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Biochemical Characterization for Lipid Synthesis in *Aspergillus niger*

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Abstract

A niger, a fungus which doesn't have high ability to production lipid, this fungus has been select to investigate the non oleaginicity. In this search, there are explorations about: i) growth profile ii) enzymes profile iii) isoforms. Growth profile shows that this fungus doesn't have ability to accumulate lipid more than 6% while bio mass are around 10g/l in spite of the presence of glucose in the media till the end of cultivation time and excision of nitrogen within 24 hrs. In enzyme study, we investigate all lipogenic enzymes Malic enzyme (ME), Fatty acid synthase (FAS), ATP: Citrate lays (ACL), NAD^+ isocitrate dehydrogenase (NAD^+ICDH), Glucose-6-phosphate (G6PD), and 6-phosphogluconate dehydrogenase (6PGD), all these enzymes show, activities till the end of cultivation time including ACL which is regarded the key enzyme to differentiate between the two species oleaginous and non oleaginous. So, there is no main reason to non oleaginicity for this fungus. A further experiment has been done using Polyacrylamide gel electrophoresis to identify ME isoforms. The result of Polyacrylamide gel electrophoresis shows multi isoforms (A, B, C, D & E), with low intensity of isoform E, the isoforms that may involve in lipid synthesis. We have now studied the biochemistry of *A.niger* grown under conditions designed to promote lipid accumulation and can now advance a coherent hypothesis to explain why *A niger* could not accumulate lipid more than 6%. So the absence of isoforme E is the main reason for non oleaginicity in *A niger.*

Key words: *A niger*, ME isoforms, lipid synthesis

Introduction:

Many microorganisms accumulate lipid as a reserve material [1]. The extent to which this lipid accumulation occurs in microorganisms varies depending on species, from

3%(w/w)to in excess of 60%(w/w)[2], N. crassa, P chrysogenum, C.tropicalis cannot accumulate mor than 10% [3,4], while *M.cirinelloides*, *M.alpina*, *C.echinulata*, *Y.lipolytica* can accumulate more than 25%[5,6,7,1]. So, what are the factors which control the amount of lipid production in different cells? Lipogenesis appears to be more closely regulated by the provision of substrates (acetyl – CoA and NADPH) then the flux of intermediates through the biosynthetic pathway. The evidence of ATP:Citrate lyase role in producing acetyl-CoA has been shown in yeasts and fungi [8,9,10]. No microbial species lacking ATP:Citrate lyase is able to accumulate more than about 10% w/w cell lipid [4].The possession of ATP:Citrate lyase alone ,however is insufficient to confer oleaginicity as several microorganisms are known to possess this enzyme and do not accumulate excessive amounts of lipids[11]. Other enzyme must be needed to ensure lipid accumulation [12].The involvement of malic enzyme in the key provision of NADPH directly for fatty acid biosynthesis has been provided[13,14]. Most previous researches have focused on the apparently crucial role malic enzyme plays in lipid metabolism in filamentous fungi[15], where it is considered to specifically provide the reductive reactions in fatty acid synthase with NADPH. Despite strong evidence that malic enzyme activity is a key factor in ensuring maximal lipid accumulation, the link between malic enzyme activity and lipid accumulation is uncertain. Fungi possessing activity of malic enzyme and ATP:Citrat Lays not certain that it could accumulate lipid, So why ? The existence of different isoforms of malic enzyme in some fungi [5,6] was the answer. Only one isoforms of malic enzyme may, therefore, be involved in lipid accumulation.

In this study we look for the biochemistry of *A niger* grown under conditions designed to promote lipid accumulation, study the lipogenic enzymes –fatty acid synthase , ATP:Citrate lyase and malic enzyme as well as to NADPH-generating enzymes-glucose-6-phosphate dehydrogenase , 6-phosphogluconate dehydrogenase, and NAD- isocitrate dehydrogenase , finally the involvement of malic enzyme isoforms, can advance a coherent hypothesis to explain why *A niger* couldn't accumulate lipid more than 6%.

Materials and Methods

Micro- organism and culture conditions

Aspergillus niger was maintained on potato dextrose agar (PDA) plates at 30ºC for 7 days. The medium for growing microbes was (modified Kendrik medium) [16] contained (g/L^{-1}) : Trace elements; $CaCl_2.2H_2O$, 0.1; $FeCl_3.6H_2O$, 0.008; $ZnSO_4.7H_2O$, 0.0001; $CuSO_4.5H_2O$, 0.001; $Co(NO_3).6H_2O$, 0.0001 and $MnSO_4.5H_2O$, 0.0001. Whereas glucose,30; Ammonium tartaret $(NH_4)_2.C_4H_4O_6$, 1; Buffer; KH_2PO_4, 7; Na_2HPO_4, 2. The activation factors were $MgSO_4.H_2O$, 1.5. Finally yeast extract, 1.5;. The pH was maintained to 6. After sterilization (121ºC for 15 minutes) the medium was inoculated with the spores at a concentration of (2×10^5) spores/ml, that produced by growing the fungi on PDA for 7 days at 30ºC. All cultivation experiments were performed in 500 ml Erlenmeyer flasks containing 200 ml of the above medium, incubated in a rotary shaker at 200 rpm and 30ºC, Figure. 1 shows *A.niger* under microscope taken from culture 7 day on PDA.

