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**Improved production of citric acid by *Yarrowia lipolytica* utilising
NBD palm olein as the carbon source**

by

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ABSTRACT

In ^{an} efforts to diversify and increase the usage of palm oil, attempts of using *Yarrowia lipolytica* to produce citric acid grown on NBD palm olein were conducted. The choice for this microorganism was due to its strong ability to produce lipase and to utilise lipids as the carbon sources. On NBD palm olein this yeast performed much better as compared to that on glucose for citric acid production. In order to improve the citric acid production, mutants were induced by treating logarithmic cells with N-methyl-N'-nitro-N-nitrosoguanidine (NTG). Initially, selection was based on sensitivity to monofluoroacetate (MFA) and an inability to grow on citrate. This indicated that the mutants have defected in the functioning of aconite hydratase activity, which can lead to accumulation of citric acid. Secondly, the strains obtained from the first selection were further tested on minimal medium incorporated with 0.1% bromocresol green as pH indicator, high acid producers formed large yellow halos.

A mutant F21, derived from ATCC 8661 (Laboratory strain no. M240) was isolated but despite its favourable characteristics, production of citric acid did not increase significantly compared to parent strain. Therefore, a second mutagenesis was carried out on F21, which led to the isolation of F21A. Strain F21A showed better growth on NBD palm olein and produced 26% (10.8 g/l) higher level of citric acid on medium containing 2% NBD palm olein compared to its parent strain of M240 (8.0 g/l). On medium containing 4% NBD palm olein F21A produced 50% (7.8 g/l) higher level of the same acid production compared to its parent strain of M240 (4.0 g/l). As comparison, strain M243 (IFO no. 1545/S-22) which has been used as a strain to produce citric acid in n-paraffin, was also grown in both concentrations (2% and 4% NBD palm olein). Other characteristics of M243 were, its sensitivity to MFA (0.1%), aconitate hydratase deficient and lower production of isocitric acid. Strain M243 showed better growth and production of citric acid on 2% NBD palm olein and produced 6% (11.5 g/l) higher level of citric acid compared to F21A. However, in medium containing 4% NBD palm olein it appeared that F21A much superior in growth and the production of citric acid, 43.6 % (7.8 g/l)

compared to M243 (4.4 g/l). Strain F21A did not perform well in medium containing glucose. The citric acid production was reduced to 2.5 g/l.

F21A showed better characteristics related to citric acid production compared to its parent strain. It was able to growth in higher concentration of NBD palm olein (4%). Apart from lipases activity, growth in high concentration of palm olein, also affected enzymes of TCA cycle and glyoxylate cycle. The activity of citrate synthase ($2.8 \mu\text{mol/mg protein/min}$) was increased by 32.8% compared to its parent strain M240.

At the maximum level of aconite hydratase activity, strain F21A showed $0.24 \mu\text{mol/mg protein/min}$ on 2% NBD palm olein and $0.19 \mu\text{mol/mg protein/min}$ in 4% NBD palm olein. Comparing with its parent strain and M243, F21A showed 20.85 and 57.2% higher level in activity of this enzyme (ACH) on 2% NBD palm olein. On 4% NBD palm olein, F21A showed 17.4% and 51.6% higher level in activity of ACH compared to its parent strain and M243 respectively.

The high activity of ACH was to fulfil high demand in the functioning of TCA and glyoxylate cycle when cells were growing actively in oil. The two enzymes of glyoxylate pathway which are exclusively localised in peroxisomes namely; isocitrate lyase and malate synthase showed high activities in all stains. The activity of isocitrate lyase in F21A was 23.5% higher ($0.98 \mu\text{mol/mg protein/min}$) on 2% NBD palm olein compared to its parent strain ($0.75 \mu\text{mol/mg protein/min}$) and 3.1% higher when compared to M243 ($0.95 \mu\text{mol/mg protein/min}$). On 4% NBD palm olein F21A ($1.5 \mu\text{mol/mg protein/min}$) showed 16.75 and 22% in acitivity of isocitrate lyase compared to M240 ($1.25 \mu\text{mol/mg protein/min}$) and M243 ($1.17 \mu\text{mol/mg protein/min}$) respectively. The maximum level attained by F21A in malate synthase was $1.88 \mu\text{mol/mg protein/min}$ on 2% NBD palm olein which was 36% higher compared to it parent strain ($1.3 \mu\text{mol/mg protein/min}$) after two days of incubation. This value was 46.3 % higher when compared to M243 ($1.01 \mu\text{mol/mg protein/min}$). On 4% NBD palm olein F21A ($2.38 \mu\text{mol/mg protein/min}$) produced 40% and 30.7% in activity of malate synthase compared to M240 ($1.5 \mu\text{mol/mg protein/min}$) and M243 ($1.63 \mu\text{mol/mg protein/min}$) respectively.

