Effect of Developed Low Cost Minimal Medium on Lipid and Exopolysaccharide Production by *Lipomyces starkeyi* Under Repeated Fed-batch and Continuous Cultivation

V. Thirumal,[a] A. Chistoserdov,[b] R. Bajpai,[a]
J. Bader,[c] M. K. Popovic,[c] and R. Subramaniam[a,*]

[a]Department of Chemical Engineering, University of Louisiana at Lafayette, Lafayette, LA 70503, USA

[b]Department of Biology, University of Louisiana at Lafayette, Lafayette, LA 70503, USA

[c]Beuth University of Applied Sciences, Life Sciences and Technology, Biotechnology, Berlin, Germany

The main objective of this study was to investigate the effects of a low cost minimal medium, developed by the UL Bioprocessing Lab, on the cultivation of *Lipomyces starkeyi* NRRL Y-11557 using repeated fed-batch and continuous fermentation strategies. The highest cell and lipid concentrations obtained were 22.7 g L⁻¹ and 11.67 g L⁻¹ under repeated fed-batch cultivation, respectively. Continuous cultivation with the dilution rate of 0.06 h⁻¹ presented the highest cell (0.401 g g⁻¹) and lipid yields (0.177g g⁻¹). Exopolysaccharide production was observed when *L. starkeyi* was cultivated in the minimal media supplemented with 90 g L⁻¹ glucose under repeated fed-batch fermentation. The produced exopolysaccharide is likely composed of 4–5 repeating sugar units, incorporating mannose and galactose and their respective uronic acids.

Keywords:
minimal medium, *Lipomyces starkeyi,* lipid, exopolysaccharide, fed-batch mode, continuous mode

Introduction

The oleaginous yeast *Lipomyces starkeyi* is a species originally isolated from soil, belonging to order Saccharomycetales. It has been shown to accumulate up to 80 % of its dry biomass as lipids under defined culture conditions[1,2]. In addition, it is an attractive microorganism for the production of microbial lipids due to its capacity to utilize a variety of carbon sources, including D-glucose, L-arabinose, D-xylose, D-galactose, D-fructose, D-mannose, D-cellobiose, and sucrose[3–5]. Furthermore, the lipid profile produced by *L. starkeyi* is optimal for the production of biodiesel, since it is mainly composed of neutral lipids[6]. Cell densities up to 85 g L⁻¹ have been reported in a repeated fed-batch cultivation of *L. starkeyi* DSM 70296, with a lipid concentration of 36.7 g L⁻¹ [7]. In addition, the cell productivity of 0.6 g L⁻¹ h⁻¹ accompanied by the lipid productivity of 0.288 g L⁻¹ h⁻¹ were reported for *L. starkeyi* DSM 70296 during continuous cultivation with 60 g L⁻¹ sugar concentration in the medium with a dilution rate of 0.06 h⁻¹ [7].

In general, the production of lipid by oleaginous microorganisms can be divided into two phases: cell growth and lipid production[8]. Since the alternatives to the batch process, such as feed batch, repeated fed-batch and continuous, make it possible to control the growth and lipid accumulation phases by modifying the feed, they are important strategies in the pursuit of economical lipid production. For example, the exhaustion of nitrogen sources during the late stages of the fed-batch process results in a reduction in cell growth rate and channeling of the carbon flux towards lipid biosynthesis[9]. Therefore, the ratio of carbon to nitrogen (C/N ratio) is an important factor in the onset and level of lipid production. As a general trend, higher C/N ratios result in increased lipid and byproduct formation[10,11]. However, nitrogen depletion in the medium can also result in the production of unwanted byproducts. *L. starkeyi* has been reported by Slodki and Wickerham[12] to be capable of exopolysaccharide production. Continuous cultivation can achieve high cell and lipid productivities with minimal exopolysac-

*Corresponding author: E-mail: raams@louisiana.edu, raams76@gmail.com

charide production[7] but has its own drawbacks in the form of a susceptibility to contamination, incomplete utilization of substrate, and increased working volumes[13].

The lipid yield, volumetric productivity, and economics of lipid production are all critically affected by the composition of the fermentation medium. Research into *L. starkeyi* cultivation has typically utilized yeast extract as a source of vitamins and trace minerals[14,15]. Naganuma *et al.*[4] demonstrated that certain constituents of yeast extract can have both positive and negative effects on lipid content and lipid yield, respectively. For example, Zn^{2+} deficiency increased lipid content by a factor ranging from 2.4 to 2.8 compared to standard zinc concentrations[4]. In contrast, a sufficient supply of Mn^{2+} increased total cell number by a factor ranging from 1.5 to 1.7 compared to standard manganese concentrations[4].

