ENHANCED PRODUCTIVITY OF LIPID AND GAMMA-LINOLENIC ACID BY Cunninghamella bainieri 2A1 IN REPEATED BATCH CULTURES

MUSLEEHAWEATEE WORNGOR, AIDIL ABDUL HAMID and WAN MOHTAR WAN YUSOFF*

School of Biosciences and Biotechnology,
Faculty of Science and Technology,
University Kebangsaan Malaysia,
Bangi, 43600, Selangor, Malaysia.
E-mail: wantar@pkrisc.cc.ukm.my

ABSTRACT

In repeated batch culture, the cultures were able to maintain their productivity higher than that obtained in batch culture where the highest lipid content and concentration were achieved at 34.36% (g lipid/g biomass) and 3.02 g/L, respectively, compared to that obtained in batch culture at 27.39% (g lipid/g biomass) and 1.87 g/L, respectively. The highest gamma-linolenic acid (GLA) content and concentration were 10.54% of total lipid and 0.28 g/L, respectively, compared to that obtained in batch culture at 9.81% and 0.16 g/L, respectively. The highest maximum biomass concentration was 14.97 g/L. This was achieved using nitrogen limited medium consisting of glucose as the carbon source and ammonium tartrate as the nitrogen source. Lipid concentration increased 14.11% after the second repeat and continued to 45.40% in the fourth repeat after 300 hr of fermentation. Therefore, cumulative productivities of lipid in each cycle of a repeated batch culture increased with increasing number of cycles. The cumulative productivity of lipid and GLA were 0.0260 g/L/hr and 0.0024 g/L/hr, respectively, which were 3.02 fold and 3.43 fold higher than that achieved from batch culture.

ABSTRAK

Kultur kelompok ulangan mampu menyelenggara produktiviti yang lebih tinggi berbanding kultur kelompok. Kandungan dan kepekatan lipid tertinggi dicapai sebanyak 34.36% (g lipid/g biojisim) dan 3.02 g/L masing-masing berbanding dengan yang diperoleh dalam kultur kelompok hanya sebanyak 27.39% (g lipid/g biojisim) dan 1.87 g/L masing-masing. Kandungan dan kepekatan GLA tertinggi adalah sebanyak 10.54% daripada lipid total dan 0.28 g/L masing-masing berbanding dengan yang diperoleh dalam kultur kelompok sebanyak 9.81% daripada lipid total dan 0.16 g/L masing-masing. Kepekatan biojisim maksimum yang tertinggi adalah sebanyak 14.97 g/L. Kesemua data dicapai dengan menggunakan medium terhad nitrogen yang mengandungi glukosa sebagai sumber karbon dan ammonium tartarat sebagai sumber nitrogen. Kepekatan lipid meningkat sebanyak 14.11% diakhir ulangan kedua dan terus meningkat sehingga mencapai 45.40% diakhir ulangan keempat selepas 300 j fermentasi. Oleh itu, produktiviti kumulatif lipid dalam setiap ulangan kultur kelompok ulangan meningkat mengikut jumlah ulangan. Produktiviti kumulatif lipid dan GLA dalam kultur kelompok ulangan adalah 0.0260 g/L/j dan 0.0024 g/L/j masing-masing dan adalah lebih tinggi daripada pencapaian kultur kelompok sebanyak 3.02 dan 3.43 ganda.

Key words: Gamma – linolenic acid, repeated batch culture, Cunninghamella bainieri 2A1

INTRODUCTION

Gamma-linolenic acid (GLA;18:3) is an essential fatty acid, which is a precursor of prostaglandins. It is essential for patients who suffer from diabetes, cancer, ageing and infection (De et al., 1999). GLA is found in certain plant seed oils (Kleiman, 1988) and algae (De et al., 1999; Hirano et al., 1990), but it can also be produced by microorganisms, e.g. by strains belonging to the fungal order Mucorales (Fukuda & Morikawa, 1987; Hansson & Dostalek, 1988; Hansson et al., 1989).