The activity of both enzymes was suppressed when the yeast was grown on medium containing glucose as the carbon source.

In the TCA cycle, the isocitrate is further oxidised by the enzyme isocitrate dehydrogenase. When F21A was grown in 2% NBD palm olein the level of this enzyme increased by 23.2% compared to M240 and 57.9% when compared to M243. On 4% NBD palm olein, F21A was 20.1% (0.1 $\mu\text{mol}/\text{mg protein/min}$) lower in activity compared to M240 (0.126 $\mu\text{mol}/\text{mg protein/min}$) and 58% higher of activity compared to M243 (0.042 $\mu\text{mol}/\text{mg protein/min}$)

There is a possibility that the changes in the characteristic of F21A was due mutation (s) in a regulatory gene(s) since it seemed to have a pleiotropic effects. It was rather unfortunate that genetic analysis of F21A could not be carried to ascertain the mutated gene(s). This study would give valuable information on the nature of the mutation. Under experimental conditions employed strain F21A failed to mate with the haploid strains ATCC 32338 A *ade* 1 or ATCC 32339 B *trp* 1 and did not show ability to sporulate. Spore analysis was not possible. The mating type of F21A was inconclusive.

The upscaling studies on F21A were also initiated. Growth capabilities in fermenter were comparable to shake flask. However, further modification and adaptation were needed to maintain production level.

The initial results on the development of the transformation system in this yeast are promising, however no doubt there are still substantial improvements to be explored. Success on attaining the expression system would enable any genes to be cloned into *Y. lipolytica*. A potential vector that has been identified to be a useful in the transformation of *Y. lipolytica* in future is pINA230.

ABSTRAK

Dalam usaha untuk memperluas dan meningkatkan penggunaan minyak kelapa sawit, kajian ke atas *Yarrowia lipolytica* untuk menghasilkan asid sitrik dengan menumbesarkannya di atas NBD palm olein telah dilaksanakan. Pemilihan organisma ini berdasarkan keupayaannya yang tinggi untuk menghasilkan lipase, dan ini membolehkan ia menggunakan lipid sebagai sumber karbon. Perbandingan penghasilan asid sitriknya telah dilakukan dengan menumbesarkan yis ini di atas NBD palm olein dan glukosa. Ia menunjukkan penghasilan asid yang jauh lebih baik jika yis ini ditumbesarkan di atas lipid. Untuk meningkatkan lagi penghasilan asid sitrik, yis ini telah diaruh dengan merawat sel-sel yang berada pada peringkat log dengan menggunakan N-metil-N'-nitro-N-nitrosoguanidin (NTG). Pengaruh ini bertujuan untuk mendapatkan mutan-mutan. Pada peringkat permulaan, pemilihan berasaskan kepada kesensitifannya terhadap monofluoroacetat (MFA) dan kebolehannya bertumbesaran di atas sitrat. Ini menunjukkan mutan-mutan kepincangan fungsi akonitat hydratas dan keadaan ini menggalakkan penghasilan asid sitrik. Strain-strain yang terpilih, pada peringkat pertama ini, selanjutnya diuji di atas bahantara minima yang mengandungi 0.1% bromokresol hijau sebagai penunjuk pH, penghasil-penghasil asid yang tinggi akan membentuk bulatan kuning yang besar pada koloni yang terbentuk.

Mutan F21, diasinkan dari aruhan ATCC 8661 (Strain makmal bernombor M240) dan memiliki ciri-ciri yang dikehendaki, walau bagaimanapun peghasilan asid sitriknya tidak meningkat jika dibandingkan dengan strain induk, M240. Oleh itu mutagenesis kali kedua telah dilakukan, dan membawa kepada pengasingan F21A. Strain F21A menunjukkan pertumbuhan yang lebih baik di atas NBD palm olein dan menghasilkan 26% (10.8 g/l) asid sitrik, yang lebih tinggi di atas bahantara yang mengandungi 2% NBD palm olein berbanding dengan strain induknya, M240 (8.0 g/l). Di atas bahantara yang mengandungi 4% NBD palm olein pula, F21A menghasilkan 50% (7.8 g/l) asid sitrik lebih tinggi, berbanding dengan strain induk, M240 (4.0 g/l). Sebagai perbandingan, strain komersial M243 (Bernombor

