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Enhancing the value of nitrogen from rapeseed meal for microbial oil production.

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Abstract

Rapeseed meal, a major byproduct of biodiesel production, has been used as a low-cost raw material for the production of a generic microbial feedstock through a consolidated bioconversion process. Various strategies were tested for the production of a novel fermentation medium, rich in Free Amino Nitrogen (FAN): commercial enzymes (2.7 $mg \cdot g^{-1}$ dry meal), liquid state fungal pre-treatment using *Aspergillus oryzae* (4.6 $mg \cdot g^{-1}$), liquid state fungal pre-treatment followed by fungal autolysis (9.13 $mg \cdot g^{-1}$), liquid state pre-treatment using fungal enzymatic broth (2.1 $mg \cdot g^{-1}$), but the best strategy was a solid state fungal pre-treatment followed by fungal autolysis (34.5 $mg \cdot g^{-1}$).

The bioavailability of the nitrogen sources in the novel medium was confirmed in fed-batch bioreactor studies, in which 82.3 g dry $cell.L^{-1}$ of the oleaginous yeast *Rhodosporidum toruloides* Y4 was obtained with a lipid content of 48%. The dry cell weight obtained was higher than that obtained using conventional yeast extract, due to a higher total nitrogen content in the novel biomedium. The Fatty Acid Methyl Esters (FAMEs) obtained from the microbial oil were similar to those derived from rapeseed oil.

Keywords: rapeseed meal; fermentation; solid state fermentation; free amino nitrogen; *Rhodosporidium toruloides*, microbial oil.

1. Introduction

The production and use of biodiesel has dramatically increased in recent years to cope with the increasing demand for fuel. It represents a promising alternative for use in compression-ignition (diesel) engines. It has some advantages over petroleum derived fuels since it is produced from renewable sources; it is biodegradable and less toxic [1]. Biodiesel production is performed after the oil extraction from various oil seeds. For this purpose, rapeseed is frequently used in Europe because of its high oil content, low-cost and abundance. In the biodiesel process from rapeseed, a protein rich solid by-product (rapeseed meal) is generated which is usually sold as animal feed [2, 3]. However, it is not an ideal animal feed and, because of the growth of the biodiesel industry, the amounts of this low value rapeseed meal are expected to increase significantly in the near future. Global rapeseed production was 47 million tons in 2010 [4], and over 25 million tons of rapeseed meal was produced. There is therefore considerable incentive to find and develop new uses for this by-product, which fit with an integrated and sustainable approach to biodiesel production.

Another by-product of the biodiesel industry, glycerol, has been shown in numerous publications to be a suitable carbon source in fermentation media for the production of various bioproducts such as succinic acid [5], microbial oil [6] and biodegradable plastics [7] or value-added metabolic products [8] and this list is far from exhaustive. However, these studies have generally relied on the use of yeast extract as a nitrogen source, which would be too expensive to use in a large biorefinery. The development of an integrated biorefinery

concept based on rapeseed requires a suitable nitrogen and nutrient source for the production of a wide range of specialty chemicals or biofuels by microbial fermentation. In this biorefinery approach it is proposed to use both by-products, glycerol and rapeseed meal, in the production of a complete generic microbial medium.

Rapeseed meal is generally used as organic fertilizer and animal feed because of its high protein content. However, the utilization of rapeseed meal in food and feed industries is limited because it contains some anti-nutritional constituents such as phytic acid, erucic acid and fibre and precursors of toxic compounds such as glucosinolate and phenol [9-11]. Moreover, rapeseed meal proteins are not easily digestible compared to other protein rich waste materials such as fish meal or soy bean meal rendering them less valuable [12].

Rapeseed meal might be used as a nutrient for fermentation processes due to its high protein, carbohydrate and mineral contents. However, microorganisms generally cannot assimilate directly these nutrients without a form of pretreatment [13]. If the rapeseed meal proteins are made accessible, many valuable products could be developed from this inexpensive and abundant waste via fermentation processes. Usual nitrogen sources for fermentation include yeast extract, peptone or inorganic nitrogen such as ammonium sulfate, but significant savings could be obtained if nitrogen was obtained from an inexpensive source.

The objective of this study was to improve the accessibility of rapeseed meal proteins by biological pre-treatments. For this purpose, enzymatic hydrolysis and fungal fermentations were conducted to convert rapeseed meal proteins into digestible components. The capacity of a lipid producing yeast to metabolize these accessible nitrogen sources was then investigated

to determine whether microbial oil production (to enhance biodiesel production) might be feasible.

2. Materials and methods

2.1. Rapeseed meal

Rapeseed meal was kindly supplied by the Oilseeds Processing Division of Cargill Plc, Liverpool, England. Its composition for relevant components was reported in a previous publication [13]. The rapeseed meal was kept in air-tight plastic containers and stored at room temperature.

2.2. Rapeseed meal pretreatments

Each pretreatment is described in Figure 1 and in the following paragraphs.

2.2.1. Commercial enzyme pretreatment (CE)

Commercial protease (P4860, Protease from *Bacillus licheniformis)* was purchased from Sigma-Aldrich. This protease is stable over the range of pH 6.0 to 10.0 and in the temperature range of 50 of 60°C. Protease activity was determined as 49 U/mL in pH 7 phosphate buffer at 55°C. An enzyme loading of 5.10^{-3} U protease.g^{-1} meal was used to hydrolyze 10 g rapeseed meal (on a dry basis) in 100 mL pH 7 phosphate buffer. Experiments were performed on autoclaved and non-autoclaved rapeseed meal.

2.2.2. Liquid state fungal pretreatment (LSF)

Liquid state fermentation tests were performed using 10 g rapeseed meal in 250 mL Erlenmeyer flasks with 100 mL liquid volume. The flasks were autoclaved and then

inoculated aseptically with 10^6 spores of *Aspergillus oryzae*.g^{-1} rapeseed meal. The isolation, purification, and proliferation of the fungus have been reported previously [13]. Fungal growth was carried out at 30°C for 72 hours.

2.2.3. Liquid state fungal pretreatment followed by fungal autolysis (LSFA)

This pre-treatment was carried out according to the previous strategy followed by autolysis of the fungus for 72 hours at 55°C. Briefly, the autolysis can be defined as the decomposition of the fungus and the release of nutrients. This step is also characterized by a higher protease activity due to the higher temperature which promotes the hydrolysis of the remaining meal components [13].

