Identification and Characterisation of an Oleaginous Fungus Producing High g-Linoleneic Acid

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Citation

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Abstract

Oleaginous fungi serve as good alternatives for the production of essential, polyunsaturated fatty acids (PUFA) such as g-linolenic acid (GLA; 18:3). In this study, a locally isolated fungus, isolate 2A1, was evaluated for its lipid and GLA production, and its identity was determined. The fungus was grown in nitrogen-limited media and the amount of lipid and GLA produced were determined every 12 hours. The highest percentage of lipid produced per cell dry weight was 38.64% (w/w) while the highest amount of GLA produced was 0.65 g/L. These observations indicate that isolate 2A1 is a good lipid producer and capable of accumulating high amounts of GLA. To identify isolate 2A1 to the species level, its morphology was observed under the light and electron microscope. Subsequently, its 18S rDNA and the internal transcribed spacer (ITS) sequences were cloned, sequenced and analysed phylogenetically to 18S rDNA and ITS1 sequences of related fungi. Microscopic observation showed that isolate 2A1 produced coenocytic hyphae and self-bearing globose sporangioles, with a diameter between 6-8 µm. Neighbour-joining tree built based on the 1.6 kb 18S rDNA region clustered isolate 2A1 with fungi of the genus Cunninghamella. Maximum parsimony tree analysis based on a 154 bp ITS1 sequence grouped isolate 2A1 together with Cunninghamella bainieri strain NRRL 1375 with 100% bootstrap value. Thus, based on morphological and molecular phylogenetic data, isolate 2A1 is designated as C. baineri strain 2A1.

INTRODUCTION

Gamma-linolenic acid (GLA: 18:3) is considered as an essential fatty acid in humans and acts as an important intermediate in the biosynthesis of biologically active prostaglandin from linolenic acid. It has been reported to be effective for the prevention or curing a variety of diseases including rheumatoid arthritis, cardiovascular diseases, hyper-cholestromia, atopic eczema and asthma [1-3]. Dietary supplement of GLA has shown a positive effect on the disorders related to the deficiencies of this fatty acid. As a result there is always considerable interest for the large scale production of GLA to support the demand of the industries.

The principal sources of GLA are the seeds of evening primrose (Oenothera biennis) with 8-10% (w/w) GLA, borage seeds (Borago oficinalis) containing 24-25% (w/w) GLA and black currant seeds (Ribes nigrum), which consist of 16-17% (w/w) GLA [4]. However, the productivity of GLA from these seed oils is still considered low, since both long periods and huge areas for plant cultivation are required. The production of GLA from plants is also dependent on the seasonal and climatic changes, which could destabilise the price of the oil in the market. In addition, with the increase interest in growing plants such as canola and soybean for biodiesel production and the need to grow crops for essential food production, stiff competition for fertile agricultural land is expected in the near future. These factors could further contribute towards the fluctuation of the production cost of GLA from plants.

To overcome these limitations, microorganisms may serve as an alternative source for GLA production. Microorganisms have several advantages over plants in the production of GLA including high growth rates, simple culture conditions, independence of seasonal and climatic changes, and can be readily grown under controlled conditions with nutritional regimes that may stimulate or repress the key steps of fatty acid formation [5]. Fungi, such as Zygomycetes have been widely reported for their competence in producing GLA. Genera of Mucor, Mortierella, Absidia and Cunninghamella had been extensively investigated as an alternative source for GLA production [6].