IFO 1545/S-22) yang telah digunakan bagi penghasilan asid sitrik dari n-paraffin, juga ditumbesarkan pada kedua-dua kepekatan (2% dan 4% NBD palm olein). Ciri-ciri lain bagi M243 ialah ia sensitif terhadap MFA, kepincangan fungsi akonitat hidratase dan rendah penghasilan asid isositrat. Strain M243 menunjukkan tumbesaran dan penghasilan asid sitrik yang lebih baik di atas 2% NBD palm olein dan pengeluaran asid ini 6% (11.5 g/l) lebih tinggi berbanding dengan strain F21A. Walau bagaimanapun di dalam bahatara yang mengandungi 4% NBD palm olein F21A menunjukkan ciri-ciri yang lebih superior dari segi tumbesaran dan penghasilan asid sitrik, iaitu 43.6% (7.8 g/l) penghasilan asid sitrik berbanding dengan M243 (4.4 g/l). Strain F21A tidak menunjukkan ciri tumbesaran dan penghasilan asid sitrik yang baik bila ditumbesarkan di dalam bahantara glukosa, malah penghasilan asid sitriknya menurun berbanding dengan bahantara yang mengandungi NBD palm olein (2% dan 4%).

F21A juga menunjukkan sifat-sifat terubahsuai yang baik untuk penghasilan asid sitrik berbanding dengan strain induk, M240. Ia berkebolehan bertumbesaran di atas kepekatan NBD palm olein yang tinggi. Kebolehannya bertumbesaran dengan mengeluarkan lipase yang tinggi, juga mempengaruhi enzim-enzimnya yang terlibat di dalam kitaran trikarboksilat (TCA) dan glioksilat. Aktiviti sitrat sintasenya telah ditingkatkan kepada 32.8% berbanding strain induknya, M240.

Pada tahap aktiviti maksima akonitat hidratase, strain F21A telah menghasilkan 0.24 $\mu\text{mol}/\text{mg protein/min}$ di dalam bahantara yang mengandungi 2% NBD palm olein dan 0.19 $\mu\text{mol}/\text{mg protein/min}$ di dalam bahantara 4% NBD palm olein. Perbandingan yang dilakukan ke atas strain induk, M240 dan M243, F21A telah menunjukkan 20.8% (M240) dan 57% (M243) lebih tinggi dalam aktiviti aconitat hidratasenya berbanding dengan strain induk dan M243, masing-masing.

Tingginya permintaan aktiviti akonitat hidratase adalah untuk memenuhi fungsi kitaran TCA dan glioksilat, dimana ketika ini sel-sel sedang membahagi secara aktif apabila ditumbesarkan di dalam lipid. Dua enzim yang telibat di dalam kitaran glioksilat yang berada di dalam peroksisom: isositrat lyase dan malat sintase menunjukkan aktiviti yang tinggi untuk semua strain yang ditumbesarkan di dalam lipid. Aktiviti isositrat lyase strain F21A adalah 23.5% (0.98 $\mu\text{mol}/\text{mg protein/min}$)

di dalam bahantara 2% NBD palm olein berbanding dengan strain induk, M240 (0.78 $\mu\text{mol}/\text{mg protein/min}$) dan 3.1% lebih tinggi jika dibandingkan dengan M243 (0.95 $\mu\text{mol}/\text{mg protein/min}$). Di atas bahantara mengandungi 4% NBD palm olein, F21A (1.5 $\mu\text{mol}/\text{mg protein/min}$) menunjukkan 16.7% dan 22% peningkatan aktiviti isositrat lyase berbanding dengan strain induk M240 (1.25 $\mu\text{mol}/\text{mg protein/min}$) dan M243 (1.17 $\mu\text{mol}/\text{mg protein/min}$), masing-masing. Tahap maksima yang dicapai oleh F21A di dalam aktiviti malat sintase pula ialah 1.88 $\mu\text{mol}/\text{mg protein/min}$ di atas bahantara 2% NBD palm olein, di mana 36% lebih tinggi berbanding dengan strain induk, M240 (1.3 $\mu\text{mol}/\text{mg protein/min}$) selepas dua hari inkubasi, dan 46.3% lebih tinggi bila dibandingkan dengan M243 (1.01 $\mu\text{mol}/\text{mg protein/min}$). Di atas bahantara 4% NBD palm olein, F21A (2.38 $\mu\text{mol}/\text{mg protein/min}$) menghasilkan 40% dan 30.7% lebih tinggi aktiviti malat sintase berbanding dengan strain induk, M240 (1.5 $\mu\text{mol}/\text{mg protein/min}$) dan M243 (1.63 $\mu\text{mol}/\text{mg protein/min}$) masing-masing. Aktiviti-aktiviti kedua enzim di tindas apabila yis ini ditumbesarkan di atas bahantara yang mengandungi glukosa sebagai sumber karbon.