2.2.4. Liquid state pretreatment using enzymatic broth (EB)

This pretreatment was carried out according to the liquid state fungal pretreatment described above followed by filtration of the broth through a qualitative filter (Whatmann 1). Ten mL of filtrate containing the active enzymes or the so-called 'enzymatic broth' was mixed with 90 mL of distilled water and 10 g of fresh rapeseed meal at 55°C in order to increase further the FAN content of the solution. This method was investigated because the fungal growth is relatively slow and recycle of the enzymatic stream for further hydrolysis would be an advantage.

2.2.5. Solid state fungal pretreatment followed by fungal autolysis (SSFA)

Firstly, a certain amount of rapeseed meal was moistened with the required amount of tap water to obtain 65 % moisture content in a 1 L bottle then sterilized at 121°C for 45 min. The meal was allowed to cool to room temperature before inoculating with approximately 10^6 spores of *A. oryzae*·g^{-1} rapeseed meal. The content was mixed by stirring with a sterile

aluminum rod and vigorous shaking. After mixing well, approximately 10-13 g of content was distributed into each 9-cm Petri dish and incubated at 30°C for 3 days.

Autolysis of fermented solids was subsequently conducted by mixing the required amount of distilled water with fermented solid to obtain approximately 55-60 $g \cdot L^{-1}$ solid concentration. The content was blended using a kitchen blender then incubated at 55°C for 3 days in a tightly capped bottle. Samples were taken periodically to measure free amino nitrogen concentration.

2.3. Bio-oil production using the nitrogen-rich media

The oleaginous yeast *Rhodosporidium toruloides* Y4, which has previously been shown by Li et al. [14] to be capable of high cell density culture was used throughout this study. Bio-oil production with *R. toruloides* Y4 was carried out using nutrient solutions obtained from different pretreatments to compare the yeast growth and bio-oil production yield.

2.3.1. Flasks experiments

For inoculum preparation, the yeast was grown for 3 days in 100 mL liquid medium composed of: 3 $g.L^{-1}$ malt extract, 10 $g.L^{-1}$ yeast extract, 10 $g.L^{-1}$ peptone, 10 $g.L^{-1}$ NaCl and 20 $g.L^{-1}$ glucose. For experiments in 500 mL Erlenmeyer flasks, the fermentation medium (100 mL) was prepared using 90 mL filtered nutrients solution obtained after the various pre-treatments and glucose was added to obtain a concentration of 50 $g.L^{-1}$. The flasks were autoclaved at 121°C for 20 minutes, and then inoculated aseptically with 10 mL of inoculum. A control with yeast extract powder (Sigma) was also carried out in parallel. Fermentations were carried out at 30°C on a 200 rpm rotary shaker.

2.3.2. Bioreactor experiments

For bioreactor experiments, the fermentation medium (1 L) was prepared using 900 mL filtered nutrients solution obtained after the various pretreatments and diluted such that an initial FAN concentration 300 $mg.L^{-1}$ was obtained. The medium was supplemented with 0.4 $g.L^{-1}$ KH_2PO_4, 1.5 $g.L^{-1}$ $MgSO_4.7H_2O$. The stock glucose solution (1000 $g.L^{-1}$) was autoclaved separately, and added at the beginning of the fermentation to obtain a concentration of 50 $g.L^{-1}$. The inoculum (100 mL) was transferred aseptically and the fermentation was performed at pH 6, 30°C and a stirring speed of 1200 rpm (Electrolab). Samples were taken at regular intervals to follow the yeast growth, glucose concentration and FAN consumption. In order to maintain a high C/N ratio, the required amount of glucose was supplemented to the medium every 24 hours.

2.4. Analytical methods

Free Amino Nitrogen concentration was analyzed by the ninhydrin colorimetric method [15]. It is a convenient method to analyze the amino nitrogen that is readily assimilated by many microorganisms. Protease activity was quantified by the formation of FAN by hydrolyzing a 15 $g.L^{-1}$ casein solution (Sigma) at 55°C in 200 mM, pH 7 phosphate buffer. One unit activity (U) was defined as the protease required for the production of 1 g FAN in one minute. Protease activity was determined in triplicate. Glucose concentration was measured in triplicate using an Analox GL6 analyser (Analox, England). Populations of fungal spores and yeast cells were microscopically quantified using a haemacytometer (Improved Neubauer, Weber England, Depth 0.1mm, $1/400mm^2$). For dry cell biomass determination, 5 mL fermentation broth was filtered through a 0.2 μm filter and dried at 60°C overnight. Oil content of dried yeast cells was determined by chloroform:methanol (1:1 v/v) extraction in a Soxtec-HT6 system (Höganäs, Sweden). The extraction time was 2 hours at 140°C, followed by 20 minutes of rinsing. The oil content was determined in triplicate.

Fatty acid analysis of lipids was conducted by gas chromatography (GC). For this, 1 mg of lipid was subjected for 30 min to methanolysis at 60°C in the presence of 15% (vol/vol) methanolic-sulfuric acid. The resulting fatty acid methyl esters were analysed on a Varian CP-3800 gas chromatograph equipped with a DB-23 capillary column (60 m x 0.25 mm; film thickness of 150 nm) and a flame ionization detector (Agilent Technologies). A 2 ml portion of the organic phase was analysed after split injection (1:50); helium (constant flow of 0.2 ml min-1) was used as a carrier gas. The temperatures of the injector and detector were 250°C. The following temperature program was applied: 50°C for 1 min, increase of 25°C min^{-1} to 175°C, increase of 4°C min^{-1} to 230°C, and 230°C for 5 min. Substances were identified by comparison of their retention times with those of a standard of fatty acid methyl esters (Sigma 18919-1AMP).

3. Results and discussion

3.1. Free Amino Nitrogen production

FAN production during various pretreatments was compared as shown in Figure 2: commercial enzyme (CE) hydrolysis with and without autoclaving, liquid state fungal pre-treatment (LSF), liquid state fungal pre-treatment followed by fungal autolysis (LSFA), liquid state pre-treatment using enzymatic broth (EB) and solid state fermentation followed by fungal autolysis (SSFA).