Research and development of GLA production from fungi is in progress and mostly aimed at improving the economic competitiveness of fungal GLA production. These include searching for high GLA producing fungi [7], optimising

growth media for optimum lipid and GLA production [8,9] and exploring the possibility of using cheaper substrates for fungal growth [10,11]. Screening of potential fungi for GLA production is an essential step, limiting the number of strains available for further improvement and practical usage. One essential aspect needs to be considered during selection for GLA production is that the selected strain should be able to produce high amounts of lipid containing high percentages of fatty acids in the form of GLA. High lipid producers alone might not be the best candidate unless they produced relatively high amounts of GLA. Although a number of filamentous fungi of the class Zygomycetes accumulate large amounts of oil, they tend to have low contents of GLA and conversely, those with high GLA content have comparatively low oil levels. Mucor circinelloides, for example, was reported to produce up to 23% lipid in the biomass and approximately 24% of the oil content is GLA [5]. Mortierella isabellina, on the other hand, produced up to 53% lipid in the biomass but with only 4.5% GLA of the total fatty acids. Hence, during screening processes, fungi producing high lipid contents with relatively high percentages of GLA should be considered.

This study reports on the characterisation of GLA production and molecular identification of a newly isolated strain of oleaginous fungus which was previously isolated from Malaysian soil [12] and could serve as a potential source of GLA production.

MATERIALS AND METHODS

FUNGAL STRAIN AND CULTIVATION

Fungal isolate 2A1 used in this study was isolated from the soil collected from Malaysian forest floor [12] and maintained on potato dextrose agar (PDA) supplied by Oxoid (United Kingdom). Prior to the isolation of genomic DNA, fungal cultures were prepared by excising 1 cm^2 pieces of mycelia from the PDA plates and were sub-cultured into 200 mL of potato dextrose broth (PDB). The culture was grown for 3 days on a rotary shaker at 250 rpm, 30°C. The mycelia were then harvested, placed into a mortar which had previously been chilled with liquid nitrogen, and grounded with a pestle in the presence of liquid nitrogen. The fine powder produced was used as a starting material for genomic DNA extraction.

For lipid and fatty acid analysis, spore suspensions were used as starter inoculums for each experiment. A total of 2×10^7 spores were inoculated into 200 mL of nitrogen limited media [13] and grown at 30°C with shaking at 250 rpm. Cultures were harvested at different time points for 7 days and filtered through Whatman No. 4 filter paper (Whatman, USA) and washed twice with deionised distiled water (ddH_2O). The cells were dried in an oven at 60-70°C for 24 to 48 hours and the dry cell weight was determined after the constant cell weight was achieved.

FUNGAL LIPID EXTRACTION AND FATTY ACID ANALYSIS

Extraction of lipids was performed according to the modified procedure of Folch et al. [14]. Prior to lipid extraction, mycelia were freeze-dried at -50°C for 24 hours to a constant weight. The dried mycelia were grounded into fine powder and added into 150 mL of chloroform/methanol (2:1, v/v). The suspension was then left overnight at room temperature. The homogenate was filtered and washed with 150 mL of 0.1% NaCl solution. The mixture was mixed and allowed to settle into two phases. The lower chloroform phase that contains lipid was recovered and rinsed twice with 150 mL distilled water. The mixture was then transferred into a rotary bottle and was evaporated using a rotary evaporator. Subsequently, the rotary bottle was washed with diethyl ether to collect the lipids and the sample was left in the fume hood to allow evaporation of diethyl ether. The lipids were weighed and analysed using a gas chromatography unit equipped with DB 23 cis/trans capilary columns. The detector FID (flame ionisation detector) was used. Fatty acids were identified based on the retention time of standard fatty acids (Sigma, USA). A total of three independent experiments were carried out to determine the lipid and the fatty acid production from isolate 2A1.

CHARACTERISATION OF FUNGAL MORPHOLOGY

Fungal strain 2A1 was identified microscopically using cellophane tape lactophenol mount method and scanning electron microscope (SEM). A strip of cellophane transparent tape was placed onto a 5-day old culture plate and samples were examined under 40× magnification of a light microscope (Olympus, Germany). The mycelia were also observed under an electron microscope (LEO Model 1450VP Variable Pressure Scanning Electron Microscope, Carl Zeiss, USA).

