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Lipid Metabolon in Non-Oleaginous Fungus *Aspergillus niger*

(Metabolon Lipid dalam Kulat Bukan Oleginous *Aspergillus niger*)

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ABSTRACT

The lipogenic multienzyme complex or lipid metabolon consisting of malic enzyme (ME), fatty acid synthase (FAS), acetyl-CoA carboxylase (ACC) and ATP:citrate lyase (ACL) was observed during lipid biosynthesis of an oleaginous fungus *Cunninghamella bainieri* 2A1. It is believed that high production of fatty acid in oleaginous species is due to the association of lipogenic enzymes forming a metabolon during lipid accumulation phase. One of the significances of lipid metabolon is that it provides efficient and rapid channel of NADPH directly from ME to FAS when lipid accumulation occurs. This leads to the assumption of low lipid yield in non-oleaginous microorganisms is due to the absence of lipid metabolon in these microorganisms. Thus, the non-oleaginous filamentous fungi *Aspergillus niger* was studied to see the association of lipid metabolon and lipid biosynthesis. The cells of *A. niger* during both lipid accumulation and lipid cessation phase were used to prepare cell-free extracts using protoplasting technique. The crude proteins were then separated by Blue Native (BN)-PAGE, associated with liquid chromatography-tandem mass spectrometry (LC-MS/MS). No high molecular mass complex was detected, speculating that no lipogenic multienzyme complex was formed in non-oleaginous fungi hence very low or no storage lipid was accumulated in this fungus.

Keywords: *Aspergillus niger*; *lipid metabolon*; *lipogenesis*; *non-oleaginous*

ABSTRAK

Kompleks multienzim lipogenik atau metabolon lipid yang terdiri daripada enzim malik (ME), asid lemak sintase (FAS), karboksilase asetil-KoA (ACC) dan ATP: sitrat liase (ACL) didapati terbentuk semasa biosintesis lipid kulat oleaginus *Cunninghamella bainieri* 2A1. Penghasilan asid lemak jumlah yang tinggi dalam spesies oleaginus dipercayai disebabkan oleh gabungan enzim lipogenik membentuk metabolon semasa fasa pengumpulan lipid. Salah satu kepentingan metabolon lipid adalah untuk membolehkan penyaluran NADPH secara berterusan dari ME ke FAS dengan cekap dan pantas semasa tempoh pengumpulan lipid. Ini membawa kepada andaian bahawa pengumpulan lipid yang rendah dalam mikroorganisma bukan oleaginus adalah disebabkan oleh ketiadaan metabolon lipid dalam mikroorganisma ini. Oleh itu, kulat berfilamen bukan oleaginus *Aspergillus niger* telah dikaji untuk melihat perkaitan metabolon lipid dan biosintesis lipid. Kultur sel *A. niger* semasa fasa pengumpulan lipid dan fasa pemberhentian lipid digunakan untuk menyediakan ekstrak bebas sel menggunakan teknik *protoplasting*. Ekstrak bebas sel kemudiannya dipisahkan menggunakan *Blue Native* (BN)-PAGE dan dianalisis menggunakan spektrometri jisim tandem kromatografi cecair (LC-MS/MS). Tiada kompleks berjisim molekul besar dikesan, mencadangkan bahawa tiada kompleks multienzim lipogenik terbentuk dalam kulat bukan oleaginus dan seterusnya membawa kepada pengumpulan jumlah lipid yang sangat rendah dalam kulat ini.

Kata kunci: *Aspergillus niger*; *lipogenesis*; *metabolon lipid*; *non-oleaginus*