A recent work by Subramaniam and Bajpai[16] has established that the yeast extract is a major cost contributor to the production of microbial lipids, and can be substituted by a combination of biotin, inositol, and calcium pantothenate. Furthermore, the concentration of phosphate salts in the medium can be reduced by 95 % without affecting cell growth, lipid production or lipid composition in a batch culture[17]. In combination, this minimal medium (MM) developed by the UL Bioprocessing Lab reduces the medium cost for lipid production by *L. starkeyi* from 3.35 US \$ L^{-1} lipids to 0.82 US \$ L^{-1} lipids[2]. The effect of the minimal medium on cell growth and lipid production by *L. starkeyi* under batch cultivation has been studied by Rahman *et al.*[2], but the effect on other modes of fermentation is lacking in the literature. Therefore, the present study focused on evaluating the effects of the minimal medium on *L. starkeyi* fermentation using different cultivation strategies, specifically repeated fed-batch cultivation and continuous cultivation, and analyzing the products.

Materials and methods

Strain and media

The oleaginous yeast *L. starkeyi* NRRL Y-11557 was used throughout this study. Lyophilized cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and revived using yeast mold (YM) medium (composition: malt extract 3 g L^{-1}, yeast extract 3 g L^{-1}, dextrose 10 g L^{-1}, and peptone 5 g L^{-1}). The cells were then cultivated on YM-agar slants (YM medium with 2 % agar) at 30 °C in an Isotemp Standard

Lab Incubator (Fisher Scientific, USA) for 48 h, and stored at 4 °C. These stock cells were propagated every month.

The composition of the MM was (per liter): 30 g of glucose, 0.35 g of KH_2PO_4, 0.125 g of $Na_2HPO_4 \cdot 7H_2O$, 0.5 g of $(NH_4)_2SO_4$, 1.5 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of $CaCl_2 \cdot 2H_2O$, 8.2 mg of $FeSO_4$, 10 mg of $ZnSO_4H_2O$, 7 mg of $MnSO_4$, 10 mg of $CuSO_4$, 9.41 mg of $CoCl_2 \cdot 6H_2O$, 3 mg of Biotin, 3 mg of Inositol, and 0.6 mg of calcium pantothenate. The listed medium composition results in a C/N ratio of 113 on a mass/mass basis. In order to prepare medium with a C/N ratio of 56, the concentration of $(NH_4)_2SO_4$ was doubled to 1 g L^{-1}.

Culture conditions

For experiments, inoculation culture was prepared by streaking freshly prepared YM-agar slants with refrigerated cells and incubating them at 30 °C for 48 h. A loop full of cells from these slants was used to inoculate 125 mL of sterile MM medium in a 500-mL baffled flask (seed flask-1). Following 48 h incubation in an orbital shaker at 140 rpm, the cells in seed flask-1 were used to inoculate seed flask-2 (at 2 % v/v) also containing 125 mL of the MM. Seed flask-2 was then incubated in the orbital shaker at 140 rpm for 30 h before being used to inoculate a fermenter with 2.5 % v/v for the repeated fed-batch and continuous cultivation. The seed culture was examined under a microscope to check for any possible contamination before inoculation. For the repeated fed-batch cultivation, a 5-L glass fermenter (New Brunswick, BIOFLO 2000) was used with the 2-L working volume. A 1.2-L glass fermenter (New Brunswick, BIOFLO 310) with the 1-L working volume was used for the continuous cultivation. In both fermenters, the air flow rate was 1 volume per volume per minute (vvm), and the temperature and pH-value were controlled at 30 °C and 5.5, respectively. An amount of 0.5 M sodium hydroxide solution was used to maintain pH-value during cultivations.

The MM was used during continuous cultivation for both the initial culture as well as the feeding media. The dilution rate was controlled by varying the medium flow rate. Dilution rates of 0.02 h^{-1} and 0.06 h^{-1} were investigated in this study. Continuous feeding was started following 24 h or 72 h of batch cultivations. The repeated fed-batch cultivation used the MM with 90 g L^{-1} glucose for the initial culture, and a pure glucose solution was used for each feeding to maintain the glucose concentration in the culture to 30 g L^{-1}. Feeding times were determined when glucose concentration fell below 1 g L^{-1}.