Production of GLA by filamentous fungi is commonly achieved in batch culture (BC) but with disadvantages such as requirement for washing and sterilizing the fermenter as well as inoculum
preparation time. The requirement can be fulfilled by repeated batch culture (RBC) technique involving taking out a fixed volume of culture and replacing it with fresh medium to increase productivity. Several reports have been cited for the improvement of productivities of L (+)-lactic acid (Yin et al., 1998), cellulolytic and xylanolytic enzymes (Srinivasan et al., 2001) bacterial cellulose (Naritomi et al., 2002), laccase (Jang et al., 2002), lipase (Yang et al., 2005), kojic acid (Wan et al., 2005), and pediocin PA-1 (Naghmouchi et al., 2008). Since some cells from the final culture broth are usually reused in RBC, it has the advantage to become the inoculum for the next RBC run. Compared to BC, RBC minimizes the time for washing and sterilizing the fermenter as well as the inoculum preparation time. In fact, the fermenter will only be washed and sterilized once and involves only one inoculum preparation.

Another important aspect of GLA production is the inherent capability of the microorganisms employed. Recently, we reported that an oleaginous fungus, Cunninghamella bainieri 2A1, isolated from soil samples has the ability to produce high lipid and GLA content (Abdul Hamid et al., 2001). So far, there is no report on lipid and GLA production using RBC. Therefore, the aim of this work was to explore the use of RBC for enhanced productivity of lipid and GLA by C. bainieri 2A1.

**MATERIALS AND METHODS**

**Microorganisms**

*Cunninghamella bainieri* 2A1 was obtained from Microbiology Laboratory (3157), School of Biosciences and Biotechnology, Faculty of Science and Technology, UKM. The culture was grown on potato dextrose agar (Oxoid) (PDA), stored at 4ºC and Technology, UKM. The culture was grown on Biosciences and Biotechnology, Faculty of Science from Microbiology Laboratory (3157), School of Technology, Switzerland for 60 hr without pH control in BC. For the RBC, culture was grown at 30ºC, and agitated at 250 rpm for 300 hr also without pH control. 10 ml samples were taken every 12 hr. After 60 hr cultivation the culture (80 ml) was transferred to another flask containing 320 ml fresh nitrogen limited medium using sterilized micro-pipette. Then, the culture was incubated at the same conditions. This procedure was repeated four times at 60 hr intervals.

**Growth Measurement**

The dry weight of biomass was determined by filtering 10 ml of cultures followed by washing with 30 ml distilled water and drying at 80ºC until constant weight of mycelium was achieved. Triplicate data are presented.

**Glucose and Ammonium Tartrate Analyse**

Glucose was assayed using GOD-PAP kit (Boehringer Mannheim). Ammonium tartrate was assayed according to the method of Chaney & Marbach (1962). Triplicate data are presented.

**Lipid Extraction**

Lipid was recovered in minimal volume of extract after the freeze dried mycelia was ground into powder in a pestle and mortar followed by an overnight extraction using chloroform 100 ml: methanol 50 ml (2:1). The extracted lipid was filtered and washed with 1% NaCl (150 ml) and distilled water (150 ml) was added twice to remove any residual methanol. Chloroform was removed by evaporation using rotary evaporator (BUCHI Rotavapor R-124, Switzerland) according to the method of Folch et al (1957). Triplicate data are presented.

**Determination of Fatty Acid Composition**

The fatty acid composition was determined by gas-liquid chromatography of the fatty acid methyl esters (FAMES) prepared by reaction with sodium methoxide. FAMES were analyzed according to IUPAC 2.301 (1987) chromatograph equipped with a flame ionization detector (FID). A capillary gas [Shimadzu GC7A] equipped with a HP23 column (30 m & 0.32 mm in length and diameter, respectively) was used. Nitrogen was used as carrier at a flow rate of 0.53 ml/min. The temperature program was as follows: an initial temperature of 90ºC, increased to 130ºC at 20ºC/min, followed by an increase at 4ºC/min to 200ºC, and at 1ºC/min to 220ºC. Peaks (methylated fatty acids) were
identified using authentic fatty acid methyl ester standard supplied by Sigma Chemical Co. (# 189-19) (Abdul Hamid et al., 2001). Triplicate data are presented.