INTRODUCTION

Oleaginous microorganisms are defined as those that can synthesise and accumulate lipid up to 20% or some even 70% lipid (g/g biomass); mostly consists of triacylglycerol (Ratledge 2004). These oleaginous microorganisms include bacteria, fungi, yeast and microalga such as *Aspergillus, Cunninghamella, Cryptococcus, Chlorophyta, Lipomyces, Mortierella, Mucor, Rhodotorula,* and *Trichosporon* (Sindhu et al. 2019; Thevenieau & Nicaud 2013). The lipid accumulation or lipogenesis is initiated by the exhaustion of nutrient: usually the limitation of nitrogen in the surrounding (Hoarau et al. 2020; Kendrick & Ratledge 1992). When the surrounding nitrogen is insufficient, the DNA replication and cell differentiation will be

interrupted, activating the biosynthesis of fatty acid. The continuous assimilation of excess carbon source occurs and is channelled into the fatty acid synthesis pathway for energy storage. Examples of these oleaginous fungi are *Mucor circinelloides*, *Mortierella alpina* and *C. bainieri* 2A1 (Ratledge 2004; Shuib et al. 2018). Whereas, in the non-oleaginous microorganisms, polysaccharides will be accumulated instead of lipids when nitrogen is limited (Carsanba, Papanikolaou & Erten 2018; Ratledge 2004).

At the onset of nitrogen exhaustion, a cascade of biochemical reaction occurs, triggering the lipogenesis. The exhaustion of nitrogen in the surrounding increases the AMP deaminase activity (to scavenge the intracellular ammonium ion for the protein and nucleic biosynthesis of cells), catalysing the conversion of AMP to IMP and NH_3. The fall of AMP concentration stops the isocitrate dehydrogenase activity and leads to the accumulation of isocitrate which is readily equilibrate with citrate via aconitase. However, this high dependency of isocitrate dehydrogenase towards AMP concentration does not happen in non-oleaginous species. The accumulated citrate is then being exported by the citrate flux system from mitochondria to cytosol and cleaved by ACL, producing acetyl-CoA which is essential for the biosynthesis of fatty acid. Meanwhile, the oxaloacetate produced is converted into malate and used for the generation of NADPH by ME (Ratledge & Wynn 2002; Zhang et al. 2016). ME, the key enzyme of lipogenesis, regulates the accumulation of lipid by providing necessary NADPH to FAS in oleaginous microorganisms (Ratledge 2014, 2004; Wynn & Ratledge 1997). Thus, Nicaud (2012) and Ratledge (2014) suggested that the extent of lipid accumulation is determined by the role of ME in one strain, in which functioning as the sole provider of NADPH in lipid synthesis.

According to the studies, it was found that the lipid accumulation in oleaginous microorganisms is regulated by ME activity (Liang & Jiang 2015; Ochoa-Estopier & Guillouet 2014). The importance of this enzyme in fatty acid synthesis by supplying NADPH to FAS has been reported. Studies such as inhibition of ME using sesamol and overexpression of gene coding for ME that significantly affected the amount of lipid accumulated in oleaginous microbes, proposed that the NADPH required by FAS during the lipid biosynthesis is provided specifically by ME and no general pool of NADPH exists (Ratledge & Wynn 2002; Shuib et al. 2018; Wynn, Kendrick & Ratledge 1997; Zhang, Adams & Ratledge 2007). In three oleaginous fungi: *C. bainieri* 2A1, *M. circinelloides,* and *M. alpina*, the cessation of lipid accumulation occurred when the ME activities decreased despite high activities displayed by other NADPH generating enzymes that which are glucose-6-phosphate dehydrogenase (G-6-PDH), 6-phosphogluconate dehydrogenase (6-PGDH) and NADP+:isocitrate dehydrogenase (NADP+:ICDH) (Hamid et al. 2014; Shuib et al. 2018; Song et al. 2001). Also, in *Cunninghamella echinulata*, activity of ME ceased along with the activities of ATP:citrate lyase and acetyl-CoA carboxylase after the cessation of lipid accumulation (Certik, Megova & Horenitzky 1999), proposing that NADPH is directly channelled into fatty acid synthase (FAS) from ME during the synthesis of lipid. This leads to the suggestion that a lipogenic multienzyme complex or lipid metabolon (involving ME and FAS) is formed during lipogenesis, to ensure proper channelling of NADPH from ME to FAS. Metabolon is a supramolecular complex of sequential metabolic enzymes and cellular structural components (Bassard & Halkier 2018; Ratledge & Wynn 2002). Metabolon forms when the enzymes that are often involved in the same pathway associated physically. This allows the direct channel of NADPH to FAS in high speed and efficiency without affecting the system of the cell where the complex is located. In Shuib et al. (2018), it was reported that the multienzyme complex involving ME and other NADPH generating enzymes of *C. bainieri 2A1* was associated during the lipid synthesis phase. Also, two different lipogenic metabolons with different group of enzymes involved in thraustochytrid was reported (Shuib et al. 2022). Hence, the existence of lipid metabolon (complexes formed between enzymes) in oleaginous microorganisms is now well documented.