Analytical methods

Cell concentration

Cell concentrations in samples were determined by measuring a culture optical density using a DR 1500 UV-Vis spectrophotometer (Hach Company, USA) at 570 nm. Samples for cell concentration measurements were collected at variable time intervals (8 to 24 h). All the measurements were conducted in triplicate. Cell dry mass (CDM) of cells was measured in cultures at the end of the cultivation, and optical density vs. CDM concentration calibration curve was prepared using appropriately diluted cell suspensions. The calibration curves were used to convert optical density into cell concentrations (g CDM L^{-1}).

Lipid analysis

Lipids in the samples were measured using the Nile red fluorescence method[18] using a Shimadzu spectrofluorophotometer (RF-1501, Shimadzu Corporation, Japan). At the end of each fermentation, cells were recovered from broth, and the Bligh and Dyer lipid extraction method was used[19]. Calibration curves relating sample lipid content vs. fluorescence intensity were then prepared for both fermenters and used to translate fluorescence into lipid concentrations (g lipid L^{-1}).

Carbohydrate separation and analysis

The culture samples were filtered through a 0.45 µm filter, and the filtrate was added to chilled ethanol at a 1:19 ratio (v/v). Following 2 h incubation in the freezer, the solution was centrifuged at 12,000 g for 10 min (SorvallStratos Benchtop Centrifuge – Thermo Fisher Scientific, U.S.A.), and the pellet (if any) was separated from the supernatant. The supernatant was analyzed for glucose concentration using the Daines colorimetric method[20], and a calibration curve relating absorbance and glucose concentration was used to report glucose concentration (g glucose L^{-1}). The pellet was suspended in deionized water and analyzed for polysaccharides using the phenol-sulfuric acid method[21]. A calibration curve was then prepared for absorbance vs. polysaccharide concentration, and used to translate the absorbance into polysaccharide concentrations (g polysaccharide L^{-1}).

Fourier Transform Infrared (FTIR) analysis

Sample was freeze dried for 48 h, before FTIR spectroscopy was conducted in the Chemistry Department at UL Lafayette on an Agilent Cary 630 FTIR spectrometer with the wave number region of 400 – 4000 cm^{-1} used to determine the functional groups of polysaccharides produced.

MALDI-TOF MS analysis

MALDI-TOF MS was performed by the Murray Mass Spectroscopy group in the Department of Chemistry, Louisiana State University.

Spheroplast production

The culture sample was centrifuged at 7500 rpm for 15 min and washed with deionized water twice, before digesting using the Zymolyase 20-T (Nacalai USA, USA) in a Tris-sorbitol buffer applying a modified method of Suzuki and Iwahashi[22].

Results and discussion

In order to ascertain the effects of the MM minimal medium as a fermentation medium for *Lipomyces starkeyi*, two cultivation strategies were investigated. Firstly, two repeated fed-batch cultivation experiments were conducted with 3 feeding pulses of glucose. For feed batch fermentations, the MM with the 90 g L^{-1} initial glucose concentration and the initial 339 C/N ratio was used. Secondly, a series of continuous cultivations was carried out using two different dilution rates (0.06 h^{-1} and 0.02 h^{-1}) and two different C/N ratios (113 and 56).

