonium sulphate medium (MGA) was used for biomass production before expression with an initial cell amount estimated by optical density at 600 nm, *i.e.* OD_{600nm}= 4. Culture in MGA medium was harvested and transferred into minimal methanol and ammonium sulphate medium (MMA) medium when the OD_{600nm} was 4 in MMA medium. A total of 0.5% (v/v) methanol was used for induction. Then, the cultures were harvested after 30 h at $3000 \times g$ at room temperature for 3 min. The supernatant was assayed by 3,5-dinitrosalicylic acid assay for detection of α-amylase activity.

Qualitative screening of recombinant α-amylase

Preliminary screening of α-amylase was performed according to a previous study (Effio *et al.* 2000). Colony 23 of *M. guilliermondii* strain SO containing recombinant plasmid, wild-type SO, and *P. pastoris* KM71H containing empty plasmid (negative control) were cultured on YPD (1% yeast extract, 2% peptone, 2% dextrose, and 2% agar) agar containing 1% (w/v) starch for 3 days. Then, Lugol solution (0.1% (w/v) iodine and 1% (w/v) potassium iodide) was flooded onto the plate for 5 to 10 min. This procedure was completed in triplicate at 30 °C with methanol induction.

Western blot analysis

Western blot analysis was performed for confirmation of the expression of extracellular His-tag α-amylase. It was completed according to the Western blot protocol (Novagen, USA). The sample (700 µL supernatant) was taken at 24 h in YPT medium and was concentrated using 700 µL of 100% (w/v) trichloroacetic acid (TCA) (Koontz 2014). The concentrated supernatant was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Bio-Rad, Philadelphia, PA, USA) for protein separation.

Then, SDS-PAGE gel was used for blotting onto a nitrocellulose membrane with an applied current of 0.45 mA for 2 h. Then, the prestained protein ladder well was cut from the membrane prior to the next steps and developing the colour. His-Tag Monoclonol Antibody and Goat-Anti-Mouse IgG HRP Conjugate (Novagen, USA) were used as primary and secondary antibodies, respectively.

3,5-Dinitrosalicylic acid (DNS) assay

The DNS assay was used to determine the reducing sugar (as a result of α-amylase reaction) content in the medium (Bernfeld 1995). The assay was conducted based on a previous study (Gandhi *et al.* 2015). A 0.5 mL enzyme s and 0.5 mL starch (1% w/v) in 50 mM phosphate buffer (pH 7.0) were mixed and allowed to react for 30 min at 60 °C. Then, same amount of DNS reagent was added to each tube and incubated at 100 °C for 10 min in order to stop the reaction. Next, the tubes were allowed to cool to room temperature and the absorbance at OD_{540nm} was taken. Reducing sugar was determined by comparing the absorbance at OD_{540nm} of the assay solution with a maltose standard curve. Colour change from orange to red-brown (540 nm) indicated the presence of α-amylase in the medium. The rate of 1% (w/v) starch converted to 1 μM maltose per minute was set to equal one unit of enzyme activity. The DNS assay was conducted in triplicate.

Effect of different media on recombinant SR74 α-amylase expression

Different media were studied to maximize SR74 α-amylase expression (Table 1). The MMAs, YPTM, and BMMY media were used for the optimization procedure. The MMG, YPTM, and BMMG were used for biomass accumulation. Then, 0.5% (v/v) methanol was used to induce the enzyme expression in cell culture every 24 h in the MMAs, YPTM, and BMMY media. The composition of each medium is listed in Table 1. The cultures were harvested after 48 h at 3000 × g at room temperature for 2 min. The samples in the form of supernatants were assayed by the DNS assay method. The growth for each culture was also monitored at OD_{600nm}.