However, is the formation of lipid metabolon, consisting of ME and other lipogenic enzymes a regulating factor leading to accumulation of high yield of lipid in oleaginous filamentous fungi? Is the low lipid percentage in non-oleaginous fungi such as *A. niger, Kluyveromyces polysporus, Saccharomyces cerevisiae,* and *Torulaspora delbrueckii* due to the absence of lipid metabolon? To date, the existence of lipid metabolon in non-oleaginous species in not evident. This research is therefore to determine the presence of lipid metabolon in non-oleaginous microorganisms. Thus, the non-oleaginous filamentous fungi *A. niger*, being one of the multipurpose industrial producers as well as a major producer of citric acid, is used in this work to determine the involvement of lipogenic metabolon in non-oleaginous species (Abghari & Chen 2017; André et al. 2010).

MATERIALS AND METHODS

MICROORGANISMS

Stock culture of *A. niger* (maintained on PDA at 4 °C) was obtained from the Department of Biological Sciences and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, Bangi, Selangor.

PREPARATION OF MEDIA AND CULTURE CONDITION

A nitrogen-limited medium was prepared for cultivation (Kendrick & Ratledge 1992). A basal medium containing 1 g/L diammonium tartrate, 7 g/L KH_2PO_4, 2 g/L Na_2HPO_4, 1.5 g/L $MgSO_4.7H_2O$, 1.5 g/L yeast extract, 0.1 g/L $CaCl_2$, 0.008 g/L $FeCl_3.6H_2O$, 0.0001 g/L $Co(NO_3)_2$.6H2O,

0.0001 g/L $ZnSO_4.7H_2O$, 0.0001 g/L $CuSO_4.5H_2O$, 0.0001 g/L $MnSO_4.5H_2O$ and 30 g/L glucose solution were prepared and sterilised at 121 °C for 15 min and 110 °C for 10 min, respectively. Seed culture was prepared, and cultures were grown in 500 mL Erlenmeyer flasks with 200 mL medium as described in Shuib et al. (2018). All cultivations were carried out at 30 °C at 200 rpm. Cultures were harvested every 24 h and analysed for biomass, lipid content, enzyme activity, ammonium and glucose concentration.

ANALYTICAL METHODS

Biomass was harvested by filtration through pre-weighed Whatman No. 1 filter paper, washed with distilled water, dried at 110 °C to a constant weight and then gravimetrically determined. The ammonium concentrations in the culture medium were determined using indophenol method and measured at 625 nm (Chaney & Marbach 1962). The glucose concentrations in the medium were determined using the GOD-PERID test kit. Lipid analysis was performed using the method of Folch, Lees and Sloane Stanley (1957). Lipid content was extracted using chloroform/methanol (2:1, v/v) and the gravimetrically determined.

PREPARATION OF PROTOPLAST AND CELL EXTRACT

Protoplast and cell extract were obtained according to the protocol reported in Shuib et al. (2018). Harvesting of mycelia were done by filtration through Whatman No.1 filter paper followed by washing with 400 mL distilled water. Filtered mycelia (0.5 g) were then suspended in the stabilising solution, in which it was prepared by adding 0.075 g lysing enzymes (β-glucanase, cellulase, protease and chitinase activities from *Trichoderma harzianum* (Sigma)) into 12.5 mL stabilising buffer (1.2 M $MgSO_4$ and 10 mM KH_2PO_4 at pH6) and filtered using 0.2 µm membrane filter. The mixture was gently shaken at 100 rpm, 30 °C for 3 h. Filtration through 3 layers of lens-cleaning tissue was performed to remove mycelia debris (de Bekker et al. 2009; Shuib et al. 2018). The protoplasts were then observed and counted before the process of centrifugation. The protoplasts obtained from centrifugation was suspended in 5 mL extraction buffer (0.1 M KH_2PO_4, 20% (v/v) glycerol, 1 mM benzamidine, 1 mM mercaptoethanol and 1 mM EDTA) for 15 min. Then, suspension was centrifuged at 4 °C, 10000 × g for 15 min to obtain the supernatant (cell extract) for subsequent experiments. Protein concentration was determined based on the method of Bradford (1976).