During commercial enzyme pre-treatment, the rate of FAN production was linear up to 8 hours reaching 2.2 mg $FAN.g^{-1}$ rapeseed meal, but it then reached a steady-state. Under the studied conditions incubation periods longer than 8 hours did not increase further the yield. When rapeseed meal was autoclaved prior to commercial enzyme pretreatment, FAN

222 production was lower. Rapeseed might contain some natural proteases, and when autoclaving
223 was applied, these proteases may have been denatured, resulting in lower yields. Another
224 possible cause is the release of inhibitory substances during heat pre-treatment that affect
225 protease activity. The FAN yield obtained with liquid state pre-treatment using enzymatic
226 broth (EB) was similar to that obtained with commercial enzyme.
227
228 The fungal pretreatment (LSF) gave better results and after 48 hours the highest FAN yield
229 became 4.5 $mg.g^{-1}$ rapeseed meal due the growth of the fungus and the release of specific
230 enzymes for this type of substrate. *Aspergillus oryzae* is known to be an excellent protease
231 producer [16]. It does not only produce protease that hydrolyze proteins into peptides and
232 amino acids, but also phytase [17], xylanase [18], β-galactosidase [19], cellulase and
233 amylolactic enzyme [20] which results in the release of phosphate and the production of
234 simple sugars to be used as carbon source for the growth of microorganisms [13]. Using
235 fungal pre-treatment followed by fungal autolysis (LSFA), intracellular enzymes were
236 released from the cells resulting in a FAN production reaching 9.13 $mg.g^{-1}$ rapeseed meal
237 after 120 hours which demonstrated a significant advantage of performing an autolysis step.
238
239 The production of FAN from rapeseed meal was carried out by stepwise solid-state
240 fermentation followed by autolysis (SSFA) of fermented solids in order to break down
241 proteins contained in the meal. During the SSFA an increased protease activity was observed
242 after the germination period and the activity of the enzyme increased rapidly until the fungus
243 entered the stationary phase of growth (data not shown). This resulted in the production of
244 15.3 mg $FAN{\cdot}g^{-1}$ rapeseed meal together with a protease activity of 118.5 $U{\cdot}g^{-1}$ rapeseed meal
245 after 72 hours. Subsequent autolysis of fermented solid boosted the production of FAN. The
246 incubation at 55°C encouraged the activity of protease to further degrade proteins which

resulted in higher production of FAN. However, enzymatic hydrolysis was not the only reaction occurring during autolysis. Blending the fermented solid also resulted in fragmentation of fungal mycelia which prevented further fungal fermentation. Moreover, the limited dissolved oxygen in the medium led to the autolysis of the fungus which encouraged the regeneration of microbial nutrients from fungal biomass [21]. FAN production after 72 hours of incubation was increased to 34.5 $mg \cdot g^{-1}$ which is equivalent to a 55 % conversion from the total nitrogen in the rapeseed meal. The remaining nitrogen may be intact proteins or peptide chains that do not react with the reagent in the ninhydrin colorimetric method. In order to assess the bioavailability of the nitrogen sources and to verify that no inhibitory substances are generated during the pretreatments, fermentations were carried using the oleaginous yeast, *Rhodosporidium toruloides* Y4 for bio-oil production.

3.3 Fermentation using the novel nitrogen-rich medium

3.3.1 Flask experiments

In this section, the yeast *R. toruloides* Y4 was used to assess the possibility of growing microorganisms on the novel fermentation medium containing FAN produced from the various pretreatments of rapeseed meal as described above. FAN was a convenient method to compare the amino nitrogen that can be readily assimilated by the yeast. Glucose was used as carbon source at a concentration of 50 $g.L^{-1}$. Figure 3A shows the growth profiles of *R. toruloides* Y4 in flasks. Yeast growth was observed in each condition indicating that amino acids produced during the pretreatments were bioavailable for the yeast metabolism. However, the growth was sluggish when commercial enzyme(CE), liquid state fungal pre-treatment (LSF) and fungal enzymatic broth (EB) were used.

Using liquid state fungal pretreatment followed by fungal autolysis (LSFA) a dry cell weight of 7.9 $g.L^{-1}$ was obtained which is similar to the value found by Wang et al. [13] with *Saccharomyces cerevisiae* growing on this type of fermentation medium. Interestingly, using the SSFA pretreatment, a final cell yield of 10.1 $g.L^{-1}$ was obtained which was more than that obtained using yeast extract powder (9.1 $g.L^{-1}$). This shows that our novel N-rich medium was at least as good as conventional yeast extract for supporting the growth of this oleaginous yeast. The better growth of the yeast on the SSFA medium was correlated by a greater consumption of FAN compared to the other media (Figure 3B) showing that the autolysis process enhanced the bioavailability of the nitrogen sources for the yeast.

At the end of the fermentation period it can be seen that the FAN concentrations remained relatively high for each condition (> 80 mg $FAN.L^{-1}$). It is not clear whether this residual FAN was bioavailable for the yeast or whether it was not consumed because of insufficient dissolved oxygen levels. To investigate this further and to confirm bioavailability of the nitrogen sources in the novel fermentation medium these experiments were scaled-up to 1 L bioreactors.

3.3.2. 1 L bioreactor experiments

In order to improve the growth conditions, fed-batch fermentations were carried out in a 1L bioreactor using high agitation speed to ensure high dissolved oxygen. The aim of the fed-batch process was to avoid substrate inhibition and enhance the production of lipids by feeding additional glucose. The culture with an initial volume of 1 L was first operated in batch mode with an initial glucose concentration of 50 $g.L^{-1}$. Then a specific volume of glucose stock solution (1000 $g.L^{-1}$) was added every 24 hours up to 120 hours of fermentation to maintain a C/N ratio in the range 70-90. Dry biomass yields increased markedly using this fed-batch technique as shown in Figure 4A. This is in accordance with several authors who

have reported that biomass yield and lipid content increase when fed-batch cultures are applied. The highest dry biomass yield (82.3 $g.L^{-1}$) was obtained using solid state fermentation followed by fungal autolysis (SSFA) confirming the results obtained in flasks. Throughout the 120 hours fermentation period, the biomass concentrations using SSFA were higher than those using conventional yeast extract (YE) powder as the nitrogen source (80.3 $g.L^{-1}$). To our knowledge this is the first report where an alternative to yeast extract and ammonium sulfate is used as nitrogen source for the growth of *R. toruloides* Y4.