Table 1. Composition for Each Medium Used in Media Optimization

Medium	MGAs	MMAs	BMGY	BMMY	YPTG	YPTM
Yeast extract (w/v)	-	-	2%	2%	1%	1%
Peptone (w/v)	-	-	2%	2%	2%	2%
Tryptic soy broth (w/v)	-	-	-	-	0.2%	0.2%
Yeast nitrogen base (w/v)	1.34%	1.34%	-	-	-	-
Biotin (w/v)	4×10^{-5}%	4×10^{-5}%	-	-	4×10^{-5}%	-
Glycerol (v/v)	1%		1%	-	1%	-
Methanol (v/v)	-	0.5%		0.5%		0.5%
Potassium phosphate buffer	-	-	100 mM, pH 6	100 mM, pH 6	-	-

MGAs: Minimal glycerol and ammonium sulphate medium; MMAs: Minimal methanol and ammonium sulphate medium; BMGY: Buffered glycerol complex medium; BMMY: Buffered methanol complex medium: YPTG: Yeast extract, peptone, tryptic soy broth, and biotin and glycerol medium; and YPTM: Yeast extract, peptone, tryptic soy broth, and biotin and methanol medium

Effect of different inducers

Different inducers were studied to optimize the α-amylase yield. Different inducers, such as 0.5% (v/v) methanol, 0.5% (v/v) methanol, and 0.25% (v/v) methylamine, and

0.25% (v/v) methylamine, were used. The YPTG medium was used in the inducer optimizations because it was able to express the highest SR74 α-amylase. The cultures were harvested at 48 h. The supernatants were assayed using the DNS assay method. The growth reading at 600 nm was examined for every culture.

Time course study

Time course study was conducted to determine the time point at which the expression was the highest. Colony 23 of recombinant *M. guilliermondii* strain SO (pFLDα₋/α-amylase), *P. pastoris* KM71H (pFLDα/α-amylase), and *M. guilliermondii* strain SO (pFLDα) were grown under the same protocol as described earlier. Amylase activity and growth at OD_{600nm} were monitored at 12 h intervals for 60 h.

RESULTS AND DISCUSSION

Quantitative and Qualitative Screening of *α*-Amylase Expression

Quantitative screening was conducted on 14 colonies. They were chosen based on the brightness of the band from colony polymerase chain reaction (PCR). Among the 14 colonies examined, colony number 23 showed the highest amylase activity. Hence, it was selected for the next optimization procedure. Figure 1 shows amylase screening agar plate for recombinant *M. guilliermondii* strain SO of colony 23, wild-type *M. guilliermondii* strain SO, and wild-type *P. pastoris* KM71H under methanol induction. Based on Fig. 1(B), wild-type SO had lower amylase activity than recombinant SO on Fig. 1(A). The larger non-blue colour (halo-zone) region observed with recombinant SO was due to the total amylase activity from both native and recombinant amylases. *P. pastoris* on Fig. 1(C) served as a negative control for the screening plate procedure. No amylase activity was observed from *P. pastoris*.
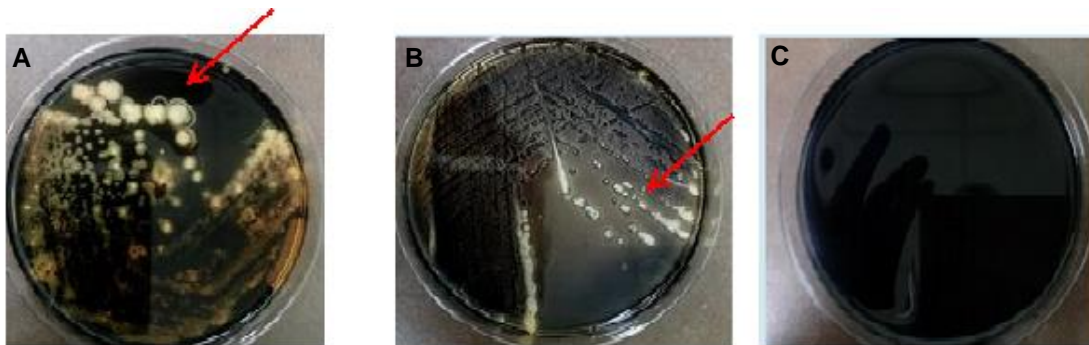

Fig. 1. Amylase plate screening of respective yeast species: (A) recombinant *M. guilliermondii* strain SO; (B) wild-type *M. guilliermondii* strain SO; and (C) wild-type *P. pastoris* KM71H with methanol induction (negative control). The screening plates were performed at 30 °C and were induced with methanol. The non-blue colour region/halo zone indicated the presence of amylase. The arrow shows a positive single colony. The recombinant *M. guilliermondii* strain SO had a larger halo zone area than the wild-type *M. guilliermondii* strain SO.