DETERMINATION OF LIPOGENIC ENZYME ACTIVITIES

All enzyme activities (ME, FAS, ACL, $NADP^+$:ICDH, G6PDH and 6PGDH) were determined using continuous assays at 30 °C based on the oxidation and reduction of NAD(P)(H) at 340 nm (Hamid et al. 2014; Wynn & Ratledge 1997).

BLUE NATIVE PAGE POLYACRYLAMIDE GEL ELECTROPHORESIS (BN-PAGE)

SAMPLE PREPARATION AND PROTEINS SEPARATION

Samples of the cultured from 24 h (during nitrogen-limited condition) as well as 72 h and 120 h (cessation of lipid biosynthesis) were obtained. The preparation of samples, buffers and separation of proteins were performed as described in Shuib et al. (2018). NativeMark™ unstained protein standard (Invitrogen) consisting of soy trypsin inhibitor (20 kDa), bovine serum albumin (66 kDa), lactate dehydrogenase (146 kDa), β-phycoerythrin (242 kDa), apoferritin band II (480 kDa), apoferritin band I (720 kDa), IgM pentamer (1048 kDa), and IgM hexamer (1236 kDa) was used as standard protein. Electrophoresis was carried out at 100 V for 3 h and subsequently run overnight at 150 V, where all steps were done at 4 °C. The exponential plot: molecular weight of standard protein against relative migration distance (R_f) of standard proteins was used to determine the molecular weight of the proteins resolved in the gradient gel.

SLICING OF THE BN-PAGE GEL

The gel was sliced into 10 groups, with an approximate length of 8 mm each, after electrophoresis. The sliced gels were then designated as group 1 (upper part of the gel) to group 10 (lowest part of the gel) (Shuib et al. 2018).

IN-GEL TRYPSIN DIGESTION

Trypsin profile IGD kit for in-gel digest was used for in-gel trypsin digestion (Shevchenko et al. 2006) according to the procedures reported in Shuib et al. (2018). Each of the gel bands (1 to 10) gained was sliced into smaller pieces, transferred into silicone tube before adding 200 µL of destaining solution (400 mM NH_4HCO_3 in 40% acetonitrile) into the tubes and incubation at 37 °C for 30 min. The step was repeated twice. Disulphite bonds of the proteins were reduced using 10 mM dithiothreitol, DTT (200 µL) followed by incubation at room temperature for 30 min. The solution was then removed and replaced with 200 µL of 55 mM iodoacetamide for proteins alkylation process. The mixture was then incubated at ambient temperature in the dark. After that, 400 µL of washing solutions (50 mM NH_4HCO_3) was added to replace the solution followed by incubation at room temperature for 15 min, and this step was repeated twice. To dehydrate the gel pieces, 100% acetonitrile (400 µL) was added into the silicone tubes, incubated at room temperature for 10 min and the supernatant was then removed. The gel pieces were dried at 50 °C for 5 min. A 20 µL (0.4 µg trypsin) trypsin (1 mM HCl and 40 mM NH_4HCO_3 in 9% of acetonitrile) was added into the silicone tubes followed by incubation for 5 min at room temperature. Next, 40 mM NH_4HCO_3 in 9% acetonitrile (50 µL) was added into the tubes, incubated overnight at 37 °C. The digested peptides were dried again

in vacuum centrifuge after transferring into new silicone tubes. The dried peptides were kept at -20 °C for further analysis (Shuib et al. 2018).