DNA EXTRACTION

Genomic DNA was isolated from mycelia using the method developed previously by Voigt et al. [15] with some modifications. Briefly, the grounded mycelia was resuspended in 700 µL of hexacetyltrimethylammonium

bromide (CTAB; Sigma Chemicals, USA) extraction buffer [100 mM Tris-HCl (pH 8.4), 1.4 M NaCl, 25 mM EDTA, 2% CTAB] and vortexed for 10 seconds. The homogenate was incubated at 65°C for 10 min. Following extraction, an equal volume of chloroform was added to the mixture, vortexed for 5 seconds and then spun for 10 min, 4°C and at 13,000 rpm using Sigma 3-18K centrifuge (Sigma, Germany). Subsequently, 500 µL of the upper phase was transferred into a new 1.5 mL tube and 5 µL of RNase A (20 mg/mL) was added to the tube. The mixture was incubated at 37°C for 30 min. DNA was precipitated with an equal volume of cold isopropanol and kept for 15 min at -20°C. After the DNA was pelleted by centrifugation at 13,000 rpm for 1 min, the supernatant was discarded and the pellet was gently washed with 70% ethanol and resuspended in 20 µL distilled water. The DNA was stored at –20°C until further application.

AMPLIFICATION AND CLONING OF 18S RDNA AND DNA REGION FOR INTERNAL TRANSCRIBED SPACERS (ITS)

To amplify the partial 18S rDNA and ITS1-5.8S-ITS2 DNA region of isolate 2A1, primers were designed using sequences from related species of Zygomycetes obtained from the National Center for Biotechnology Information (NCBI) database. For the amplification of 18S rDNA region, the forward primer, 5'-AAGGCCTGACTTCGGGAG-3', and the reverse primer, 5'-TCCTCTAAATAATCTAGTTTGCCAT-3' were used. The forward primer, 5' AGGTGAACCTGCGGAAGGATCATTA 3' and the reverse primer, 5' ATTGATATGCTTAAGTTCAGCGGT 3' in turn were used to amplify ITS1-5.8S-ITS2 DNA region. PCR amplifications were carried out in a total volume of 20 µL. The reaction mixture contained 500 ng of genomic DNA, 1x PCR buffer (Promega, USA), 2.5 mM MgCl, 10 µM each of dNTP (dGTP, dATP, dCTP and dTTP), 20 pmol of each primer and 0.5 units of Taq DNA polymerase (Promega, USA). The PCR cycling profile was as follows: 95ºC for 5 minutes (one cycle), followed by 29 cycles of 95ºC denaturation for 20 seconds, primer annealing for 30 seconds at 60ºC for 18S rDNA and 65ºC for ITS, and elongation at 72ºC for 2 minutes. The final primer extension continued for an additional 10 minutes.

The amplified products were analysed by gel electrophoresis on a 1.0% agarose gel containing 10 mg/mL of ethidium bromide and visualised under UV light. The PCR products were then gel purified using QIAquick gel extraction kit (Qiagen, Germany) and ligated into pGEM-T Easy vector (Promega, USA).

SEQUENCING AND PHYLOGENETIC ANALYSIS

Sequencing of clones was performed on both strands using BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, USA). Sequencing was carried out from the 5' end using the T7 primer (5'-GAGTAATACGACTCACTATAGGG-3) and from the 3' end using SP6 primer (5'TATTTAGGTGACACTATAG-3'). The reaction contained 3.2 pmol of each primer with a reaction volume of 10 µL and 1 µL of Big Dye solution. The cycle sequencing reaction was 96°C for 5 minutes (one cycle), 35 cycles of 96°C for 30 seconds, 50°C for 15 seconds and 60°C for 5 minutes. The sequences were deposited in the GenBank database. The accession number of 18S rDNA of isolate 2A1 is EF562534 and ITS1-5.8S-ITS2 DNA fragment is EF562535.