Western Blot Analysis

Western blot was used to identify the protein of interest in a protein complex. Western blot has been used widely for the detection of the protein of interest in methylotrophic yeast, especially for *P. pastoris* (Linder *et al.* 1997; Gandier and Master

2018). Hence, Western blot analysis was chosen to validate the expression of extracellular His-tag SR74 α-amylase in the supernatant. Trichloroacteic acid (TCA) precipitation method was chosen to concentrate the recombinant α-amylase in the supernatant. The TCA precipitation method was chosen to concentrate the SR74 α-amylase due to its simplicity and simple and reliable process. His-tagged SR74 α-amylase was identified based on the dark purple colour that formed on the nitrocellulose membrane. As displayed in Fig. 2, the purple colour band was developed at a protein size in the range of 48 to 63 kDa, which corresponded to the size of the SR74 α-amylase (59 kDa) on L2. Thus, it was confirmed that extracellular SR74 α-amylase was successfully expressed and secreted into the culture medium. *P. pastoris* has the ability to secrete the foreign proteins using both native host signal sequence and α-factor secretion signal from *S. cerevisiae* (which was presence in *Pichia* expression vectors). Paifer *et al.* (1994) reported that the secretion thermostable of bacterial α-amylase was higher in *P. pastoris* when using the α-factor secretion signal from *S. cerevisiae* than when the native host signal sequence was used. This feature was also demonstrated by other foreign protein expression in *P. pastoris* (Latiffi *et al.* 2013). It was confirmed that *P. pastoris* could be used to secrete foreign proteins using both signal sequences.
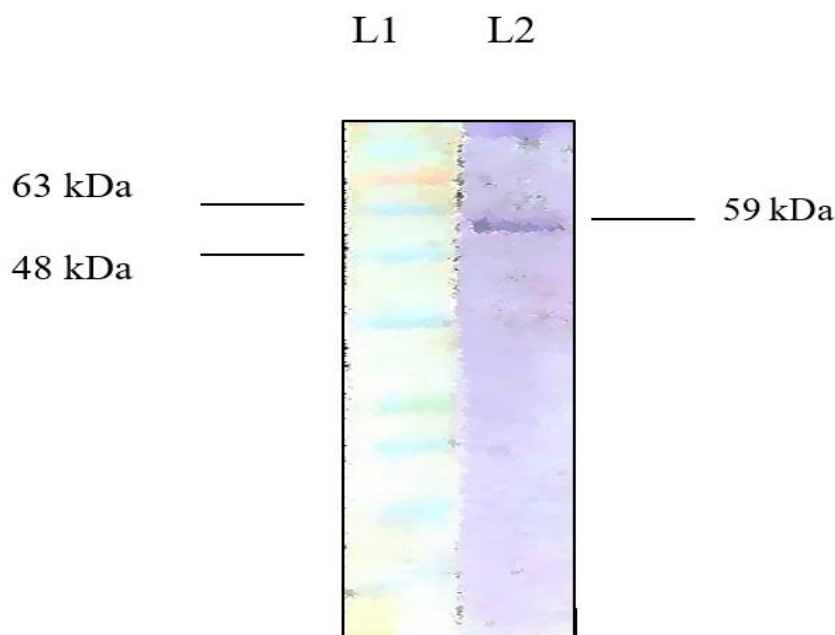

Fig. 2. Western blot analysis extracellular SR74 α-amylase: L1: SMOBio tri-colour pre-stained protein ladder pre-stained protein marker; and L2: crude extract of α-amylase. The size of the amylase was approximately 59 kDa. Colour of the membrane for L1 and L2 was different due to the multiple washing and staining steps used to detect the present of His-tagged protein where these steps were not applied to the prestained protein marker.