IDENTIFICATION OF PROTEIN USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY (LC-MS/MS)

Proteins were identified as described in Shuib et al. (2018). Samples were analysed using Agilent 6520 Accurate Mass Q-TOF LC/MS-Nano ESI (ChipCube) (Agilent Technologies). Peptides identification was carried out by Spectrum Mill MS Proteomics Workbench (Rev B.04.00.127; Agilent Technologies), where cysteine carbamidomethylation was set as fix and variable modification. Search was done online for *Mucorales* at UniProtKB/Swiss-Prot database.

RESULTS

GROWTH AND LIPID ACCUMULATION PROFILES

A. niger was cultivated in nitrogen-limiting medium with glucose as the sole carbon source. Based on the growth profile obtained (Figure 1), the nitrogen concentration was depleted within the first 24 h of cultivation while glucose was still in excess (22 g/L), coincident with an increase in the biomass concentration, achieving 6.29 g/L. However, although the lipid content increased from 48 to 72 h, the highest lipid content achieved was only 6.9% g/g biomass as depicted in Figure 1. This does not achieve the minimum amount of lipid content accumulated as defined by the oleaginous microorganisms, i.e., 20% g/g biomass, indicating that *A. niger* is a non-oleaginous strain.

SPECIFIC ACTIVITIES OF LIPOGENIC ENZYMES

As depicted in Figure 2, all lipogenic enzymes displayed significant activities yet no particular correlation was observed between the enzyme activities and lipid accumulation of *A. niger*. Conversely, the ME activity of *A. niger* was detected between 15.8 and 18.5 nmol/min/mg protein after 72 h until the end of the cultivation, which was different from oleaginous microbes that has pronounced decreased in ME activity when lipid accumulation ceased. The specific activities of FAS, ACL and other NADPH generating enzymes (G-6PDH, 6-PGDH and $NADP^+$:ICDH) showed pronounced activity throughout the cultivation, however, with no correlation to the lipid accumulation profile.

