

# Lipid Turnover During Inverse Flocculation in *Saccharomyces cerevisiae* UOFS Y-2330

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## ABSTRACT

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In this study we uncovered that *Saccharomyces cerevisiae* UOFS Y-2330 does not only demonstrate inverse flocculation, but is also characterised by two different lipid turnover patterns. During Flo1 phenotype flocculation, this yeast showed two neutral lipid accumulating stages (i.e. at 8 h and from 12 h). This is probably triggered by flocculation, which can be regarded as a survival mechanism where cells accumulate predominantly neutral lipids as a reserve energy source – a similar mechanism is probably operative when cells enter stationary growth. Contrary to Flo1 behaviour, this strain in NewFlo phenotype mode demonstrates only a single lipid accumulation phase i.e. when cells enter stationary growth, which coincides with increase in flocculation. In addition, an increase in phospholipids was experienced during active growth in both flocculation behaviours i.e. Flo1 and NewFlo probably as a result of active membrane production.

**Key words:** Flo1, flocculation, lipid turnover, NewFlo, *Saccharomyces cerevisiae*.

## INTRODUCTION

Flocculation is of prime importance to the beer industry and other biotechnological processes where it functions as a means to separate suspended cells from the medium, before further processing. In yeasts, flocculation may occur as a prelude to sexual reproduction or as a means of protection during adverse environmental conditions<sup>18</sup>.

Even after extensive research, the exact mechanisms of flocculation onset and of flocculent bond formation are still poorly understood. Currently the Lectin Theory is accepted to explain flocculation<sup>15</sup>, although an increase in cell wall carboxyl groups and thus cell surface hydrophobicity (CSH) at the onset of flocculation was observed<sup>2</sup>. Here, a threshold value for carboxyl group density was observed. In cases where this threshold value was exceeded, flocculation was initiated. Consequently, CSH has been identified as a major factor responsible for flocculation onset<sup>1,20–22</sup>.

This observation was further supported by Kock et al.<sup>13</sup> who observed the accumulation of hydrophobic carboxylic acids (3-hydroxy oxylipins) on the cell surfaces of a *Saccharomyces cerevisiae* strain during the initiation of flocculation.

It is proposed that 3-hydroxy oxylipins are produced by incomplete  $\beta$ -oxidation probably in the mitochondria of fungi<sup>8</sup>. Strikingly, mitochondrial function also appears to be important for flocculation since flocculation induction is repressed in the presence of uncouplers as well as glycolytic and respiratory inhibitors<sup>17</sup>.

With this as background it became the purpose of this study to follow the lipid turnover during the growth cycle of a yeast strain showing both Flo1 and NewFlo behaviour<sup>19</sup>. This phenomenon may shed more light on the role of lipids in yeast flocculation.

## MATERIALS AND METHODS

### Strain used

*Saccharomyces cerevisiae* UOFS Y-2330 was used throughout this study and is held at the University of the Free State (UFS), South Africa.