Effect of Different Media on Recombinant α-Amylase Production

Media optimization is one of the basic elements in the optimization procedure. It had to be conducted prior to large-scale fermentation. According to the Invitrogen manual (Invitrogen 2012) and a previous study (Gandhi *et al.* 2015), three types of media were chosen in media optimization. Fig. 3 displays the graph of the effect of different media on recombinant α-amylase production and cell growth. The YPTM produced the highest cell density and α-amylase expression than the others. The YPTM is a complex medium rich

in trace elements, amino acids, peptides, vitamins, and carbon, as well as an energy source (Tao *et al.* 1999). Cell density increased rapidly, and expression of genes was elevated while the biosynthesis pathway was turned off in the rich media (Tao *et al.* 1999; Kim and Kim 2017). A similar trend was observed in the YPTM medium. Cell growth is connected to protein production (Oslan *et al.* 2015). An increase in cell density led to an increase in protein production. Therefore, YPTM was the best medium for the expression of SR74 *α*-amylase. From the finding, MMA did not promote high cell density of yeast growth as well as the recombinant SR74 *α*-amylase expression level in strain SO. Because MMA is a minimal medium, only expression of housekeeping genes involving in biosynthetic pathways are up-regulated to build the building blocks from single energy and carbon source in response to stress (Tao *et al.* 1999) and survive (Kim and Kim 2017). There was also protein instability caused by extracellular proteases. It is essential to supply casamino acid in the minimal medium for the prevention of protein degradation (Clare *et al.* 1991). In contrast, BMMY did increase the cell density but did not promote *α*-amylase expression. Generally, there should be a correlation between cell density and gene expression. With respect to the BMMY medium, the pH might be the factor that led to the low expression of amylase even though the cell density was high. At a pH 6.0, the medium is optimum for the production of the protein using BMMY medium (Clare *et al.* 1991; Maleki *et al.* 2010). Hence, it was used in this study. However, pH 6.0 might not be a suitable pH for extracellular *α*-amylase, which led to the low amylase activity. Environmental pH is one the factors that affects protein structure (Monahan *et al.* 1995). The appropriate protein structure is crucial for the protein to function (Sharma 2013). Thus, pH optimization was crucial for SR74 *α*-amylase production in the BMMY medium.
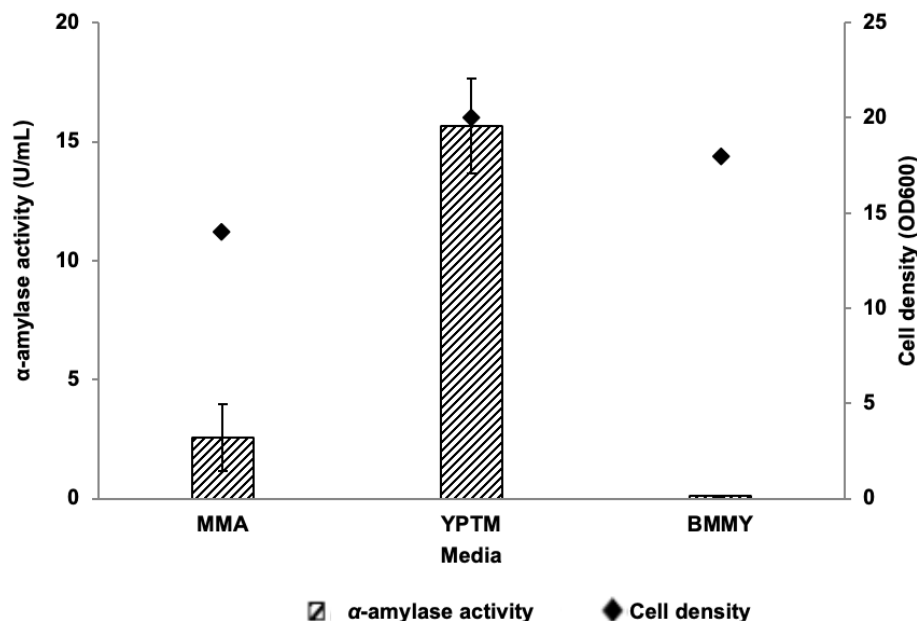