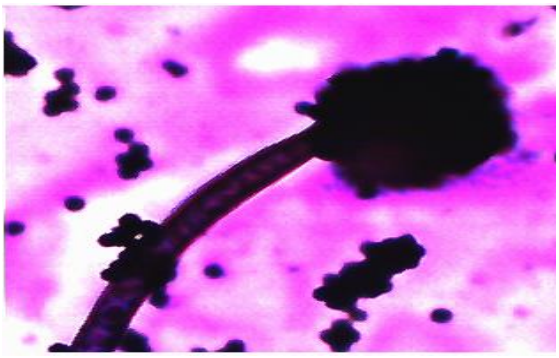

Fig. (1): *A.niger* under microscope taken from culture 7 day on PDA.

Analytical Methods

Total biomass (x) was harvested by filtration with a Whatman No. 1 filter paper, washed with distilled water at 4ºC temperature, frozen at – 20ºC, using lyophilizer to freeze–dried the mycelium, and then gravimetrically determined. The dry mycelium was homogenized, and lipids were extracted and separated with chloroform / methanol (2:1, v/v) following Folch method [17]. Total lipids were determined gravimetrically, while fatty acids were determined by gas chromatography (GC).

Cell–free Extracts Preparation

Cold distilled water (4ºC) was used for washing harvested mycelia, and enzyme extraction for the assay of enzyme activities were done by disrupting mycelia in an extraction buffer [18]. Centrifugation at 10,000 for 15 minutes at 4ºC. The protein in the clear supernatant was assayed following the protocol of Bradford [19]

Analysis of the Culture Supernatant

GOD kit has been used for determination of glucose concentration in the culture medium according to the manufacturer's instructions. Whereas indophenols test has been used for determined ammonium concentration in the culture [20].

Estimation of Enzyme Activities

The activities of glucose-6-phosphate dehydrogenase 6-PGDH, NAD-isocitrate dehydrogenase NAD-ICDH, fatty acid synthase FAS and ATP:citrate lyase ACL, were assayed as described by Wynn et al 1997[14].

Detection of Malic Enzyme Activity and its Isoforms

The activity of Malic enzyme in cell extracts was assayed according to the method of Has and Lardy [21]. Malic enzymes isoforms were distinguished by activity staining of gels from non-denaturing (native) polyacrylamide gel electrophoresis (PAGE). The gel was prepared following Hames in 1985[22] by fixing the concentration to 10% (W\V) acrylamide with completely absence of sodium dodecyl sulphate (SDS). Enzyme activity has been determined according to the method of Chang et. al. in 1991[23]. Activity staining of malic enzyme isoforms was performed by immersing the gel in to a solution containing phosphate buffer pH 7.4, 0.47 Mm $NADP^+$, 17.2 Mm L-malate, as a substrate 0.55 mg/ml nitroblutetrazolium and 0.097 mg/ml phenazine methosulfate for staining. The reaction later stopped by replacing the stain solution with 5% acetic acid.

Results and Discussion

Growth and Lipid Synthesis in *A. niger*.

Lipid synthesis in *A.niger* occurred within the 24 hrs of cultivation in closed system (batch culture). At the early few hours of culture, the mycelium accumulated as the culture period follow-up Figure.(2) Lipid yield showed increment in the concentration quickly from the first day of cultivation to the third day in maximum level 6%, where this might have been due to exhaustion of nitrogen, followed by using glucose to synthesize lipids. *A.niger* entered the logarithmic phase after a short time of growth, where it began to use the nitrogen sources and other compound inside the medium to reproduce mycelium. During this period, biomass accumulated around (6.5 g/l).

After depletion of nitrogen sources (at about 24 hours after inoculation), just (4%) of total lipid were synthesized at this time. Culture with presence of glucose and depletion of nitrogen, is the perfect culture for accumulate lipid [1,24 and 25], in spite of this, conditions *A.niger* could not accumulate lipid more than 6%.