Calculation of Productivity

Productivities of lipid and GLA in BC and RBC were calculated using Equation (1) and (2) (Naritomi et al., 2002), respectively.

\[
P_{\text{batch}} = \frac{C_f - C_i}{T_p + T_m + T_r}
\]

Equation (1)

Where \(C_f\) is the final lipid or GLA concentration in BC (g/L); \(C_i\) the initial lipid or GLA concentration in BC (g/L); \(T_p\) is the operation time prior to batch culture including seed preparation, washing and sterilizing shake flask or fermenter (hr); \(T_m\) is the cultivation time (hr); and \(T_r\) is the time for product recovery (hr).

\[
P_{\text{repeat batch}} = \frac{C_{n} + C_{n+1} + \ldots + C_{n}}{(T_p + T_{m1} + T_{m2} + \ldots + T_{mn}) + T_r)
\]

Equation (2)

where \(C_{n}\) is the final lipid or GLA concentration at \(n\)th cycle in RBC (g/L); \(C_{n}\) is the initial lipid or GLA concentration at \(i\)th cycle in RBC (g/L); \(T_p\) is the operation time prior to the first BC including seed preparation, washing and sterilizing shake flask or fermenter (hr); \(T_{mi}\) is the culture time at \(i\)th cycle in RBC (hr); and \(T_r\) is the time for product recovery (hr).

In Equation (1) and (2), \(T_p\) and \(T_r\) were assumed at 60 hr and 97 hr, respectively based on the actual time in this study.

Statistical Analysis

Statistical analysis was done using One-Way ANOVA to compare means between data. Duncan analysis was done using program SPSS 11.5 for Windows \((P<0.05)\) to determine the significant differences among the means.

RESULTS AND DISCUSSION

Lipid and Gamma-linolenic acid Production in Batch Culture (BC) Compared to Production in Repeated Batch Culture (RBC)

Growth profile of \(C. bainieri\) 2A1 from BC is shown in Fig. 1. The BC performed for 60 hr showed that maximum biomass concentration was 12.87 g/L with biomass yield \((Y_{x/s})\) amount of lipidless biomass per substrate utilized at 0.411. Glucose was consumed at about 13.66 g/L after 60 hr cultivation. Whilst, ammonium tartrate depleted after 24 hr. Lipid content increased 2.64 fold after 24 hr compared to lipid content obtained at 12 hr. This indicated that the rate of lipid accumulation increases when concentration of ammonium tartrate reduces. Maximum lipid content and concentration were 27.39\% (g lipid/g biomass) and 1.87 g/L, respectively. Lipid yields, \(Y_{L/s}\) (amount of lipid per substrate utilized) and \(Y_{L/x}\) (amount of lipid per biomass produced) were 0.140 and 0.240, respectively, with productivity of lipid at 0.0086 g/L/hr. Whilst, maximum GLA content and concentration were recovered at 9.81\% of total lipid and 0.16 g/L, respectively. GLA yields, \(Y_{G/s}\) (amount of GLA per substrate utilized), \(Y_{G/x}\) (amount of GLA per biomass produced) and \(Y_{GL}\) (amount of GLA per lipid produced) were 0.012, 0.021 and 0.086, respectively, with productivity of GLA at 0.0007 g/L/hr.

Profile of biomass production from RBC is shown in Fig. 2. The RBC performed over 300 hr showed that maximum biomass concentration achieved in each cycle was between 11.63 to 14.97 g/L while biomass yield \((Y_{x/s})\) in each cycle was between 0.282 to 0.413. This represented an achievement of biomass yield compared to that from BC five times in a row. Glucose was consumed by \(C. bainieri\) 2A1 in each cycle at about 15.00 g/L maintained after 60 hr. This indicates that the rates of utilizing of glucose were not affected. This is also true in nitrogen assimilation, which ammonium tartrate was depleted in each cycle after 24 hr. This showed that repeated cultivation did not affect growth. This is further supported by data that showed similar concentration of maximum biomass achieved. The biomass yield achieved in the RBC is similar to that obtained in BC. This showed that the efficiency of glucose utilized for biomass growth was also not affected even after the forth cycle.