Moreover, the remaining FAN concentrations were lower than 50 $mg.L^{-1}$ in the bioreactors after 72 hours (Figure 4B), which meant that FAN consumption and therefore growth were enhanced in the oxygen rich medium. The rate of FAN uptake was higher in the bioreactor than in flasks with more than 80% of the FAN being consumed within 24 hours for each nitrogen source.

The amount of biomass produced using solid state fermentation followed by fungal autolysis (SSFA) and liquid state fungal pre-treatment followed by fungal autolysis (LSFA) and commercial enzymes(CE) pretreatments were significantly higher than those produced by liquid state fungal pre-treatment (LSF) or liquid state pre-treatment using enzymatic broth (EB). In order to shed more light on these differences, total nitrogen in each medium was measured.

3.4. FAN and Total Nitrogen (TN) content of fermentation media

The initial FAN concentrations from each pretreatment were adjusted before the fermentation to be equal and it was observed that the FAN consumption rates were similar to each other. However, the initial total nitrogen concentrations from solid state fermentation autolysis

pretreatment (SSFA), fungal autolysis pretreatment (LSFA) and commercial protease(CE) pretreatment were 1747, 1675 and 1604 $mg.L^{-1}$, respectively, giving rise to an initial C/N ratio of approximately 15. TN concentrations of the solutions coming from fungal enzyme broth (EB) and liquid state fungal (LSF) pretreatments were only 762 and 730 $mg.L^{-1}$, respectively (Figure 5), corresponding to an initial C/N ratio of approximately 32. The difference in growth profile in flasks and bioreactor was therefore due to the difference of TN in these two latter media. This shows that the yeast requires not only amino acids, but that the growth was significantly enhanced when other sources of nitrogen were also present, such as inorganic nitrogen, peptides or proteins. The results have demonstrated that the media from SSFA and LSFA have a good balance of amino nitrogen, organic and inorganic nitrogen for the growth of the yeast compared to the media from LSF and EB. Figure 5 also shows that the autolysis step carried out for the SSFA and LSFA media was essential for the release of these beneficial nitrogen sources. The high amount of nitrogen was beneficial for the rapid growth of *R. toruloides* Y4.

3.5 Lipid content and composition

In order to investigate the production of lipids in *R. toruloides* Y4 growing in these nitrogen-rich fermentation media, lipid content and composition of the fatty acids were analyzed.

At the end of the 120-hours-long fed-batch fermentation in the 1L bioreactor, dry cell biomass reached 82.3 $g.L^{-1}$, while intracellular lipid content was 48.1% (w/w) using the SSFA medium (Table 1). The dry cell biomass and lipid content from the YE medium was 80.3 $g.L^{-1}$ and 65%, respectively. It should be borne in mind that the lipids start to accumulate within the cells when the nitrogen source is exhausted. Since the TN content in YE was lower than that in the media from LSFA and SSFA it is possible that the yeast started to accumulate lipids

sooner, which is why their lipid content was slightly higher. However, the total dry cell weight was lower in the YE medium. This shows that there is a trade-off between the number of cells, which depends on the initial nitrogen content, and the lipid content that these cells can accumulate given the final assimilable nitrogen and carbon content, i.e. the C/N ratio.

There are several publications describing fed-batch cultivation for microbial lipid production using yeast extract, peptone, nitrate or ammonium sulphate. A rich medium containing yeast extract and/or peptone is generally used to grow the inoculum, while the production medium generally contains an inorganic source because it is much cheaper [22]. However, it has been found that *R. toruloides* Y4 accumulated more lipids when an organic nitrogen source was employed [23]. Organic nitrogen improves both cell growth and lipid accumulation, while inorganic nitrogen is more beneficial in biomass accumulation [24].

Pan et al. [25] used a feeding medium containing glucose (600 $g.L^{-1}$) and yeast extract (20 $g.L^{-1}$) and obtained a final lipid content of 40%. In another study, an initial medium of glucose (32.5 $g.L^{-1}$) and ammonium sulphate (6.1 $g.L^{-1}$) was used in a fed-batch process using *Cryptococcus curvatus* and a cell density of 70 $g.L^{-1}$ and a lipid content of 53% were obtained after 172 hours fermentation. Zhu et al. [26] used a fed-batch technique to grow *Mortierella alpina* with an initial medium containing 50 $g.L^{-1}$ glucose and 3 $g.L^{-1}$ nitrate and achieved a cell density and lipid content of 35.6 $g.L^{-1}$ and 35%, respectively. Some of these fed-batch processes simultaneously introduced a carbon and nitrogen source, which may not provide an optimal C/N ratio for lipid accumulation.

Li et al. [14] obtained a cell density and lipid content of 106.5 $g.L^{-1}$ and 67.5% (w/w), respectively, over a 134-h fermentation using *R. toruloides* Y4 in a 15 L-bioreactor. They

used a synthetic medium consisting of glucose (60 $g.L^{-1}$) and a very high initial concentration of peptone (15.7 $g.L^{-1}$) and yeast extract (15.7 $g.L^{-1}$) which explains why their cell density was so high. In our study a relatively high cell density of 82.3 $g.L^{-1}$ was obtained with only 1.7 g TN. L^{-1} in the initial SSFA medium. Our cell density was similar to that obtained by Zhang et al [22] who cultivated *Cryptococcus curvatus* in fed-batch mode with an initial medium of glucose (60 $g.L^{-1}$), peptone (10 $g.L^{-1}$) and yeast extract (10 $g.L^{-1}$).

Fakas et al. [27] have used agro-industrial wastes containing organic nitrogen such as tomato waste, corn gluten, corn steep liquor or whey concentrate to grow *Cunninghamella echinulata* while synthetic glucose was supplemented for lipid accumulation. They observed that organic nitrogen favoured glucose uptake and lipid accumulation. However, an acid hydrolysis step and a neutralisation step were required to make the organic nitrogen from tomato waste assimilable. The authors pointed out that an essential condition for lipid accumulation in oleaginous micro-organisms was the use of nitrogen sources in limiting concentrations or the use of nitrogen sources having low availability [28]. The medium derived from rapeseed seems to be an ideal nitrogen source because it contains assimilable FAN required for growth and also complex organic nitrogen sources. Further growth depends on the ability of the microorganism to break down these sources, so as to release assimilable nitrogen. This process allows for keeping a high C/N ratio for lipid production.