Repeated fed-batch cultivation

The repeated fed-batch cultivation resulted in a maximum cell density of 22.7 g L^{-1} (306 hours) and a maximum lipid concentration of 11.67 g L^{-1} (330 hours), respectively. Maximum cell density of 20.5 g L^{-1} (144 hours) was achieved utilizing the initial glucose in the medium; subsequent additions of glucose resulted in variable production of cell mass and lipids (Fig. 1). Each feeding reduces the concentration of cells and lipids due to the increase in volume, but both cells and lipids generally increase to their previous concentrations. An extended lag phase of 24 h exhibited by both cultures, which is a contrast with 16 h observed for batch culture by[2], could be an indication that the yeast cells were experiencing a shock resulting from increased substrate concentrations. Such a culture shock can explain the production of a byproduct[23]. The production of a byproduct results in a discoloration of the culture and an increased viscosity of the medium. Similar behavior of discoloration and increased viscosity changes was also observed by Koller *et al.*[24] with the bacterial strain *Haloferax mediterranei* during the synthesis poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) and extracellular polysaccharide. The byproduct concentration reaches a maximum of 39.5 g L^{-1}. SEM imaging of the freeze-dried broth indicated that the produced substance is localized outside the cells, and treatment with the zymolyase
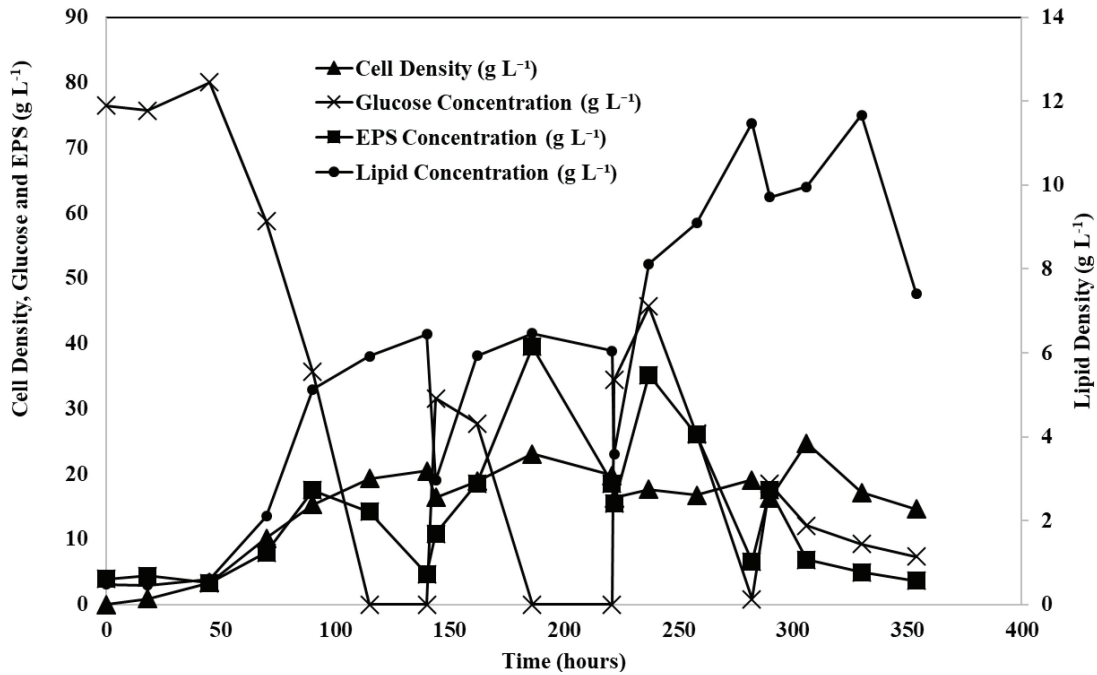
F i g . 1 – *Cell density, lipid concentration, glucose concentration, and polysaccharide production by L. starkeyi during repeated fed-batch cultivation in the MM with glucose*

enzyme cocktail results in its almost complete breakdown and removal (Fig. 2). Interestingly, there is almost a 10-fold reduction in the fluorescence intensity observed in the presence of the byproduct even though the cell lipid content does not decrease. There is no literature evidence on an impact of other chemicals on the cell permeability to Nile Red, but the difference in fluorescence intensity for similar lipid content depending on the presence or absence of the byproduct lends credence to the idea.

The finding that zymolyase enzyme can breakdown the produced compound, in combination with the FTIR analysis (Fig. 3) indicates that the produced material is an exopolysaccharide (EPS). The FTIR peaks of interest include 3500 – 3200 cm⁻¹ (–OH group), 3000 – 2850 cm⁻¹ (C–H stretch), 1470 – 1450 cm⁻¹ (C–H bend), 1370 – 1350 cm⁻¹ (C–H rock), and 1320 – 1000 cm⁻¹ (C–O stretch), all indicating a possible saccharide[25]. Comparison with FTIR spectra of common mono- and di-saccharides reveale that the polysaccharide possibly contains mannose and galactose due to peak similarity. Alternative methods of polysaccharide extraction were considered, including water boiling and acid bath[26]. Both methods were evaluated by SEM imaging, as indicated in Fig. 2. Based on the images, it can be concluded that the acid bath did not digest the polysaccharide, but did digest the cellular matter, resulting in the pockmarked texture. The water boiling method only partially removed polysaccharide surrounding the cells. This could be an indication that measurement of the polysaccharide, following fil-

tration of cells, does not necessarily capture the total content of produced exopolysaccharides.