Lin et al. (2001) reported that cell density was stable when using repeated batch culture in the production of lipase by Acinetobacter radioresistens which was similar to that obtained in this work. On the other hand, other research showed that cell growth increased with number of batch cycle (Sakai et al., 2005). Wan et al. (2005) also reported that the dry cell weight increase when using repeated batch culture at 25\% and 75\% medium replacement. Whilst, dry cell weights were maintained in repeated batch culture both using 5\% and 40\% broth as the seed (Huang et al., 2008).

Fig. 3. (a) and (b) shows comparison between lipid contents and concentrations from the RBC experiment. Lipid contents in each cycle was recovered to be between 26.83 to 34.36\% (g lipid/g biomass) compared to that obtained from BC and showed an increase of 28.07\% in the fourth cycle.
Fig. 1. Profile of biomass, glucose and ammonium tartrate concentrations and lipid content of *Cunninghamella bainieri* 2A1 in 400 ml nitrogen limited medium in 1 L shake flask containing glucose as a carbon source and ammonium tartrate as a nitrogen source were used in batch culture. The cultures were grown at 30°C, shaken at 250 rpm for 60 hr.

Fig. 2. Profile of biomass, glucose and ammonium tartrate concentrations of *Cunninghamella bainieri* 2A1 in 400 ml nitrogen limited medium in 1 L shake flask containing glucose as a carbon source and ammonium tartrate as a nitrogen source were used in repeated batch culture. The cultures were grown at 30°C, shaken at 250 rpm for 300 hr.

*1st repeat is in 60 to 120 hr, 2nd repeat is in 120 to 180 hr, 3rd repeat is in 180 to 240 hr, 4th repeat is in 240 to 300 hr.*
Lipid content at RBC0 was not significantly different with others except for RBC4. Lipid content of RBC4 was also not significantly different with others except for RBC0, but between RBC0 and RBC4 lipid content was significantly different (P<0.05). Concentrations of lipid in each cycle were also comparable to that achieved in BC between 2.05 to 3.02 g/L. Lipid concentration at RBC0 was not significantly different with others except at RBC4. Whilst, lipid concentration for RBC4 was significantly different with others except RBC3, lipid concentration for RBC0 and RBC4 were significantly different (P<0.05).

Fig. 3. Lipid contents (a) and concentrations (b) produced by Cunninghamella bainieri 2A1 in a 4 cycle repeated batch culture maintained at 30°C, shaken at 250 rpm for 300 hr. RBC0 is the batch culture. * a and b showed different significantly (P<0.05).

Lipid content and GLA content until the fourth repeat clearly proved it is superior compared to BC. In addition, lipid content and GLA content varies inversely in BC. The performance of RBC technique could reduce seed culture preparation time (from stock culture) and the time for adaptation (lag phase) (Stanbury et al., 1995). Hence, RBC allows microorganisms to continue with lipid synthesis.
immediately after each addition of fresh medium. This enhancement by acclimatization improves the synthesis efficiency in the second to fourth repeat. The drawing out and replacement of fresh medium may also have reduced end-products that inhibited lipid synthesis. The added dissolved oxygen in the fresh medium is important for electron transport in desaturase-6 enzyme for desaturated linoleic acid to GLA further contributed to the enhancement and maintenance of lipid and GLA content. This is evident from the lipid and GLA concentration data that showed an increase after the second repeat by 45.40% and 56.66%, respectively compared to the KKU0.