Fatty acid compositional analysis revealed that three major constituent fatty acids were palmitic acid, oleic acid and linoleic acid. Individual fatty acid distribution varied slightly according to the nitrogen sources (Table 2). The oleic acid content was above 60% when a medium derived from rapeseed meal was used, except with the CE medium, while with yeast extract the oleic acid content was 56 %. A higher palmitic acid content was obtained when YE was used compared to content in the range 14-16 % with the other media. It can be seen from

Table 2 that the composition is close to that of rapeseed oil. Based on the fatty acid analysis data, FAMEs made from microbial oil by using rapeseed meal as N source have therefore good potential for enhancing overall rapeseed biodiesel production.

To our knowledge this is the first time that such high cell densities with high lipid content are reported with the yeast *R. toruloides* Y4 growing on a nitrogen source derived from rapeseed meal. This shows that the rapeseed meal produced in high quantities each year from biodiesel plants around the world could be used to grow an oleaginous microorganism for the production of oil. This could be a more sustainable option and will offer a cheaper alternative than expensive nitrogen source for microbial cultivation to be cost-efficient. The use of waste glycerol, another by-product from biodiesel production, would reduce further the fermentation costs and could increase the overall biodiesel production from rapeseed.

In conclusion, the accessibility of the N source was improved via biological pre-treatments in this study. The best strategy consisted in hydrolyzing rapeseed meal using *A. oryzae* followed by fungal autolysis. When this novel nitrogen rich fermentation medium was used in a fed-batch process for lipid production using *Rhodosporidium toruloides* Y4, high microbial lipid concentrations were obtained with a potential for biodiesel production.

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***Highlights**

Highlights

- Solid state fermentation and autolysis were used to obtain FAN from rapeseed meal.
- The novel fermentation medium was at least as good as conventional yeast extract.
- The Fatty Acid Methyl Esters (FAMEs) were similar to those derived from rapeseed.

Figure 1

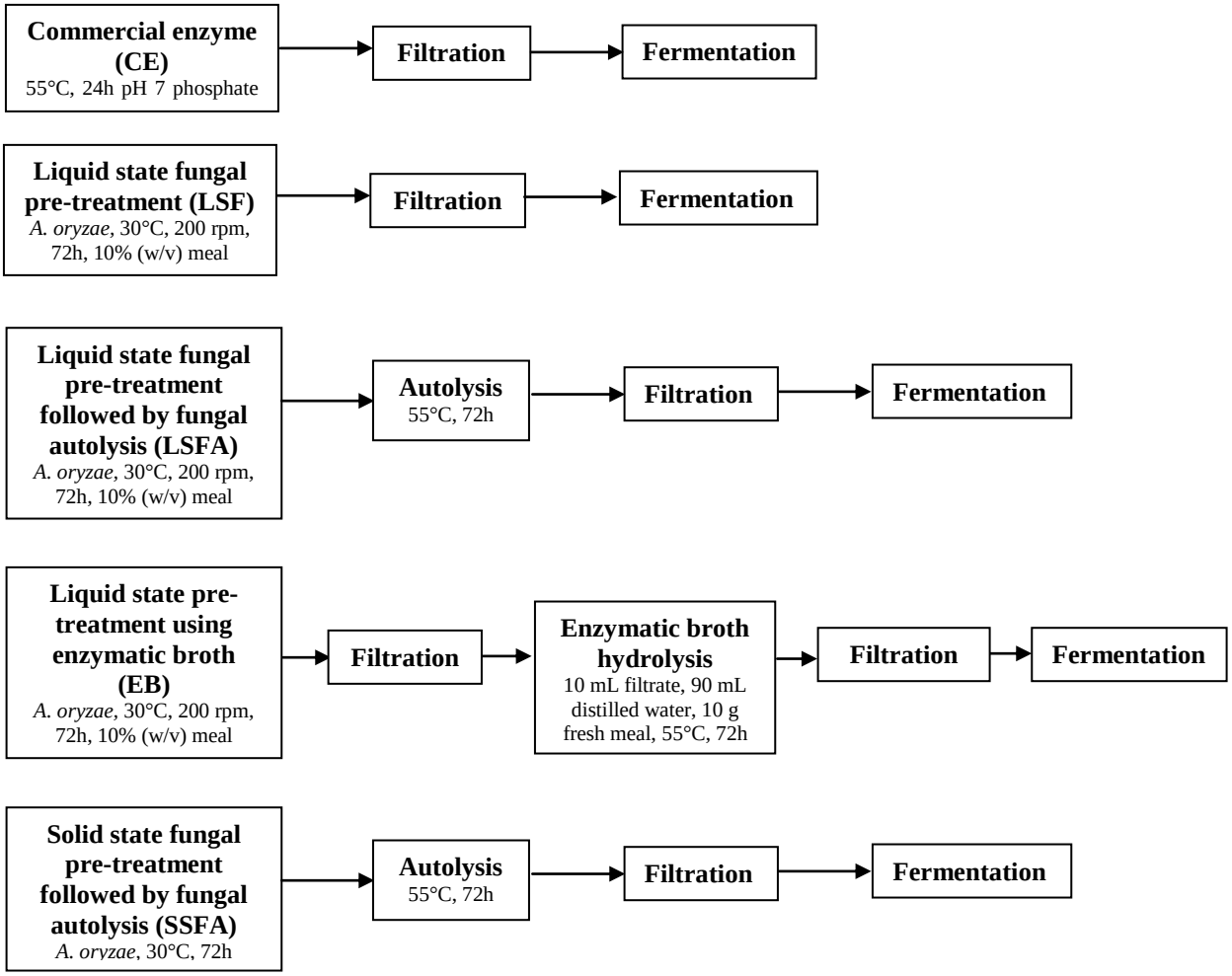


Figure 1. Pretreatments used in this study to improve the accessibility of nitrogen from rapeseed meal.