Sequences of 18S rDNA and ITS1 of isolate 2A1 were analysed along with the 18S rDNA sequence of 35 Zygomycetes and ITS1 of 13 Zygomycetes. Sequences for Zygomycetes 18S rDNA and ITS1 for the phylogenetic analysis were retrieved from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). The sequences were aligned using ClustalW program [16]. Phylogenetic analyses were carried out using both neighbour-joining (NJ) and maximum parsimony (MP) methods by using PAUP*4.0 (http://paup.csit.fsu.edu/downl.html). Both analyses were assessed for support by bootstrap analyses of 1000 replicates. To root the trees, the 18S rDNA sequence of a few species of Mortierellales and ITS1 sequence of Absidia glauca and Absidia coerulea were retrieved from the GenBank sequence database.

RESULTS AND DISCUSSION

LIPID AND GLA PRODUCTION OF STRAIN 2A1

In an effort to identify potential GLA producers, fungi isolated from soil collected at various sites in Malaysia were screened for their cellular lipid and GLA content [12]. Subsequently, several fungal isolates, which contain more than 25% of their biomass in the form of lipids, were identified (data not shown). Preliminary analysis showed that isolate 2A1 was able to produce lipid up to more than 30% of its dry weight. In order to further evaluate its capability to produce lipid and GLA, isolate 2A1 was cultivated in a nitrogen limited media and the production of

lipid as well as the composition of different fatty acids was observed. Nitrogen limited media was used in this experiment as this medium was shown to enhance lipid accumulation in fungi [13]. In oleaginous fungi, nitrogen limitation prevents cell proliferation and allows the conversion of the available carbon source into storage lipid.

In this growth media, the biomass of isolate 2A1 gradually increased until 72 hours of growth and then started to be consistent (Table 1). The highest biomass yield was 4.12 g/L which was recorded after 72 hours of growth. The percentage of total lipid produced over dry weight also increased until 96 hours of growth before it started to be consistent. Lipid synthesis in isolate 2A1 was correlated with the production of its biomass. The highest production of lipid produced per cell dry weight was approximately 38.64% (w/w) after 144 h of growth (Table 1). The amount of lipid production in this fungus is comparable to the lipid produced by other oleaginous fungi. For example Mortierella ramanniana var. ramanniana produced 54.2% (w/w) lipid over cell dry weight, Mucor sp LB-54 produced 20.73% [17], Conidiobolus nanodes produced 34% (w/w) [5], Mucor rouxii produced 32% (w/w) [18] and Cunninghamella strain LGAM produced 28.1% [19]. Hence, this data indicate that isolate 2A1 is a good lipid producer and the amount of lipid produced per cell dry weight was comparable or, in some cases, better compared to other reported oleaginous fungi.

Figure 1

Table1: Yield of cellular biomass, lipid and GLA produced by fungal isolate 2A1 grown in nitrogen limited media

Incubation time (hrs)	Biomass (g/L)	Lipid (g/L)	Total lipid/dry weight (% w/w)	GLA/ total fatty acids (% w/w)	GLA (g/L)
12	1.72 ± 0.017	0.19 ± 0.06	10.88 ± 3.61	15.64 ± 0.81	0.12
24	2.59 ± 0.097	0.61 ± 0.08	23.79 ± 1.74	10.53 ± 1.39	0.26
48	3.45 ± 0.015	1.09 ± 0.19	31.61 ± 5.02	9.02 ± 0.72	0.39
72	4.12 ± 0.051	1.35 ± 0.18	32.75 ± 4.71	9.25 ± 0.12	0.49
96	3.55 ± 0.011	1.36 ± 0.00	38.21 ± 0.61	8.95 ± 0.15	0.48
120	3.52 ± 0.036	1.09 ± 0.06	31.16 ± 1.02	9.99 ± 0.30	0.44
144	3.77 ± 0.058	1.46 ± 0.14	38.64 ± 4.03	11.06 ± 0.21	0.65
168	3.47 ± 0.064	1.09 ± 0.06	28.56 ± 2.86	11.48 ± 0.93	0.50