Several reports with similar result in their RBC work further supports our results. It was reported that the production of laccase from RBC of free mycelia of *Trametes* sp. in shake flask was increased from 12,000 U/L to 20,000 U/L during a 40 day fermentation has been reported (Jang, 2002). The total acetate production by *Moorella* sp. was 840 mmol/L which was 17 fold higher than that in the BC (Sakai, 2005). Although, the Dye-Decolorizing Peroxidase (rDyP) activity were stable in RBC both using wheat bran powder and rice bran powder. However, average rDyP productivities in fed-batch cultures were slightly lower than those in RBC (Shakeri et al, 2007). In addition, Naghmouchi et al. (2008) reorted that the pediocin PA-1 production by free *Pediococcus acidilactici* UL5 cells pH controlled BC has reached 2,048 and 4,096 AU/ml after 11 and 12 hr of incubation in Whey Permeate (SWP) medium and Man Rogosa and Sharpe (MRS) broth, respectively. Whilst in RBC, immobilized cells reached a maximum concentration of 7.3±0.2 x 10^{10} and 4.3±0.9 x 10^{10} cfu/g of beads in MRS and
Fig. 5. Lipid and GLA cumulative productivities of each cycle of the repeated batch culture of *Cunninghamella bainieri* 2A1 in 400 ml nitrogen limited medium in 1 L grown at 30ºC, shaken at 250 rpm for 300 hr. RBC0 is the batch culture.

* a, b, c, d and e showed significantly different (P<0.05).

SWP media. The maximum pediocin PA-1 activity obtained during RBC fermentation was 4,096 AU/ml, it was attained after only 0.75 and 2 hr of incubation in MRS and SWP media, respectively. The highest lipid yield $Y_{L_A}$ and $Y_{L_A}$ were 0.23 and 0.42, respectively and the highest GLA yield $Y_{G_A}$, $Y_{G_A}$ and $Y_{G_A/L}$ were 0.024, 0.039 and 0.105, respectively. All data showed significant increment compared to the BC.

Lipid cumulative productivities of each cycle was recovered to be between 0.0096 to 0.0260 g/L/hr. Lipid cumulative productivity increased with increasing number of batch cycles as shown in Fig. 5 (a). Lipid cumulative productivity in RBC was 3.02 fold higher than that in BC. Whilst, GLA cumulative productivities were recovered to be between 0.0009 to 0.0024 g/L/hr. GLA cumulative productivity also increased with increasing number of batch cycles as shown in Fig. 5 (b). GLA cumulative productivity was 3.43 fold higher than that in BC. However, there were many reports that represented similar result in this work such as the production of hyaluronic acid (HA) by *Streptococcus zooepidemicus* in an operation that seed 31% cell, the volumetric production rate (productivity) of the RBC (0.59 gHA/L/hr) was found to be 2.5-fold of the BC (0.24 gHA/L/hr) (Huang *et al.*, 2008). In a report by Naghmouchi *et al.* (2008) the production of pediocin PA-1 by *Pediococcus acidilactici* UL5 in BC the volumetric productivities were 187 and 342 AU/ml/hr in Whey Permeate (SWP) medium and Man Rogosa and Sharpe (MRS) media, respectively. Whilst in RBC, the volumetric productivities were 5,461 and 2,048 AU/ml/hr in MRS and SWP media, respectively. These results confirmed that the RBC could enhanced productivity.
and presented a viable technique to reduce time of cultivation, preparation of inoculum medium and equipments.

CONCLUSION

In lipid and GLA productions, lipid content and concentration were 34.36% (g lipid/ g biomass) and 3.02 g/L, respectively. GLA content and concentration of 10.54% of total lipid and 0.28 g/L, respectively were attained using RBC. Lipid cumulative productivity in RBC was 3.02 fold higher than that in BC. Whilst, GLA cumulative productivity was 3.43 fold.

ACKNOWLEDGEMENTS

The authors would like to thank the Ministry of Science, Technology and Innovation for financial support under IRPA 09-02-02-0001 (BTK/TD/001) and the laboratory staff from the School of Biosciences and Biotechnology, Faculty of Science and Technology, University Kebangsaan Malaysia.

REFERENCES