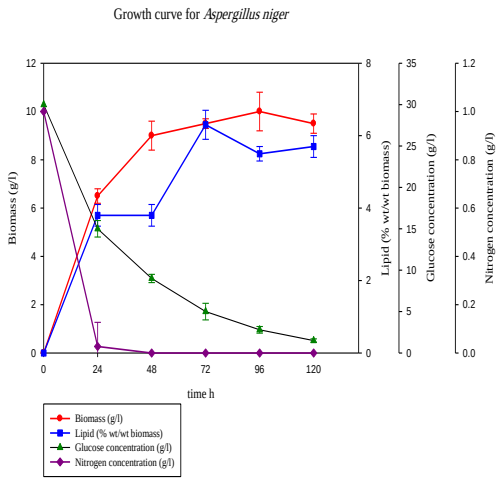

Fig.(2) Profiles of biomass g/L, lipid wt/wt biomass, glucose g/L and nitrogen g/L.for *A. niger* in nitrogen limited medium. The experiment was performed at 30°C for 120 hrs. with the culture being agitated at 250 rpm.

As well as it is could not produce more than 1.2% GLA (C18:3) as shown in Figure. (3), the principal cellular fatty acid was the (C18:1), the concentration of which changed within the growth, while (C18:2, C16 and C18) remained almost constant during growth as Figure. (3) shows.

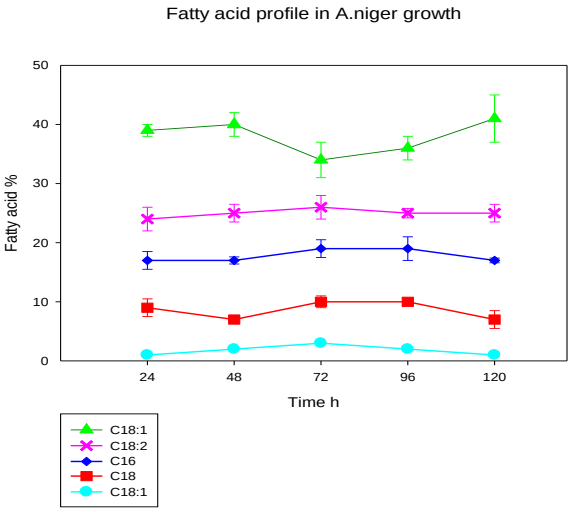

Fig. (3) Determination of fatty acids during growth of *Aspergillus niger.*

Activity of Key Enzymes During Lipid Synthesis in *A.niger*.

A range of enzymes involved, or potentially involved, in the lipid synthesis were studied. These enzymes included those belonging to the lipid biosynthetic pathway itself, FAS and ACL, which is implicated in the production of cytosolic acetyl-CoA for lipid synthesis in microorganism, and a range of enzymes potentially involved in the generation of NADPH for fatty acid synthesis, ME, NAD-ICDH, G-6-PDH and 6-PGDH. All those enzymes possessed specific activity in *A.niger* and most of the enzymes demonstrated a similar developmental profile of activity throughout the cultivation time as Figure. (4) shows. FAS and ACL showed activity in all the cultivation time, so theoretically the production of acetyl-CoA occured in all the cultivation time, in the other hand in this type of fungus, ACL was not regarded as precursor for lipid synthesis. The enzyme was present in all yeasts capable of accumulating lipid to 20% or more of their biomass while it was inactive in yeasts and could not accumulate lipid [4]. So, we should look for other enzymes, which may cause, the non oleagincity of *A.niger*. The NADPH generated enzymes were studied, and no

any evidence was involved to those enzymes in inhibits lipid synthesis in this fungus. NAD-ICDH, 6GPD and G-6-PD showed high activity, also ME shows activity in all cultivation time, all the previous studies proved that ME was the major source of NADPH for lipid accumulation.

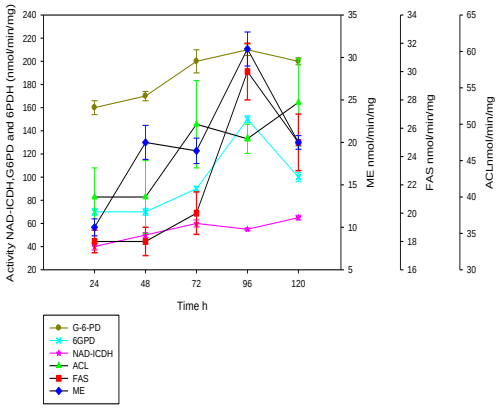

Fig.(4) Activity of key enzymes during lipid synthesis in A.niger

Malic Enzyme Isoforms in Non Oleaginous Species *A. niger* and it Is Related to Lipid Biosynthesis.