To determine the fatty acid profile in lipid extracted from isolate 2A1, lipid extracted at different time points of fungal growth was analysed using gas chromatography. The presence of twelve fatty acids in the lipid samples, namely lauric acid (C12:0), myristic acid (C14:0), pentadecanoic acid (C15:0), palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), -linolenic acid (C18:3G), -linolenic acid (C18:3A) and behenic acid (C22:0), were investigated and examined. Data presented in Table 2 shows the result of changes in the amounts of fatty acids in the lipid produced by isolate 2A1 during growth in shake flasks at different time points. Fatty acid analyses indicate that the lipid of isolate 2A1 was rich in five fatty acids, namely palmitic acid with the highest production of 24.2 % (percentage of fatty acid from the total lipid), stearic acid with the highest production of 8.6%, oleic acid with the highest production of 41.6%, linoleic acid with the highest production of 18.7% and -linolenic acid with the highest production of 15.64% (Table 2). There was very minimal presence of alpha-linolenic acid (18:3) (Table 2) and other beneficial, long polyunsaturated fatty acids such as dihomo--linolenic acid (20:3), arachidonic acid (20:4) and eicosapentaenoic acid (20:5) detected in lipid of isolate 2A1 (data not shown). Amongst fatty acids that were present in this analysis, -linolenic acid (GLA), an important fatty acid due to its tremendous commercial potential was chosen for further analysis. To analyse further on the GLA production, the amount of GLA produced per liter of medium was measured at different growth points. The highest amount of GLA produced by isolate 2A1 was 0.65 g/L which was produced after 144 hours of growth (Table 1). Although the highest percentage of GLA over lipid production was 15.64% after 12 hours of growth, their production of GLA per liter was the lowest. This is because at this stage the amount of the biomass produced was the least amongst the biomass taken from different time points. It also should be noted that at this stage, the conidia of isolate 2A1 started to germinate and produce germ tubes. Khunyoshyeng et al. [20] reported that the level of GLA is high during germ tube emergence of Mucor rouxii. Therefore, the high percentage of GLA over lipid production detected at 12 hours could coincide with the germination of the conidia of isolate 2A1 and not represent the storage GLA.

Figure 2

Table 2: Fatty acids composition of the cellular lipids produced during growth in nitrogen limited medium at various time points.