The solubility testing with common organic solvents revealed that the polysaccharide is soluble in water, but insoluble in methanol, ethanol, toluene, tetrahydrofuran, dimethyl sulfoxide, and dichloromethane. The effect of selected solvents (THF and toluene) in the cell broth is presented in Fig. 2, showing that the solvents do not significantly change the surface of the freeze-dried cells. MALDI-TOF MS analysis of the precipitated EPS (Fig. 4) indicates peaks primarily in the 680–880 Dalton size. Considering 180 Dalton molecular mass per hexose, and 194 Dalton molecular mass for uronic acids, approximately 4–5 repeating units are expected to form the structure of the polymer. The peaks from 850 cm⁻¹ to 1100 cm⁻¹ in the FTIR spectra also indicate that that some of the moieties are uronic acids[27].

The polysaccharide and lipid production generally seems to share an inverse relationship. Although both increase following an addition of glucose, eventually, the EPS quantities seem to be declining, and the lipid quantities increasing. One possible explanation is that the yeast is actively consuming the EPS. *L. starkeyi* has the capability to produce polysaccharide hydrolyzing enzymes such as dextranase and amylase[28]. These enzymes could potentially be cleaving the polysaccharide back into its constituent monosaccharides (including mannose and galactose), which are then utilized by the yeast in lipid production. However, there is a fluctuation in EPS concentrations. One possible explanation is
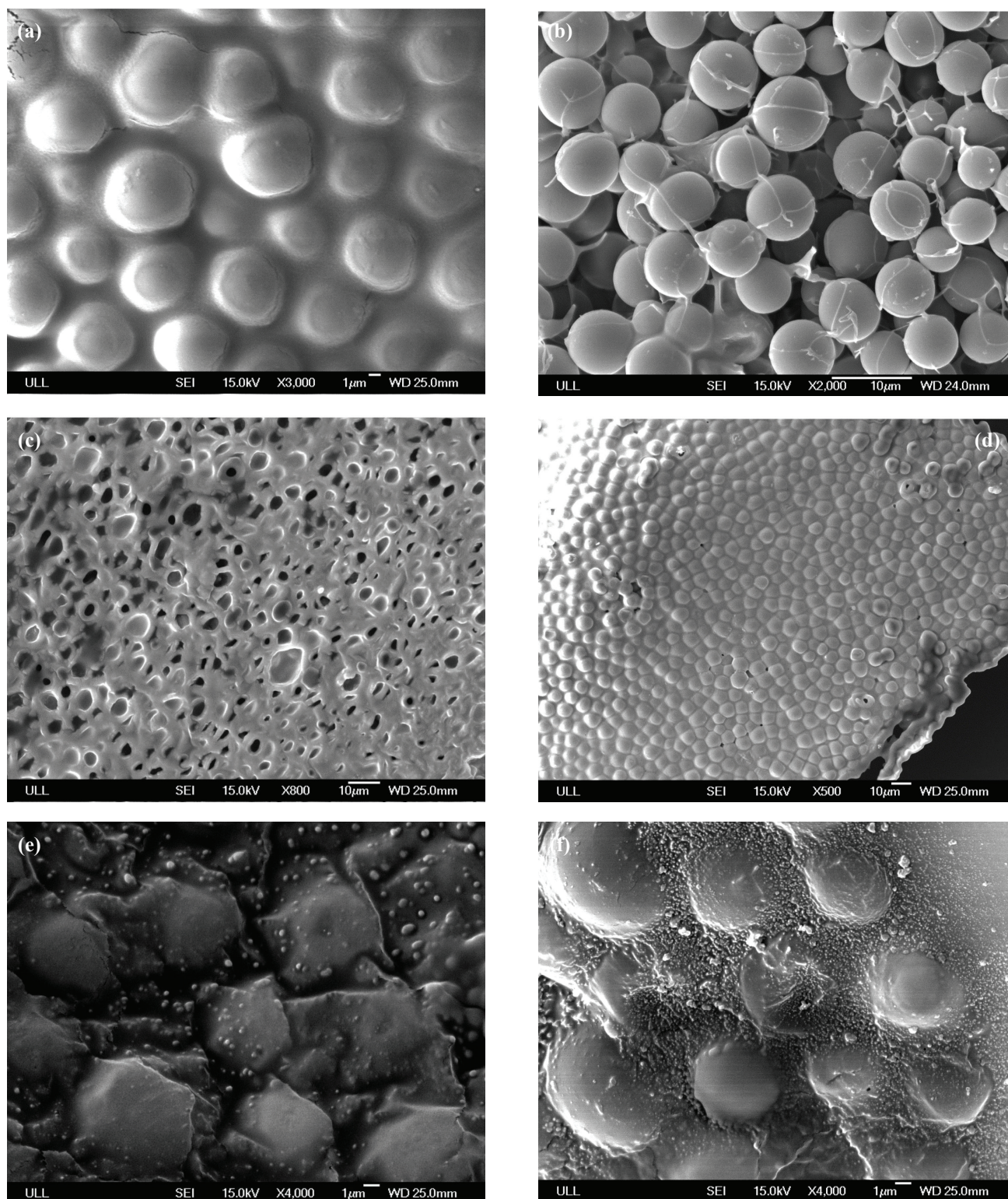
Fig. 2 – *(a) SEM image of freeze-dried cells collected after repeated fed-batch experiment. Cells are seen encased in the EPS connective matter. (b) SEM image of freeze-dried, zymolyase-treated cells collected after repeated fed-batch experiment. Cells are stripped of the EPS connective matter. (c) SEM image of freeze-dried, acid-bath-treated cells collected after repeated fed-batch experiment showing EPS remaining after cell digestion. (d) SEM image freeze-dried, boiling-water-treated cells collected after repeated fed-batch experiment showing EPS remaining intact. (e) SEM image of freeze-dried cells mixed with THF. (f) SEM image of freeze-dried cells mixed with toluene.*