Fig. 3. Effect of different media on *α*-amylase production and cell density of recombinant *M. guilliermondii* strain SO. The reading was taken at 48 h with 0.5% (v/v) methanol induction. The initial biomass was in the range of OD_{600nm} of 14 to 16. MMA: minimal methanol medium; YPTM: yeast extract, peptone, tryptic soy broth, and biotin and methanol medium; and BMMY: buffered minimal methanol yeast extract. All data are presented as mean of ± standard deviation of triplicates. The bar chart represents *α*-amylase activity and the rhombus symbol indicates cell density.

Effects of Different Inducers

The P_{FLD} is the alternative to the P_{AOX1}. Both promoters are strongly inducible and able to produce high foreign protein production. The ability of the P_{FLD} to respond to methylamine makes it an attractive promoter over the P_{AOX1}. Methylamine is less expensive and easier to handle than methanol. The P_{FLD} can be induced by methanol and/or methylamine in *P. pastoris*. Methylamine was the best inducer for foreign protein production in *P. pastoris* (Shen *et al.* 1998). However, the best inducer for the P_{FLD} in *M. guilliermondii* strain SO is still unknown. Hence, it was essential to identify the best inducer for the promoter before proceeding to large-scale fermentation.

Figure 4 shows the effect of different inducers on α-amylase production and cell growth. Initially, the culture with no inducer was a negative control for this optimization procedure. It was unexpected that culture with no inducer was able to express the highest amylase activity than culture with inducer(s). This is the first study to date that reports the ability of P_{FLD} to produce foreign protein without any external inducer. Its expression was the highest (23 U/mL). The P_{AOX1} in a similar host also was able to express foreign protein without methanol induction, but at low yield. A mere 2 U/mL lipase was produced after optimization using response surface methodology (RSM) (Abu *et al.* 2017). The study reported by Abu *et al.* (2017) had used *M. guilliermondii* strain SO to express recombinant T1 lipase and had shown similar feature to this study where the methanol inducible promoters (P_{AOX1} and P_{FLD}) could express the recombinant proteins without the present of methanol. But, no activity of P_{FLD} was observed with 0% methanol induction (without inducer) in *Pichia methanolica*. Additionally, studies were also conducted with *P. pastoris*, *Hansenula polymorpha*, and *Candida boidinii*, where the studies' focus was more on the comparison between different inducers than with or without inducer. Comparison with and without induction between *M. guilliermondii* strain SO and well-known expression hosts could not be performed. To date, the expression of recombinant proteins with the uninduced inducible promoters were only present in *M. guilliermondii* strain SO.
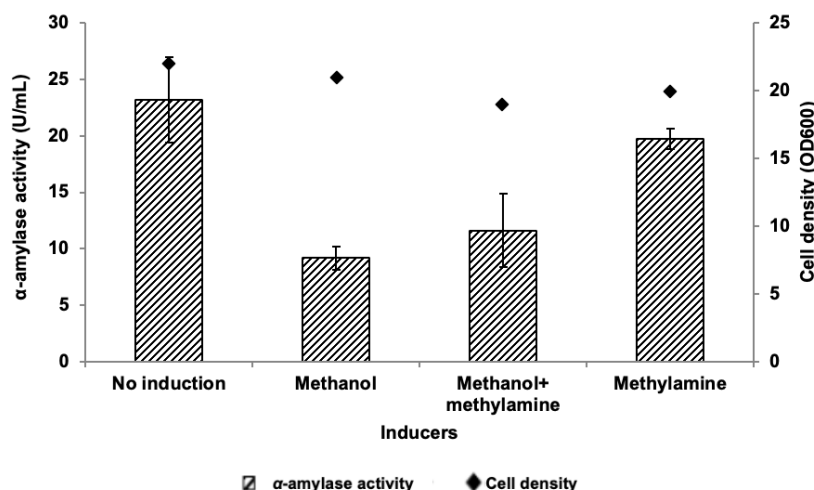