Incubation time (hour)	Fatty acids (% in total lipid)										
	Lauric Acid (C12:0)	Myristic Acid (C14:0)	Penta-decanoic Acid (C15:0)	Palmitic Acid (C16:0)	Palmitoleic Acid (C16:1)	Stearic Acid (C18:0)	Oleic Acid (C18:1)	Linoleic Acid (C18:2)	γ-Linolenic Acid (C18:3G)	α-Linolenic Acid (C18:3A)	Behenic Acid (C22:0)
12	0.03 ± 0.03	0.64 ± 0.04	0.08 ± 0.01	17.04 ± 0.55	0.40 ± 0.31	8.63 ± 1.28	40.69 ± 0.49	13.49 ± 1.16	15.64 ± 0.82	0.11 ± 0.01	0.32 ± 0.05
24	0.13 ± 0.17	0.74 ± 0.14	0.06 ± 0.04	20.37 ± 2.72	0.50 ± 0.15	7.83 ± 1.00	41.63 ± 1.79	13.85 ± 0.87	10.53 ± 1.39	0.12 ± 0.07	0.22 ± 0.20
48	0.01 ± 0.02	0.68 ± 0.02	0.04 ± 0.01	24.28 ± 0.71	0.56 ± 0.02	5.86 ± 0.15	41.13 ± 0.67	14.82 ± 0.22	9.02 ± 0.72	0.04 ± 0.04	0.27 ± 0.02
72	0.03 ± 0.03	0.75 ± 0.06	0.06 ± 0.02	24.06 ± 0.94	0.68 ± 0.05	4.89 ± 0.26	40.59 ± 0.61	15.82 ± 0.17	9.26 ± 0.12	0.06 ± 0.02	0.26 ± 0.04
96	0.09 ± 0.13	0.62 ± 0.01	0.04 ± 0.03	23.16 ± 0.42	0.51 ± 0.04	5.51 ± 0.07	41.61 ± 0.51	16.58 ± 0.44	8.95 ± 0.15	0.06 ± 0.01	0.28 ± 0.01
120	0.03 ± 0.03	0.67 ± 0.06	0.07 ± 0.01	23.23 ± 0.33	0.64 ± 0.11	4.72 ± 0.36	40.66 ± 0.93	17.42 ± 0.45	9.99 ± 0.30	3.33 ± 5.66	0.24 ± 0.02
144	0.03 ± 0.01	0.63 ± 0.05	0.06 ± 0.01	20.66 ± 0.56	0.60 ± 0.10	4.15 ± 0.14	40.14 ± 0.23	18.52 ± 0.63	11.06 ± 0.21	0.05 ± 0.01	0.23 ± 0.02
168	0.05 ± 0.02	0.59 ± 0.01	0.06 ± 0.01	20.39 ± 0.67	0.53 ± 0.05	4.24 ± 0.31	40.81 ± 2.13	18.75 ± 0.55	11.45 ± 0.93	0.07 ± 0.11	0.17 ± 0.12

The data represent the mean values from three independent cultures and fatty acid determination were carried out in duplicate.

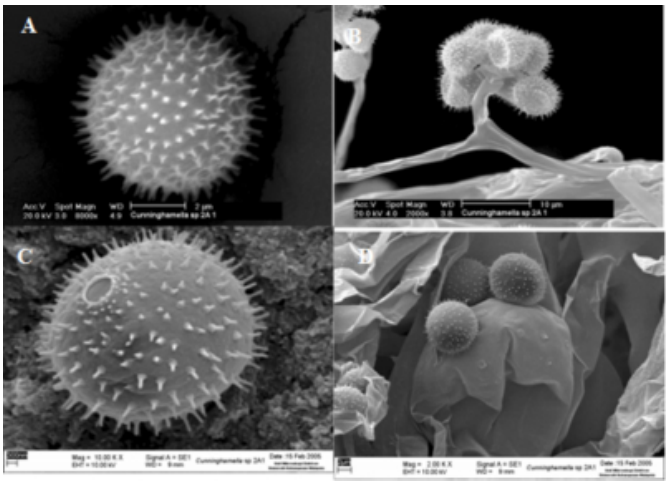
However, it is difficult to make a direct comparison in GLA production between isolate 2A1 with published GLA production data of other fungal species. This is due to the differences in media formulation and growth conditions employed by different studies. However, based on the data obtained, it can be concluded that isolate 2A1 is a good candidate for GLA production since it can accumulate high amounts of lipid (up to 38.2% lipid/ dry biomass) with satisfactory content of GLA (11% GLA/total lipid). Further analysis, such as the usage of cheap substrates for growth and variations in growth media formulation, should be explored in order to maximise the production of lipid and GLA from isolate 2A1, which in turn will increase its industrial competitiveness.