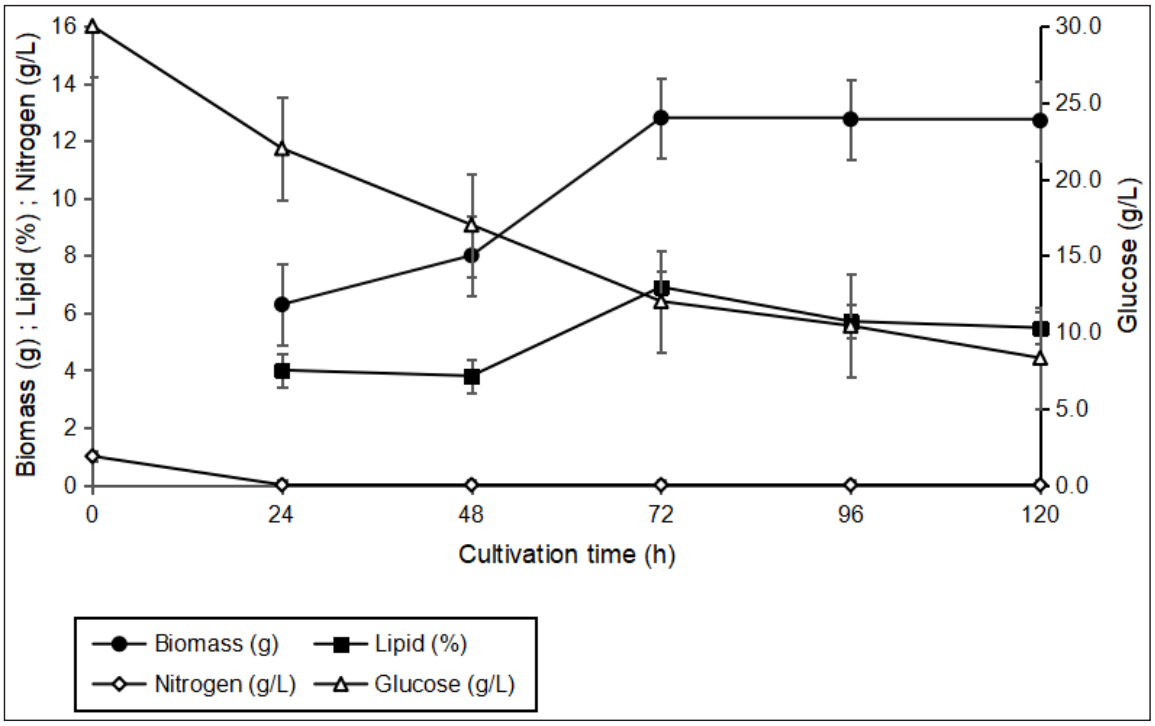
LC-MS/MS OF LIPOGENIC ENZYMES

Samples of the culture were obtained at 24 h, 72 h, and 120 h, which the nitrogen depletion began at 24 h, indicating the starting of nitrogen-limited condition. In addition, the lipid accumulation peaked at 72 h, followed by a cessation of lipid biosynthesis at 120 h. Based on the tandem mass spec result (Table 1), no evidence of the presence of any complex involving any of the lipogenic enzymes FAS, ME, ACL, ACC, pyruvate carboxylase (PC) and malate dehydrogenase (MDH) was observed. All the lipogenic enzymes were observed as separate bands corresponding to their molecular weight, indicating that no physical association was evident.

DISCUSSIONS

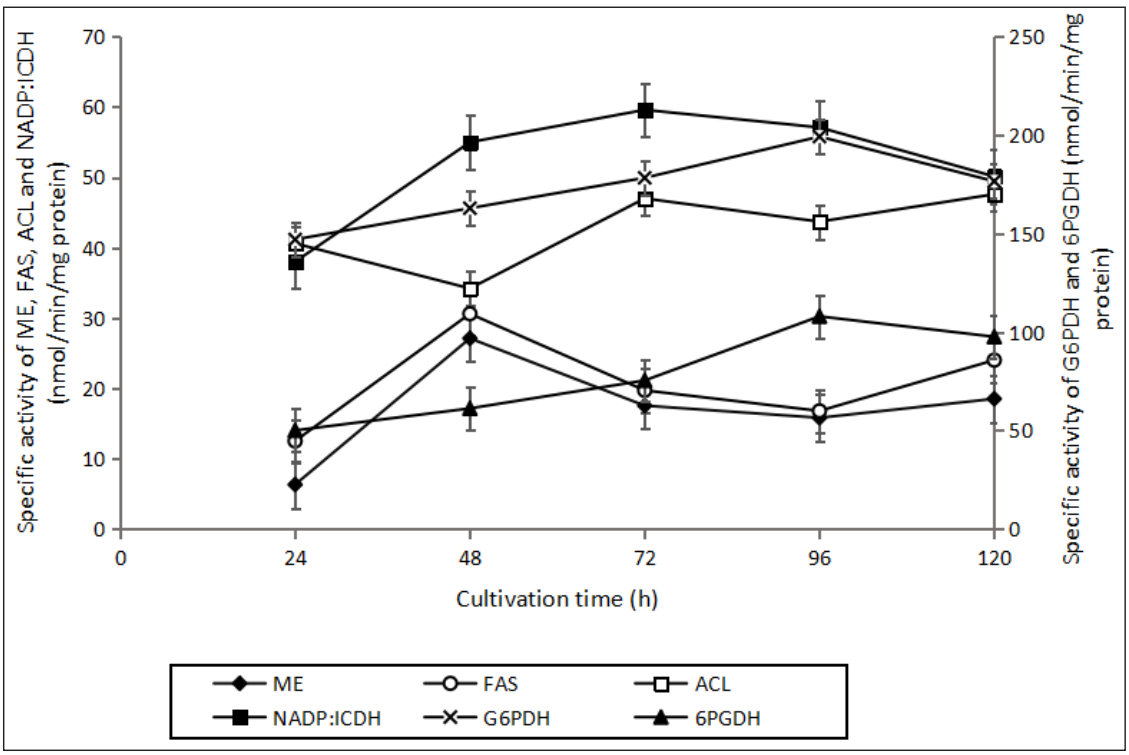
In oleaginous microorganisms, exhaustion of nutrient especially nitrogen is known to initiate lipid biosynthesis where the cells will assimilate and channel the excess glucose into lipid as storage (Ratledge 2004; Zhang et al. 2016). Hence, the limited nitrogen condition with excess glucose achieved in the medium provided an optimal condition and favoured the lipid accumulation to occur in oleaginous microorganisms (Furlan et al. 2017). Oleaginous microorganisms have been defined as those which accumulate more than 20% lipid (g/g biomass) consisting mostly of triacylglycerols (Ratledge 2004; Ratledge & Wynn 2002). Whereas most non-oleaginous species are reported to contain between 5 and 10% lipid which formed the structural components of the cells, even though lipogenic enzymes are also being expressed in non-oleaginous organisms. For instance, non-oleaginous yeasts such as *K. polysporus, S. cerevisiae,* and *T. delbrueckii* are reported to contain lesser lipid content i.e., between 2-5% lipid (g/g biomass) when cultured on the same medium as used for oleaginous yeast (Kolouchová et al. 2016). Thus, the low quantity of lipid accumulated (with highest lipid content of 6.9% g/g biomass) indicated that the *Aspergillus niger* studied is a non-oleaginous strain.