Figure 2

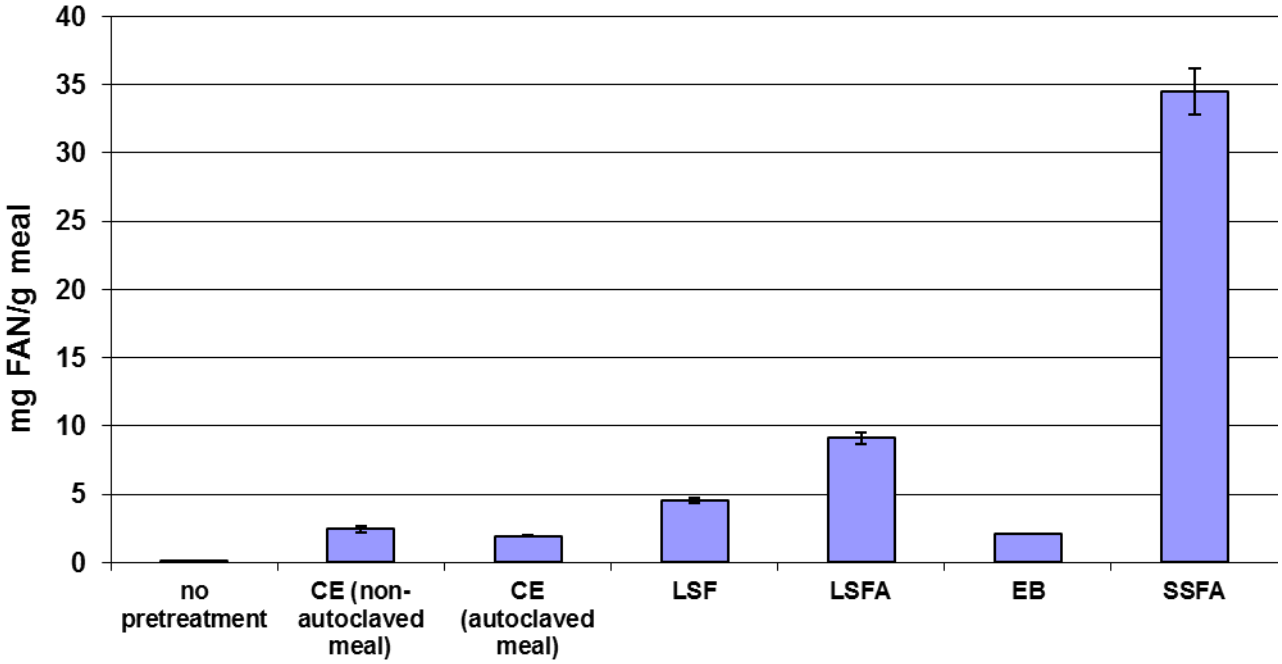


Figure 2. Effect of various pretreatments on the FAN yield