MOLECULAR IDENTIFICATION AND PHYLOGENETIC ANALYSIS OF NUCLEAR RDNA SEQUENCE

In an effort to identify isolate 2A1 to the species level, detailed microscopic observation and analysis of the 18S rDNA and the ITS sequences of the fungus were performed. Both 18S rDNA and the ITS sequences have shown great variables and were frequently used to differentiate many fungal species [21, 22]. The morphology of the fungus was examined under light and scanning electron microscopes. One obvious characteristic of the fungus was the absence of septate hyphae, which indicates that it is a Zygomycete. This correlates with the findings associating Zygomycetes such as Mortierella, Mucor, Rhizopus and Cunninghamella with high amounts of cellular lipid and GLA [5, 19, 23]. In addition, the presence of globose sporangioles with spines was observed. These sporangioles were attached to a vesicle which was located at the tip of the sporangiophore (Figure 1). The size of a sporangiola was approximately 6-7 µm in diameter. Sporangioles are structures that contain sporangiospores and the spines present on the outer surface mainly consist of calcium oxalate dihydrate [24]. Based on these morphological structures it was deduced that isolate 2A1 is a Zygomycete and may be a member of the genus Cunninghamella.

Figure 3

Figure 1: Structure of isolate 2A1 under Scanning Electron Microscopy (SEM)



A. Spiny sporangiola. B. Sporangioles attached to a shrunken vesicle located at the tip of sporangiophore. C: Released mature sporangiola with a scar represented the attachment site to the vesicle. D. Sporangioles attached to a vesicle

To identify the fungus at the genus and species level, the 18S rDNA was sequenced and compared with the 18S rDNA of other Zygomycetes. The primers used to amplify the 18S rDNA was designed based on the sequence of conserved region of 18S rDNA of several species of Zygomycetes. A total of 1613 bp of the 18S rDNA sequence were amplified, cloned and sequenced. The sequence obtained was submitted to GenBank with the accession number, EF562534. Initial analysis of 18S rDNA sequence using BLAST program from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) showed 99% identity to Cunninghamella polymorpha, C. elegans and C. bertholletiae. Using the 18S rDNA sequence, phylogenetic trees that include 18S rDNA sequences of 35 Zygomycetes

that are available from GenBank, were constructed. Neigbour-joining using 18S rDNA and bootstrap analyses support that the isolate 2A1 sequence falls within species of Cunninghamella genus (Figure 2). Similar analysis using maximum parsimony generated the same result (data not shown).

Based on the observations from both morphological characterisation and 18S rDNA sequence analysis, the rDNA region comprising the 5.8S rDNA and two adjacent internal transcribed spacers (ITS1 and ITS2) was sequenced and compared with ITS1 sequences of other Cunninghamella species. The sequence data for the ribosomal internal transcribed spacers for most members of the genus Cunninghamella was available from work described by Liu et al. [25]. PCR amplicon containing the ITS1-5.8S rDNA-ITS2 with the size of 706 bp was amplified, cloned and sequenced. The sequence obtained was submitted to the GenBank with accession number, EF562535. BLAST analysis showed 97% identity between isolate 2A1 to C. bainieri strain NRRL 1375 [25] for both ITS1 and ITS2 regions. Using a 154 bp of the ITS1 sequence, a phylogenetic tree was constructed. The aligned sequence was 154 bases with gaps. The maximum parsimony tree grouped the isolate 2A1 sequence together with C. bainieri with 100% bootstrap support (Figure 3). Similar relationship was obtained when phylogenetic analysis was carried out using neighbour-joining (data not shown). Thus, the isolate 2A1 is designated as Cunninghamella baineri strain 2A1 based on the molecular phylogenetic inferences that provide strong support for the close relationship of isolate 2A1 to C. baineri.

Figure 4

Figure 2: Neighbour-joining tree showing the phylogenetic relationship amongst Zygomycetes based on their 18S rDNA sequences. 18S rDNA sequence of three species were used as outgroups to root the tree.