The specific activity of lipogenic enzymes in Figure 2 showed no correlation with the cessation of lipid accumulation. This is different from what was reported to occur in oleaginous filamentous fungi such as *M. circinelloides*, *M. alpina,* and *C. bainieri* 2A1 where a profound decrease and diminishing ME specific activity occurred coincident to the cessation of lipid accumulation (Ratledge & Wynn 2002; Shuib et al. 2018; Wynn, Kendrick & Ratledge 1997). However, the high specific activity of ME was found throughout the whole cultivation time (120 h). It was contrast with what was reported in Chen et al. (2015) where high ME activity of oleaginous species was detected in the early phase of lipid biosynthesis (24 h) and then decreased in the later phase (68 h). This suggests that ME in non-oleaginous strains such as *A. niger* may not be directly involved in the lipid biosynthesis, where ME was reported as the key enzyme for NADPH generation that is essential for synthesis and accumulation of lipid in oleaginous strains (Liang & Jiang 2015; Wynn & Ratledge 1997). In many oleaginous filamentous fungi and yeast, the lipid content accumulated were significantly affected, either decreased or increased, when ME was inhibited or overexpressed, proving the crucial role of ME in lipogenesis (Li et al. 2013; Zhang, Adams & Ratledge 2007).



Errors bars based on three biological independent experiments

FIGURE 1. Profiles of growth and lipid accumulation in *A. niger* cultivated in 200 mL nitrogen-limited medium in 500 mL shake flask for 120 h, 200 rpm at 30 °C



Errors bars based on three biological independent experiments

FIGURE 2. Profiles of specific enzyme activity in *A. niger* cultivated in 200 mL nitrogen limited medium in 500 mL shake flask for 120 h, 200 rpm at 30 °C