Di dalam kitaran TCA, isositrat akan dioksidasikan selanjutnya oleh enzim isositrat dehidrogenase. Apabila F21A telah ditumbesarkan di atas bahantara 2% NBD palm olein, tahap enzim ini meningkat sebanyak 23.2% berbanding dengan strain induk, M240 dan 57.9% jika di bandingkan dengan M243. Di atas bahantara 4% NBD palm olein, F21A telah menunjukkan 20.1% (0.1 $\mu\text{mol}/\text{mg protein/min}$) lebih rendah aktiviti berbanding dengan strain induk, M240 (0.126 $\mu\text{mol}/\text{mg protein/min}$) dan 58% lebih tinggi aktiviti berbanding dengan M243 (0.048 $\mu\text{mol}/\text{mg protein/min}$).

Ciri-ciri yang terdapat pada strain F21A kemungkinan di sebabkan oleh mutasi yang berlaku ke atas gen regulasinya, yang membawa kepada kesan-kesan pleiotropik. Walau bagaimanapun, kajian sehingga ketahap gen termutasi itu tidak dapat ditentukan kerana kegagalan meneruskan analisis genetik bagi F21A. Pembelajaran ini amat berharga kerana maklumat yang diperolehi akan menerangkan keadaan mutasi tersebut. Di dalam keadaan ujikaji yang dilakukan,

strain F21A gagal untuk dikacukkan samada terhadap strain ATCC 32338 A *ade1* atau ATCC 32339 B *trp1*, kegagalan untuk mengacuk, menyebabkan spora tidak terbentuk. Oleh itu, analisis spora tidak dapat dijalankan dan jenis kacukan (mating type) F21A tidak dapat ditentukan.

Perancangan bagi menghasilkan asid sitrik pada jumlah yang tinggi dengan menggunakan fermentor juga telah dilakukan. Perbandingan kebolehan yis ini bertumbesaran di dalam fermentor dan kelalang bergoncang telah dilakukan. Bagaimanapun perubahan dan penyesuaian diperlukan apabila fermentasi dilakukan di dalam fermentor, supaya tahap penghasilan asid sitrik yang terbentuk dari kelalang bergoncang dapat dikekalkan dalam sistem fermentor.

Keputusan awal ke atas perkembangan sistem transformasi yis ini boleh menjanjikan sesuatu, bagaimanapun penemuan-penemuan lain yang memantapkan lagi maklumat untuk menyumbang ke arah ini masih perlu diterokai. Kejayaan sehingga ke tahap ekspresi, membolehkan sebarang gen diklonkan ke dalam *Y. lipolytica*. Vektor yang berpotensi yang dikenal pasti berguna dalam transformasi pada masa akan datang adalah pINA230.

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ABBREVIATIONS

ACH	Aconitate hydratase
BPB	Bromophenol blue
BSA	Bovine serum albumin
CIAP	Calf intestinal alkaline phosphatase
DTNB	5,5'-dithio-bis(2-nitrobenzoic acid)
EDTA	Ethylenediaminetetra-acetic acid
EMS	Ethylmethane sulphonate
HPLC	High performance liquid chromatography
ICDH	Isocitrate dehydrogenase
MFA	Monofluoroacetate
MFC	Monofluorocitrate
MgSO ₄ .7H ₂ O	Magnesium sulphate 7-hydrate
µM	Micromolar
NBD	Neutralised bleached deodorised
NTG	N-methyl-N'-nitro-N-nitrosoguanidine
PEG	Polyethylene glycol
PVA	Polyvinyl alcohol
SDS	Sodium dodecyl sulphate
TBE	Tris-Boric-EDTA
TE	Tris-HCL-EDTA
TPN	Triphosphopyridine nucleotide
µg	Microgram
YEPA	Yeast extract peptone potassium acetate
YEPD	Yeast extract peptone glucose

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