from rapeseed meal.

Figure 3

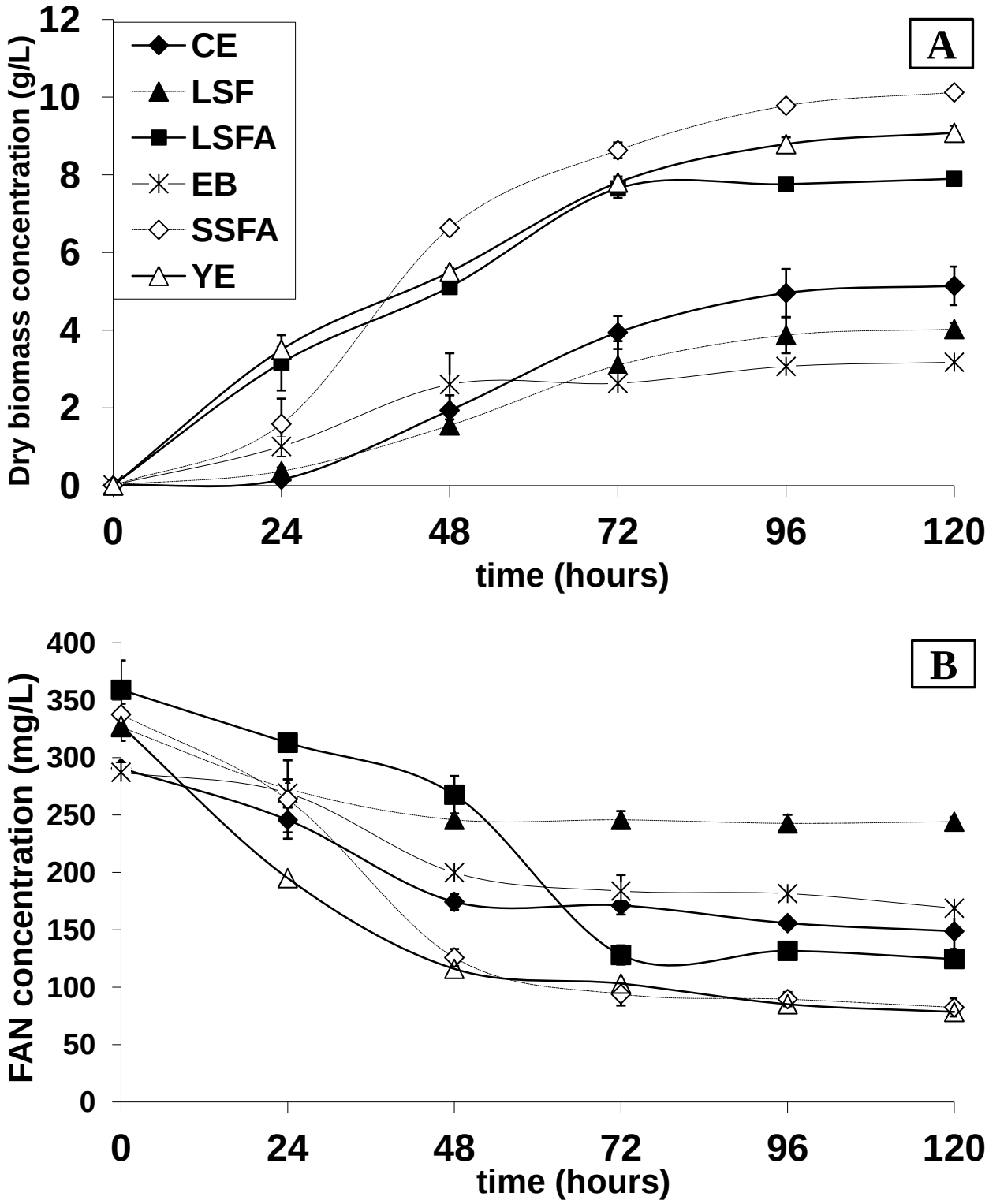
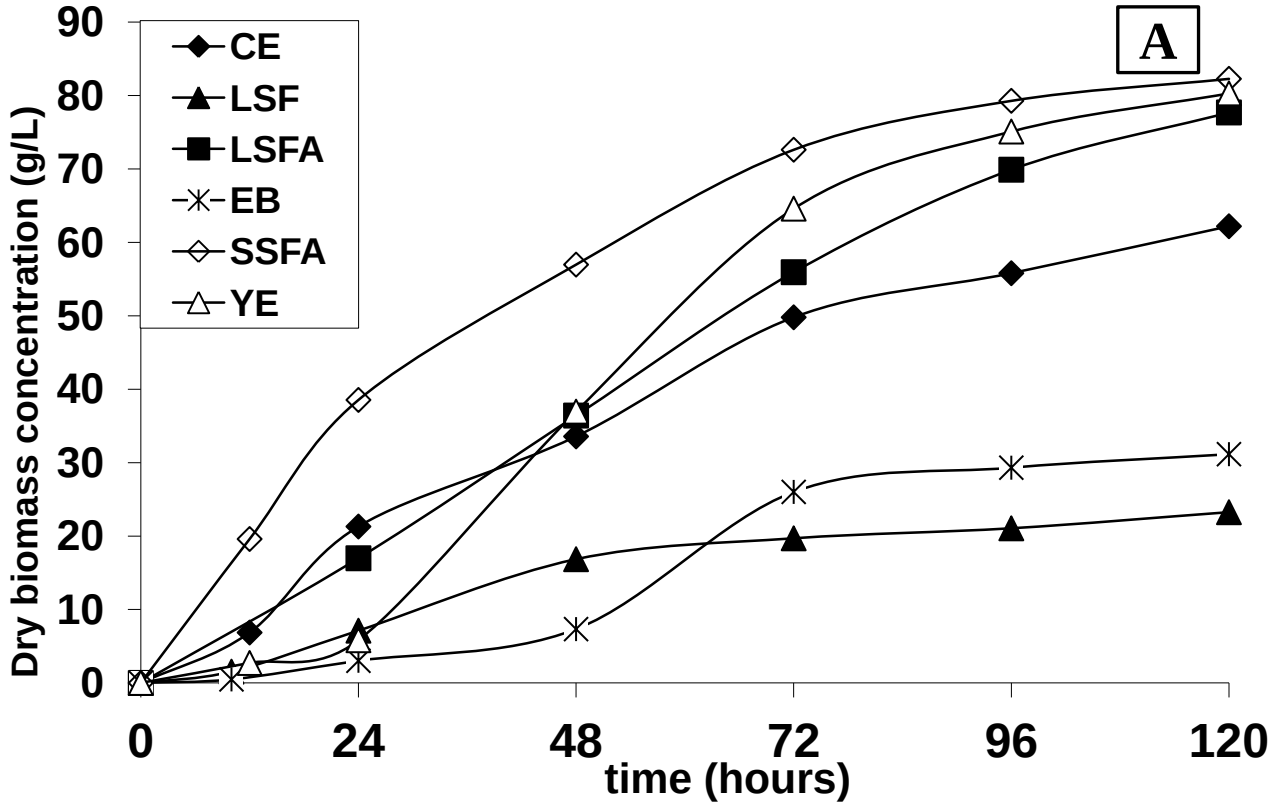