F i g . 3 – *FTIR spectra of precipitated EPS from repeated fed-batch cultivation*

the dilution of EPS during the addition of new batch of sugar. A second possible explanation could be consumption of EPS when glucose in the medium is limited, and the third possible explanation could be that the yeast species might be capable of producing both water soluble and water-insoluble polysaccharides, as has been reported for other species[29]. While transitions between the two polysaccharides can explain the changes in concentrations, the driving force behind this possible mechanism is unclear.

Continuous cultivation

The fermentation profiles obtained from the continuous cultivation of *L. starkeyi* in the MM are shown in Fig. 5. At the lower dilution rate and C/N ratio, 83 % of the glucose was consumed resulting in cell growth to 5 g L^{-1} with the lipid content of 40 %. At a higher dilution rate and C/N ratio, only 43 % of the glucose was consumed, reaching a cell mass of 5 g L^{-1} and lipid content of 46 %. Although Huang *et al.*[30] have indicated that the dilution rate lower than 0.06 h^{-1} is normally required for optimal substrate-to-product conversions, our results show higher lipid concentrations at the 0.06 h^{-1} dilution rate. In contrast to the results of Anschau *et al.*[7], the change in dilution rates does not seem to affect the stability of the cell concentration, but does affect the stability of the glucose concentration. Papaniko-
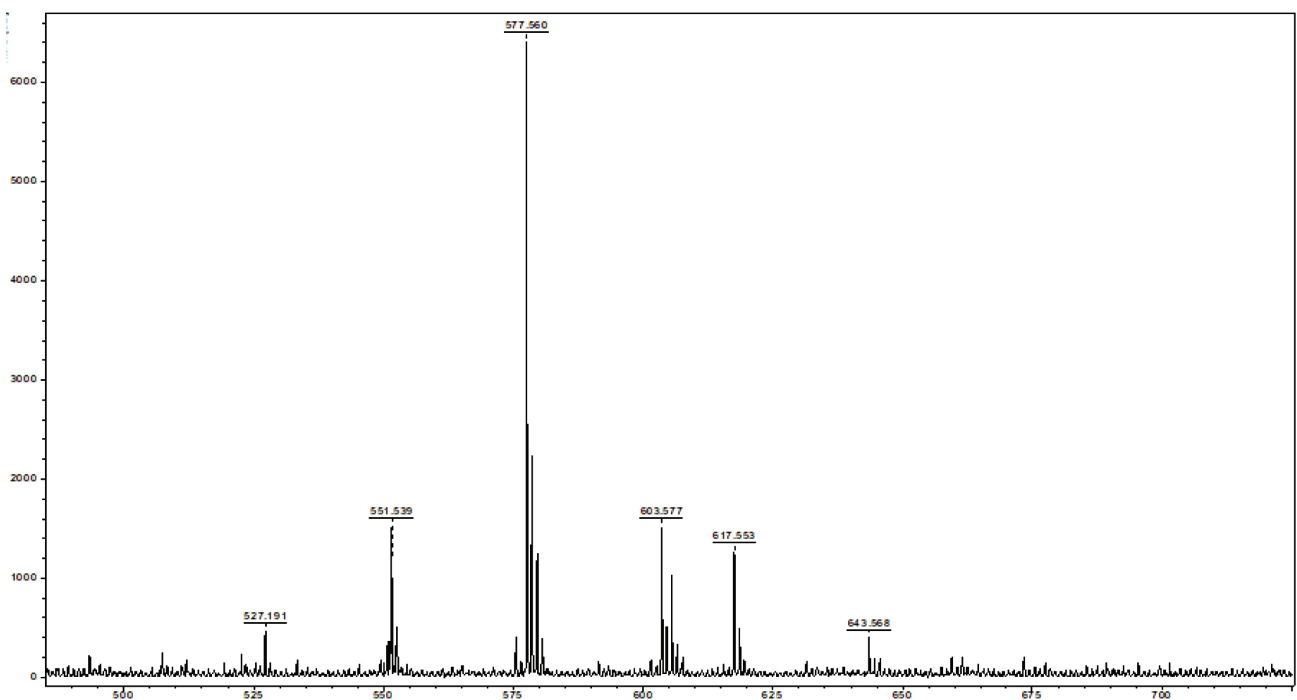