### Growth experiments

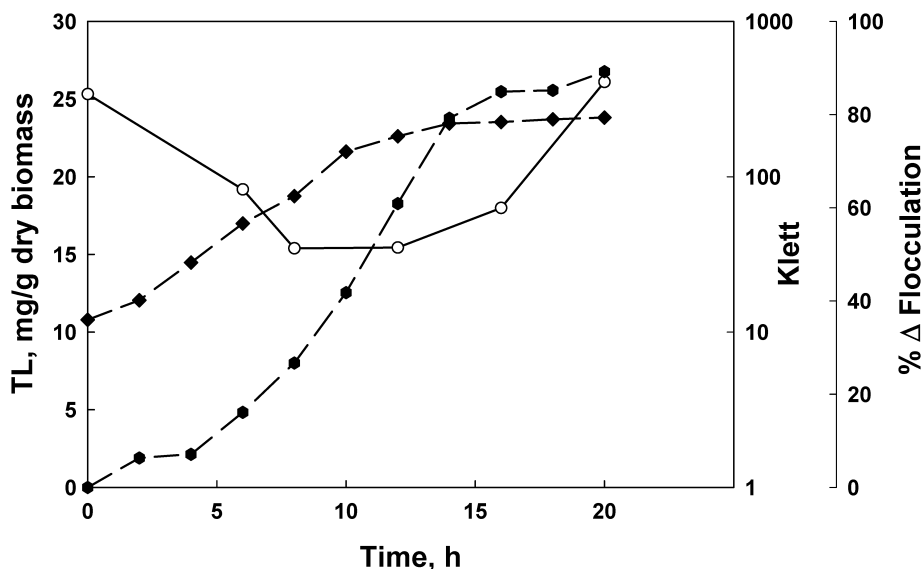
*Saccharomyces cerevisiae* UOFS Y-2330 was cultivated as described by Strauss et al.<sup>19</sup> In short, cells were inoculated from YM<sup>24</sup> agar slants into 250 mL conical flasks containing 50 mL of glucose-YM broth (12g/L glucose, 5 g/L peptone, 3 g/L yeast extract, 3 g/L malt extract) and incubated at 30°C while shaking (160 rpm) for 20 h until late exponential phase was reached. Appropriate volumes were then transferred to several 500 mL side-arm conical flasks containing 100 mL of the same complex medium to yield a final absorbency of 10 Klett units. These cultures were incubated at 30°C while shaking (160 rpm) for 20 h. Growth was measured at regular intervals using a Klett Summerson colorimeter (red filter) in one of the flasks equipped with a side arm. The degree of flocculation (i.e. %  $\Delta$  flocc) in this culture was measured throughout growth by calculating the decrease in cell turbidity<sup>4</sup> while the pH was constantly monitored in the cultures harvested. Several flasks to yield sufficient biomass and lipids were harvested at different time intervals as indicated in Fig. 1, followed by immediate freezing and freeze-drying.

This experiment was repeated when the yeast was grown for 20 h in chemically defined media i.e. glucose–YNB (Yeast Nitrogen Base, Difco Laboratories) medium

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**Fig. 1.** Changes in growth (Klett), %  $\Delta$  flocculation and total lipid content over the growth cycle of *Saccharomyces cerevisiae* UOFS Y-2330 when cultivated in a complex medium (NewFlo behaviour). (--- $\blacklozenge$ ---) Optical density; (--- $\bullet$ ---) %  $\Delta$  flocculation; (— $\square$ —) Total lipids (TL). This experiment was repeated and produced an SE < 5%.

(10 g/L glucose, 6.7 g/L YNB) under similar growth conditions. All experiments were performed at least in duplicate.

#### Harvesting of cells, lipid extraction and fractionation of extracted lipids

Cells were harvested by centrifugation at 9000 rpm for 10 min and then rapidly frozen at  $-70^{\circ}\text{C}$  followed by freeze-drying. Total lipid (TL) extraction was performed on freeze-dried cells according to Folch et al.<sup>9</sup> In short, lipids were extracted with a mixture of chloroform and methanol (2:1, v/v) and then washed with  $\text{dH}_2\text{O}$ . Next<sup>12</sup>, the extracted lipids were dissolved in chloroform and applied to a column of activated silicic acid (by heating overnight at  $110^{\circ}\text{C}$ ). Neutral lipid (NL), glycolipid (GL) and phospholipid (PL) fractions were eluted from the column by successive applications of organic solvents of different polarities. The extracted TLs and lipid fractions were finally dissolved in a minimal volume of diethyl ether and transferred to pre-weighed vials followed by drying over a stream of nitrogen gas. In order to quantify the TL, NL, PL and GL fractions, samples were dried to a constant weight in a vacuum oven at  $50^{\circ}\text{C}$  over  $\text{P}_2\text{O}_5$  before they were finally weighed.

#### Fatty acid analysis

The fatty acid (FA) compositions of the different fractions were determined after transesterification by the addition of trimethyl sulphonium hydroxide (TMSOH) according to Butte<sup>3</sup>. Subsequently, the FA methyl esters were analysed using a Hewlett Packard 5890 gas chromatograph, equipped with a Supelcowax 10 polar/capillary column (30 m  $\times$  0.53 mm) with nitrogen as carrier gas – set at a flow rate of 4 mL/min. The inlet temperature was set at  $180^{\circ}\text{C}$  with the initial column temperature set at  $145^{\circ}\text{C}$  – increasing at  $3^{\circ}\text{C}/\text{min}$ , followed by the same temperature

increment to a final temperature of  $240^{\circ}\text{C}$ . The FA peaks were detected using a flame ionisation detector (FID) set at  $300^{\circ}\text{C}$ . Peaks were identified using suitable standards.