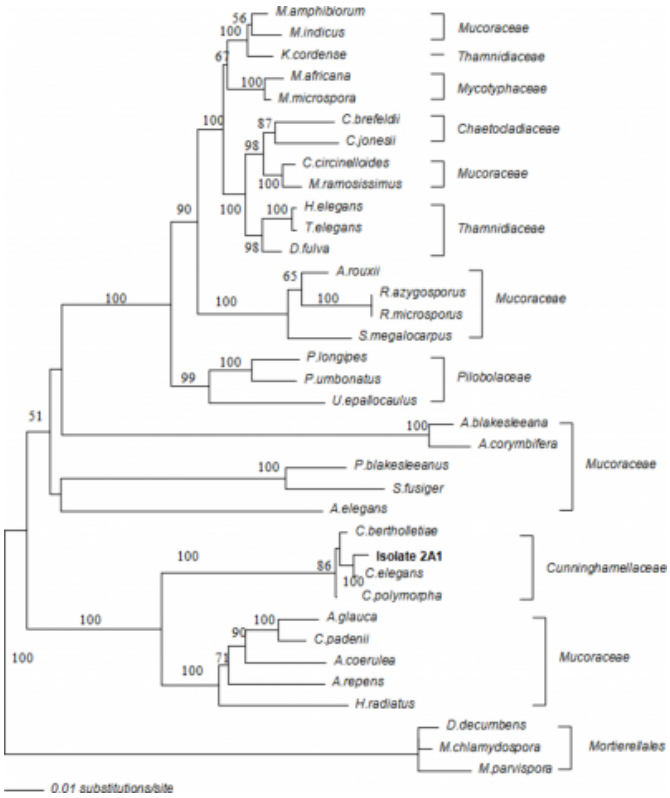
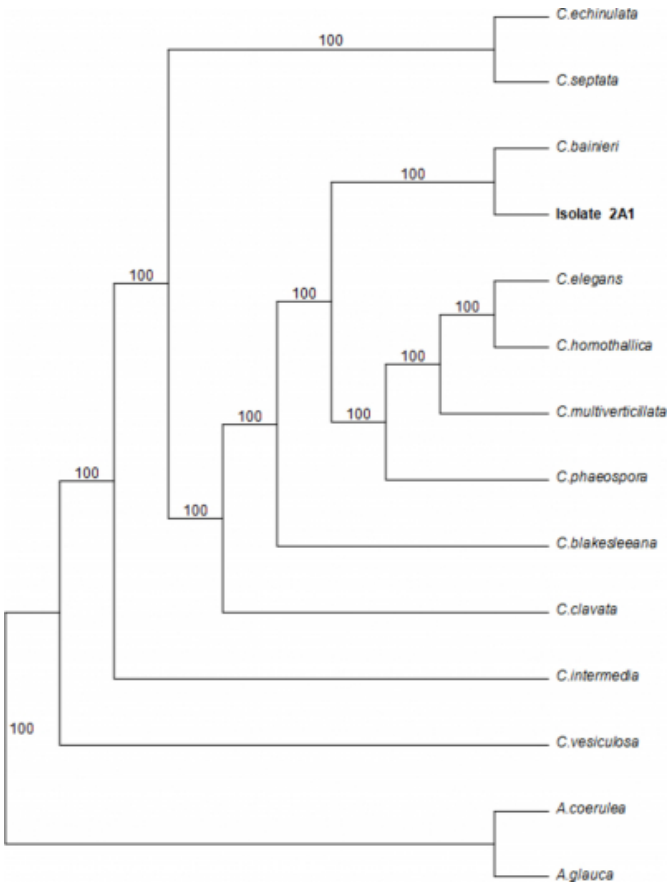


Figure 5

Figure 3: Maximum parsimony tree showing the phylogenetic relationship between species based on their ITS1 sequences. The ITS sequence of two species were used as outliers.



The identification of C. bainieri as a potential GLA producer, as described in this work, is not uncommon since a number of Cunninghamella species such as C. elegans [10], C. echinulata [26] and Cunninghamella strain LGAM [19] has been reported to produce high lipid and GLA. The production of lipid and GLA from C. bainieri 2A1 is comparable, if not better, to other Cunninghamella species. Further analysis involving optimisation of growth media and utilisation of cheap substrates should be explored in the future so that the potential of this strain to produce GLA at the industrial level will be materialised.

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