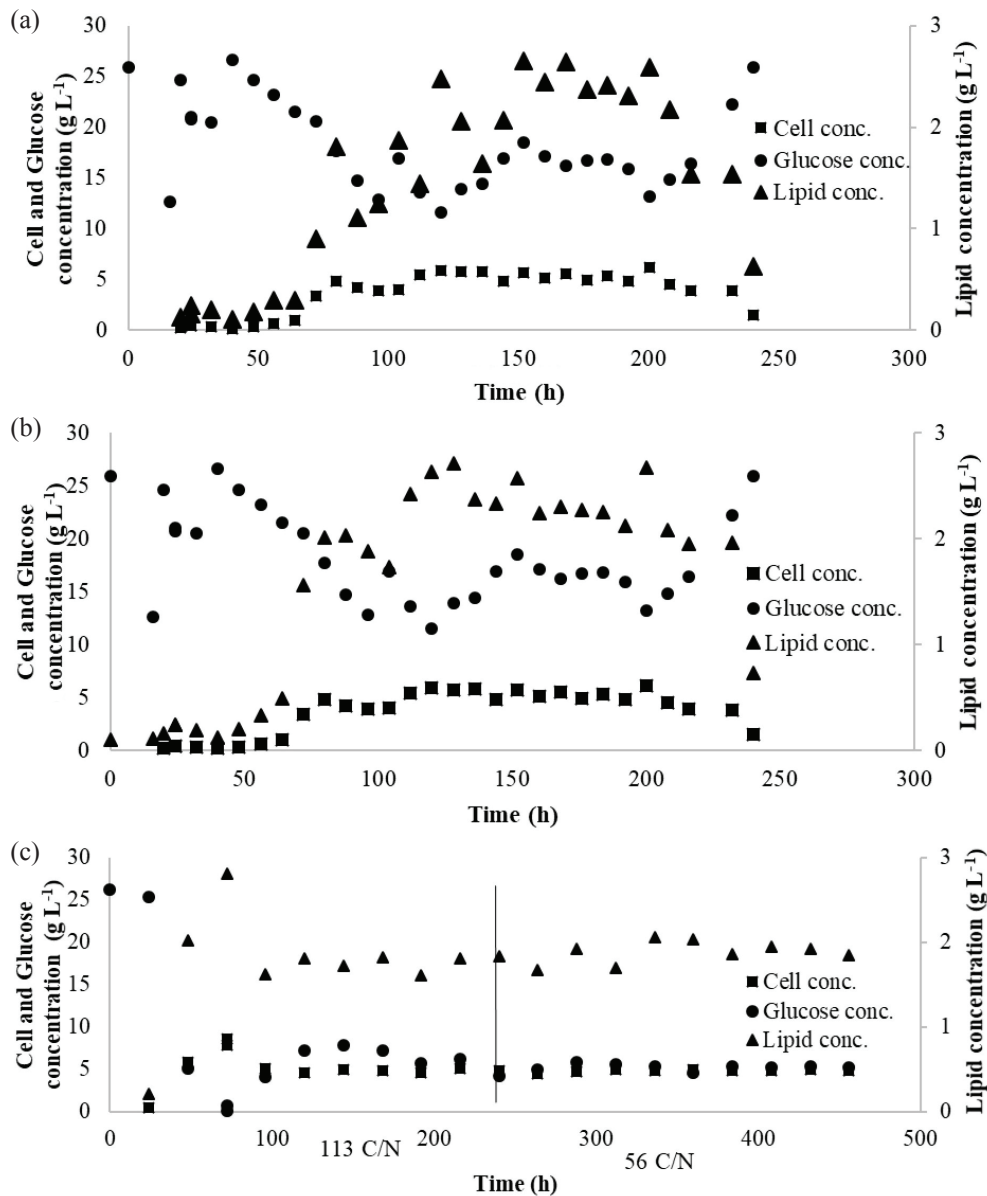F i g . 4 – *MALDI-TOF MS spectra of EPS separated from cell-broth by ethanol precipitation*