Figure 3. (A) *R. toruloides* Y4 growth in flasks using FAN produced during various pretreatments. Data points show the averages from triplicate fermentations. (B) FAN concentration during the fermentation using N rich solutions from various pretreatments. Data points show the averages from triplicate fermentations.

Figure 4



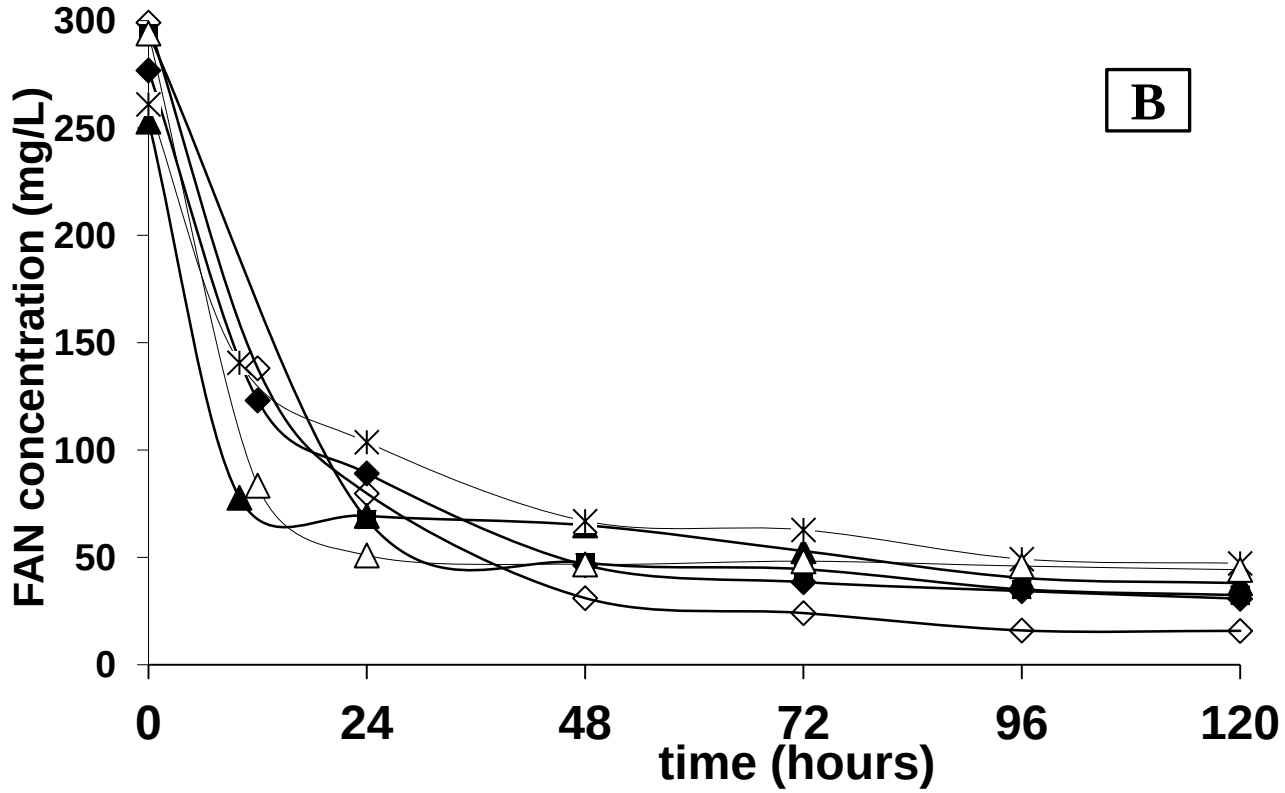


Figure 4. (A) *R. toruloides* Y4 growth profiles in bioreactors using FAN produced by different pre-treatments. Data points show the averages from duplicate fermentations. (B) FAN concentration during the fermentation using N rich solutions from various pretreatments. Data points show the averages from triplicate analyses.

Figure 5

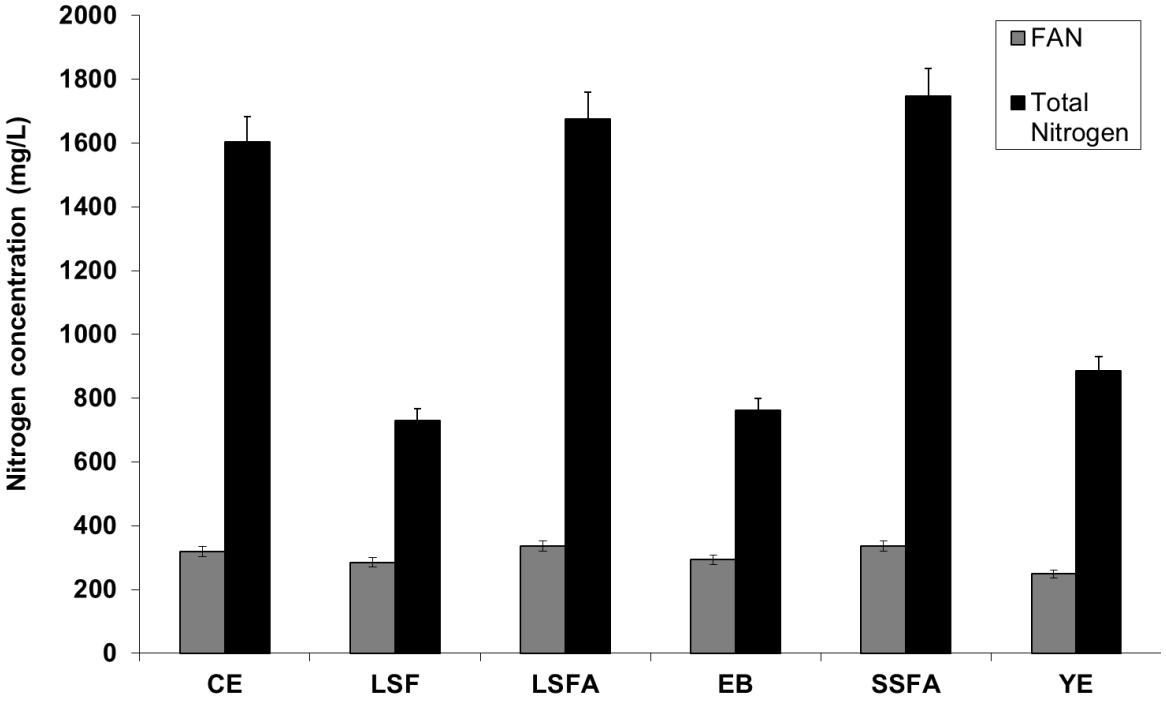


Figure 5. FAN and total nitrogen concentrations of N sources which were obtained from different pretreatments. Data points show the averages from at least duplicate measurement.

Table 1. Dry biomass, lipid content (% of dry biomass) and lipid yield (g lipid/g glucose consumed) obtained at the end of the fermentation in a 1 L bioreactor with different nitrogen sources.

	Dry biomass (g biomass/L)	**Lipid content (%) (g lipid/g biomass)**	**Lipid yield (g lipid/L)**	**Lipid yield (g lipid/g glucose)**
CE	62.2±3.0	18.3±0.2	29.4±0.1	0.29
LSF	34.8±1.7	44.2±7.0	15.4±2.4	0.15
LSFA	77.7±3.9	54.4±3.7	42.3±2.9	0.30
EB	31.2±3.1	41.3±5.5	12.9±1.7	0.13
SSFA	82.3±4.3	48.1±2.5	39.6±2.1	0.28
YE	80.3±4.0	65.5±1.6	52.5±1.3	0.24

Table 2

Table 2. Fatty acids composition of FAME derived from lipids of *R. toruloides* Y4 using glucose and novel nitrogen rich media derived from rapeseed meal.

	Content (%, w/w)						
	CE	**LSF**	**LSFA**	**EB**	**SSFA**	**YE**	**Rapeseed oil [29]**
Myristic acid (C14:0)	0.8	0.7	0.8	0.6	0.7	1.1	0.0
Palmitic acid (C16:0)	14.8	14.7	16.2	14.0	15.8	18.3	4.2
Palmitoleic acid (C16:1)	0.4	0.3	0.4	0.3	0.4	0.5	0.1
Stearic acid (C18:0)	12.9	10.0	9.8	10.8	9.7	9.1	1.6
Oleic acid (C18:1)	56.4	63.3	62.1	64.2	61.6	59.9	59.5
Linoleic acid (C18:2)	12.1	8.1	8.7	8.1	9.3	8.8	21.5
Linolenic acid (C18:3)	2.6	2.0	1.7	2.0	1.7	1.9	8.4
Arachidic acid (C20)	-	0.4	0.2	-	0.1	0.3	0.4
Behenic acid (C22)	-	0.4	-	-	0.2	-	0.3
Lignoceric acid (C24)	-	0.4	-	-	0.4	-	0.1