#### Chemicals used

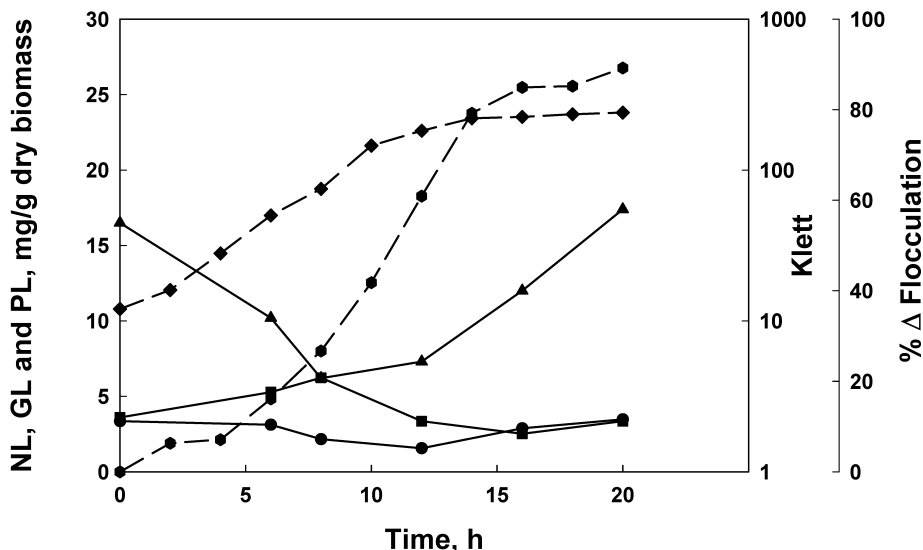
All chemicals and organic solvents used were of highest purity and analytical reagent grade and obtained from major retailers. All standards were from Sigma, while silicic acid (100 mesh) was from Aldrich.

## RESULTS AND DISCUSSION

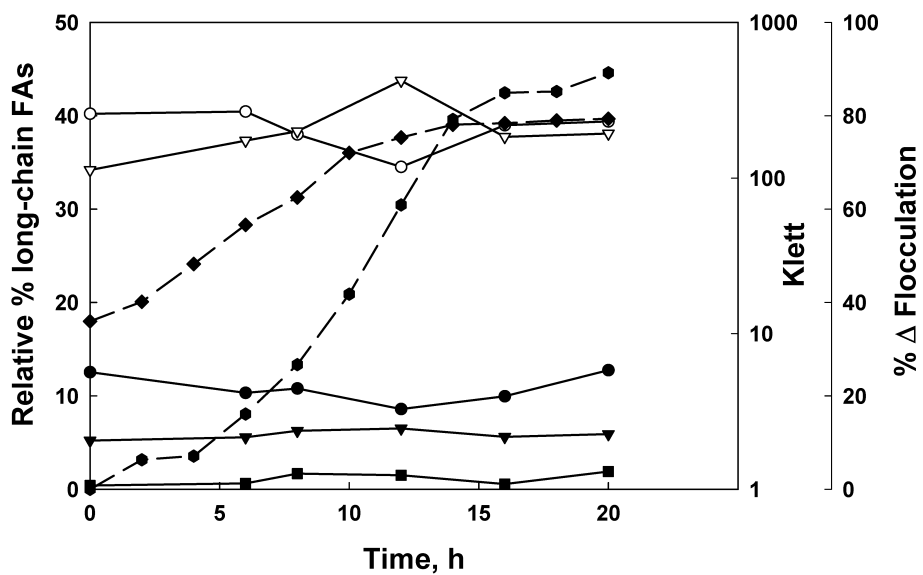
#### Lipid turnover in NewFlo phenotype behaviour