Fig. 4. Effect of different inducers on α-amylase production and cell growth. The reading was conducted at 48 h starting with initial biomass of OD_{600nm} 18 to 20. Concentration of inducers was: methanol 0.5% (v/v) and methylamine 0.25% (v/v). Culture with no inducer produced the highest α-amylase expression compared to methanol, methanol and methylamine, and methylamine. All data are presented as mean of ± standard deviation of triplicates. The bar chart represents α-amylase activity and the rhombus shapes indicate cell density.

Time Course Study

A time course study was conducted to determine at which particular time the production of α-amylase was the highest. Figure 5 shows the growth study and expression of α-amylase at optimum conditions. Generally, the growth of microorganisms consists of four main stages: lag phase, log phase, stationary phase, and death phase. With respect to *M. guilliermondii* strain SO, 0 to 24 h was the log phase, 24 to 36 h was the stationary phase, and 36 to 60 h was the death phase (lag phase is where this yeast was grown in the YPTG medium for 24 h). The expression level of α-amylase in *M. guilliermondii* strain SO seemed to be related to the growth phase. α-Amylase expression increased as the cell density increased during the log phase. The log phase is a phase where all cellular constituents are synthesized at a constant rate (Monod 1949). Protein synthesis mostly occurs at this stage (Monod 1949).

The expression was the highest (26 U/mL) at the beginning of the stationary phase. Moreover, the highest production of foreign protein under different promoter (P_{AOX1}) was also at the beginning of the stationary phase (Oslan *et al.* 2015). During the stationary phase, the cell growth starts to slow down until it is in balance with cell deaths (Albertson *et al.* 1990; Kolter *et al.* 1993). The slowing of cell growth is due to the excretion of the toxic compounds into the medium, depletion of nutrients and oxygen, and accumulation of carbon dioxide. These factors are intensified during the death phase. During the death phase, cell growth decreases drastically. The declining of viable cells reduces protein expression (Albertson *et al.* 1990). This explains the expression level of α-amylase that started to drop after 36 h. Wild-type *M. guilliermondii* strain SO infrequently showed any amylase activity. As a conclusion, α-amylase activity was solely from recombinant SR74 α-amylase and native/inherent amylase was a non-thermostable (DNS assay was performed at 65 °C) extracellular amylase.

This newly developed expression system using *M. guilliermondii* strain SO as a host and P_{FLD} as a chosen promoter was able to produce a comparable yield of α-amylase to the established *P. pastoris* expression system at one-fifth the fermentation time grown without any external inducer. Specifically, the newly developed system was able to produce 1.08 U/mL of α-amylase per hour. In contrast, *P. pastoris* expression system could only produce 0.24 U/mL of α-amylase per hour. There was a 16-fold increase in terms of production rate in this newly developed expression system. Therefore, this newly developed system is beneficial for the mass production of SR74 α-amylase for its commercialization purposes in industrial applications.

CONCLUSIONS

1. Recombinant SR74 α-amylase was expressed extracellularly in a new expression system using the yeast expression host of recombinant *M. guilliermondii* strain SO/pFLDα/α-amylase.

2. *M. guilliermondii* strain SO isolated from spoiled orange was shown to produce α-amylase at a comparably higher yield than the commercial *P. pastoris* expression system without any external inducer (methanol and/or methylamine) at a much shorter fermentation time.

3. The production cost of *α*-amylase from strain SO is expected to be appreciably lower due to its shorter fermentation time and without any need for inducers. Thus, a new expression system with higher production rate of SR74 *α*-amylase was developed.

4. SR74 *α*-amylase has a higher chance for application in food, feed, and pharmaceutical industries because its optimum production does not require any chemical/inducer, with high potential for toxicity.

ACKNOWLEDGMENTS

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