A. niger, this species proved that it could not accumulate lipid more than 6% as Figure.(2) shows, while malic enzyme activity was detected until the end of cultivation time as well as the presence of ACL and FAS, those results have been shown in Figure. (4) .*A. niger* manifested five isoforms with low intensity of E band as appeared in Figure. (5). Our previous results suggested the involvement of this isoform in lipid synthesi in *C. bainieri* 2A1. Similar observation was reported in *Mucor circinelloides and Mortierella alpine* by Song et al. in 2001 and Zhang et al. 2008 [5,6] which proved that this band is related to lipid synthesis in oleaginous fungi. When we compared this result with our previous result on *Cunninghamella bainieri* in nitrogen limited media in Figure.(5), we strongly suggest that the key lipogenic isoform was isoform E, this result agree with Aidil abdulhameed results in 2014 [26]. It is hypothesis that, the *A.niger* didn't shows good intensity of key isoform (E), the isoform that related to lipid biosynthesis pathway in lipid accumulation with its relations on gene expression [27,28]

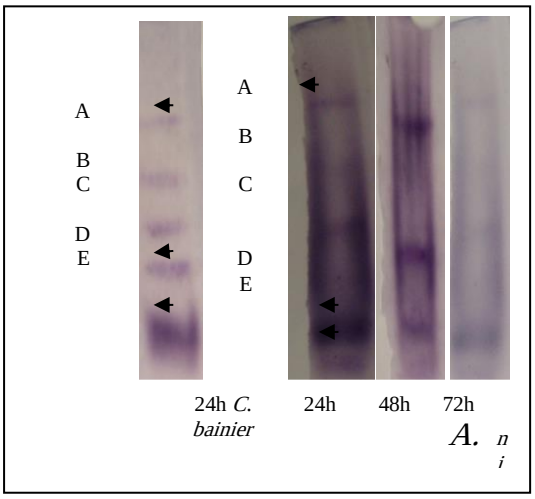

Fig. (5) Isoforms of malic enzyme in *A. niger* at different growth stages, 24-72 hrs. in 500 ml Erlenmeyer flasks containing 200 ml of growth medium nitrogen limited. The isoforms were separated by native PAGE then visualized using activity stain. The arrows indicate isoforms of ME present at different culture times.

Conclusion

Finally we conclude a hypothesis that the non oleagnecity of *A.niger* species relate to lock of isoform E (one of the malic enzyme isoforms
which is previously proven its role in lipid synthesis).

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التوصيف الكيميائي الحياتي لانتاج الدهون في فطر الاسبارجيلس

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الخلاصة

فطر الاسبارجلس فطر معروف بعدم قابليته على انتاج الدهون، اختير هدا الفطر لدراسة اسباب عدم انتاج الدهون في بعض الفطريات. تم التطرق الى منحنى نمو الفطر، منحنيات الانزيمات مع النمو، المتناضرات الانزيمية لانزيم المالك. منحنى النمو لفطر الاسبارجلس اظهر عدم قابلية هدا الفطر على تجميع الدهون اكثر من 6% في حين كتلة الوزن الجاف 10 g/l ،على الرغم من وجود الكلوكوز في الوسط الزرعي حتى نهاية وقت النمو ونفاد وجود النايتروجين حلال ال 24 ساعة الاولى من النمو. تم دراسة الانزيمات المسؤوله عن ايض الدهون انزيم المالك، انزيم الفاتي اسد سينثيتيز، و ال ائ تي بي ستريت لايس، الايزوستريت ديهايدروجينيز، كلوكوز 6 فوسفيت ديهايدروجينيز و6 فوسفوكلوكونيت ديهايدروجينيز. اظهرت هده الانزيمات فعالية حتى نهاية فترة النمو بما فيها انزيم ال اي تي بي ستريت لايز الدي يعتبر المفتاح للتمييز بين انواع الفطريات المنتجه للدهون والفطريات الغير منتجه للدهون. لدالك لا يوجد سبب رئيسي لعدم انتاج الدهون في فطر الاسبارجلس . تجارب اضافية نفدت باستخدام هلام البولي اكريلامايد في تقنية الترحيل الكهربائي لتشخيص المتناظرات الانزيمية للانزيم المالك. اظهرت النتائج العديد من متناظرات الانزيم A,B,C,D & E مع ظهور شده واطئة من المتناضر الانزيمي E ،المتناظر الدي ممكن ان يكون السبب الرئيس في ايض الدهون. لذلك تم دراسة الكيمياء الحيوية لنمو فطر الاسبارجلس تحت ظروف صممت الى انتاج الدهون للوصول الى الاسباب الرئيسة في عدم قابلية هدا الفطر على انتاج الدهون وكان الاستنتاج هو غياب المتناظر الانزيمي E يعد السبب الاساسي في عدم قابلية الفطر على الانتاجية.

الكلمات المفتاحية: فطر الاسبارجلس، متناظرات انزيم المالك، بناء الدهون