TABLE 1. The results of liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Gel group	Protein detected after cultivation period of the culture (h)		
	24	72	120
Group 1 (4600-10,000 kDa)	Uncharacterised protein (A0A100IQY3)	Uncharacterised protein (A0A100IQY3)	Uncharacterised protein (A0A100IQY3)
Group 2 (1200-4600 kDa)	40S ribosomal protein S122 (A0A370CAD1	40S ribosomal protein S122 (A0A370CAD2	40S ribosomal protein S122 (A0A370CAD3
	FAS subunit alpha (A0A100IU03)	FAS subunit alpha (A0A100IU03)	FAS subunit alpha (A0A100IU03)
	FAS subunit beta (A0A370BIB1)	FAS subunit beta (A0A370BIB1)	FAS subunit beta (A0A370BIB1)
Group 3 (950-1200 kDa)	Uncharacterised protein (G3XT52)	Uncharacterised protein (G3XT52)	Uncharacterised protein (G3XT52)
Group 4 (830-950 kDa)	Phosphofructokinase (A0A254TU37)	Phosphofructokinase (A0A254TU37)	Phosphofructokinase (A0A254TU37)
Group 5 (650-830 kDa)	Glutamine synthetase (A0A319ADM1)	Glutamine synthetase (A0A319ADM1)	Glutamine synthetase (A0A319ADM1)
Group 6 (400-650 kDa)	Multifunctional tryptophan biosynthesis (A2QRH6)	Multifunctional tryptophan biosynthesis (A2QRH6)	Multifunctional tryptophan biosynthesis (A2QRH6)
Group 7 (245-400 kDa)	ATP citrate lyase (A0A117E036)	ATP citrate lyase (A0A117E036)	ATP citrate lyase (A0A117E036)
	Pyruvate carboxylase (A0A3F3RQ07)	Pyruvate carboxylase (A0A3F3RQ07)	Pyruvate carboxylase (A0A3F3RQ07)
	Acetyl CoA carboxylase (Q1JTV6)	Acetyl CoA carboxylase (Q1JTV6)	Acetyl CoA carboxylase (Q1JTV6)
	Pyruvate kinase (A0A254UBY1)	Pyruvate kinase (A0A254UBY1)	Pyruvate kinase (A0A254UBY1)
Group 8 (130-245 kDa)	Glucose 6 phosphate (P48826)	Glucose 6 phosphate (P48826)	Glucose 6 phosphate (P48826)
	Malic enzyme (A0A100ITQ3)	Malic enzyme (A0A100ITQ3)	Malic enzyme (A0A100ITQ3)
	NADP:isocitrate dehydrogenase (P79089)	NADP:isocitrate dehydrogenase (P79089)	NADP:isocitrate dehydrogenase (P79089)
	Glyceraldehyde-3-phosphate dehydrogenase (A0A254U566)	Glyceraldehyde-3-phosphate dehydrogenase (A0A254U566)	Glyceraldehyde-3-phosphate dehydrogenase (A0A254U566)
Group 9 (30-130 kDa)	6 phosphogluconate dehydrogenase (A0A100IJ08)	6 phosphogluconate dehydrogenase (A0A100IJ08)	6 phosphogluconate dehydrogenase (A0A100IJ08)
	Malate dehydrogenase (A0A370BUC0)	Malate dehydrogenase (A0A370BUC0)	Malate dehydrogenase (A0A370BUC0)
Group 10 (15-30 kDa)	heat shock protein (A0A100IIS3)	heat shock protein (A0A100IIS3)	heat shock protein (A0A100IIS3)

Lipid metabolon formed during lipogenesis helps in high lipid production of oleaginous organisms. This allows direct channel of NADPH needed to FAS for the accumulation of fatty acid in cells. This has been shown further to be achieved through the formation and dissociation of a multienzyme complex of six enzymes i.e., FAS, ME, ACL, ACC, PC, and MDH. The multienzyme complex was shown to be transient where its formation and dissociation were correlated to active and cessation of lipid biosynthesis, respectively (Shuib et al. 2018). In order to investigate the possibility of such existence (lipid metabolon) in *A. niger*, attempts were carried out to detect the metabolon by BN-PAGE coupled with LCMS/MS using cell free extracts prepared by gentle cell breakage i.e., protoplasting. According to the results, it indicated that no physical association was evident at any time point throughout the cultivation, as all the enzymes were observed to be as separated bands according to their molecular weight. However, in *C. bainieri* 2A1, these enzymes have been shown to be detected as a single band of approximately 3.2 MDa molecular weight, regardless of the individual size, when the cell free extracts of cultures obtained at the earlier phase of nitrogen limitation was subjected to similar procedures (Shuib et al. 2018). These suggest that there are no lipogenic metabolon formed in *A. niger*, which may be related to its non-oleaginous properties, even though all the lipogenic enzymes activities were detected. However, to validate the importance of lipogenic metabolon in oleaginous species more thorough studies are required.

CONCLUSIONS

A. niger displayed all the lipogenic enzyme activities, including ME, FAS, ACL, G6PDH, 6PGDH, and $NADP^+$:ICDH, yet lacking the ability to accumulate high lipid content. No evidence of lipogenic multienzyme complexes formed during lipogenesis was shown in the low lipid yield *A. niger*. This study suggests the significance of lipid metabolon association in accumulating high lipid content in oleaginous microorganisms.

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