Fig. 5 – *(a) Continuous cultivation of L. starkeyi at 0.06 h⁻¹ dilution rate with C/N ratio of 113; (b) Continuous cultivation of L. starkeyi at 0.06 h⁻¹ dilution rate with C/N ratio of 56; (c) Continuous cultivation of L. starkeyi at 0.02 h⁻¹ dilution rate with C/N ratio of 113 and 56, respectively*

laou and Aggelis[31] indicated that a higher concentration of sugars that can be detected at high dilution rates results in an increased cell mass yield, but reduced lipid fraction.

The parameters characterizing the continuous cultivation are presented in Table 1. The lag phase varied for the cultures from 24 h to 80 h. Since this was longer than that for the batch culture, it could be an indication that the cells were undergoing some additional stress during the inoculation. The cell concentration, lipid concentration, cell productivity, and lipid productivity at the 0.06 h⁻¹ dilution rate and 56 C/N ratio are comparable to that of Anschau *et al.*[7], after adjusting for the difference in starting sugar concentration by a factor of 1:2. The

effect of the change in C/N ratios is seemingly negligible. Both the 113 and 56 C/N ratio media compositions show no markedly different changes in fermentation parameters. This finding is contrary to previous research, where the C/N ratio had a noticeable impact on cell growth[10,32]. One potential explanation could be that the nitrogen supplied in the media is not consumed completely at the tested dilution rates, resulting in similar cell concentrations for the two C/N ratios. Since the rate of cell growth is driven primarily by the availability of nitrogen for the production of important macromolecular components[33], the continued availability of nitrogen might result in similar cell growth profiles. Another possibility could be that, although the nitrogen in

Table 1 – *Cell productivity, lipid productivity, and glucose consumption for continuous cultivation of L. starkeyi*

C/N ratio	113	113	56	56
Dilution rate (h⁻¹)	0.06	0.02	0.06	0.02
Stable cell concentration (g L⁻¹)	5	5	5	5
Stable lipid concentration (g L⁻¹)	2.3	1.9	2.3	2
Stable glucose concentration (g L⁻¹)	17	7	15	5
Cell productivity (g L⁻¹ h⁻¹)	0.313	0.0971	0.307	0.0987
Lipid productivity (g L⁻¹ h⁻¹)	0.138	0.0468	0.124	0.0410
Glucose consumption rate (g L⁻¹ h⁻¹)	0.78	0.46	0.90	0.50
Cell yield (g g⁻¹)	0.401	0.211	0.341	0.197
Lipid yield (g g⁻¹)	0.177	0.102	0.138	0.082

the media is consumed completely, the activity of AMP deaminase is sufficient at the culture conditions to maintain nitrogen availability[34].

Conclusions

The study investigated the cell mass and lipid production of *L. starkeyi* using minimal medium under repeated fed-batch and continuous cultivation strategies. A maximal cell mass of ~26 g L⁻¹ and lipid concentration of ~13 g L⁻¹ were achieved using repeated fed-batch cultivation, along with exopolysaccharide production (max of ~50 g L⁻¹). The produced exopolysaccharide was found to likely be composed of 4–5 repeating units, with mannose and galactose. Continuous cultivations at 0.06 h⁻¹ presented the highest yields and productivities and were comparable to *L. starkeyi* grown on media containing yeast extract. The results indicate that the developed minimal media is a comparable low-cost alternative to the Suutari media[15] in continuous cultivation as well as repeated batch cultivation. However, the repeated fed-batch cultivation process, using the MM minimal medium, will need to be further investigated and adjusted, in order to overcome byproduct exopolysaccharide formation, or the economics of exopolysaccharide production process needs to be investigated since it has commercial value, before the minimal medium can be utilized in lipid production by *L. starkeyi*.

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