**Changes in total lipid (TL) content.** When *Saccharomyces cerevisiae* UOFS Y-2330 was grown in complex glucose containing medium, this yeast reached stationary growth phase after about 14 h (Fig. 1). The degree of flocculation (%  $\Delta$  flocc) increased sharply towards late exponential phase, which is in accordance with literature for NewFlo phenotypes<sup>11</sup> as well as that previously reported by us<sup>19</sup>. During the first 8 h of growth the TL content decreased from 25.3 mg/g dry biomass to a minimum value of 15.4 mg/g dry biomass, where-after it remained relatively constant for the next four hours. As this NewFlo yeast entered stationary phase (from 14 h onwards), the TL content increased to a value of 26.1 mg/g dry biomass after 20 h of growth. When this experiment was repeated, similar patterns were observed.

**Composition of total lipids (TLs) over the growth cycle.** Changes in the NL content during growth were similar to those found for the TLs (Fig. 2). During the first 8 h of growth the cellular NLs decreased from 16.5 to 6.2 mg/g dry biomass probably to serve as energy source for growth, through oxidation of stored FAs<sup>23</sup> and/or PLs synthesis via diacylglycerol (DAG)<sup>6</sup>. Following this, the



**Fig. 2.** Changes in growth (Klett), %  $\Delta$  flocculation and different lipid fractions over the growth cycle of *Saccharomyces cerevisiae* UOFS Y-2330 when cultivated in a complex medium (NewFlo behaviour). (---◆---) Optical density; (---●---) %  $\Delta$  flocculation; (—▲—) Neutral lipids (NL); (—■—) Phospholipids (PL); (—●—) Glycolipids (GL). This experiment was repeated and produced an SE < 5%.



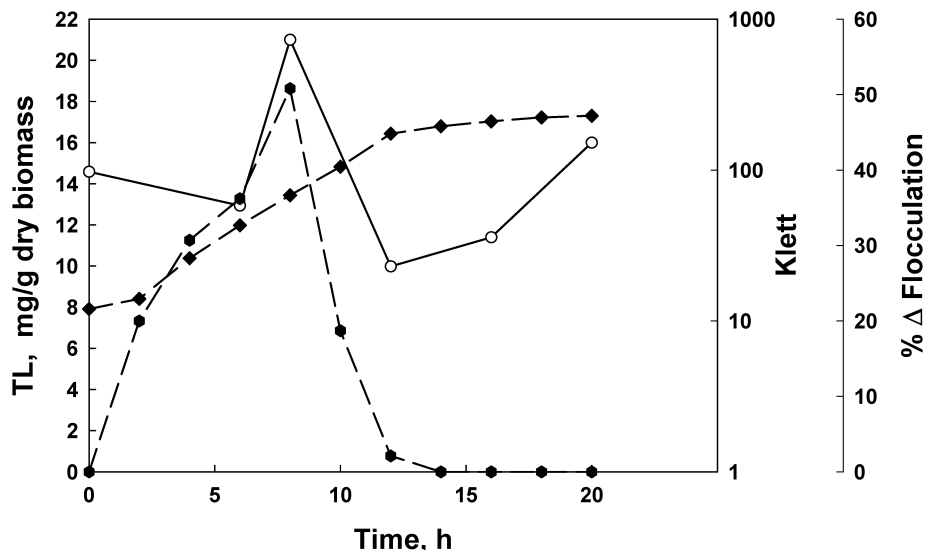
**Fig. 3.** Changes in growth (Klett), %  $\Delta$  flocculation and % long-chain fatty acids of NLs over the growth cycle of *Saccharomyces cerevisiae* UOFS Y-2330 when cultivated in a complex medium (NewFlo behaviour). (---◆---) Optical density; (---●---) %  $\Delta$  flocculation; (—●—) 16:0 (palmitic acid); (—○—) 16:1 (palmitoleic acid); (—▼—) 18:0 (stearic acid); (—▽—) 18:1 (oleic acid) and (—■—) 18:2 (linoleic acid). This experiment was repeated and produced an SE < 5%.

NLs started to accumulate from 8 h (6.2 mg/g dry biomass) to 20 h (17.4 mg/g dry biomass) of growth as the cells reached the stationary growth phase. These NLs are probably stored as an energy source for later use<sup>23</sup>.

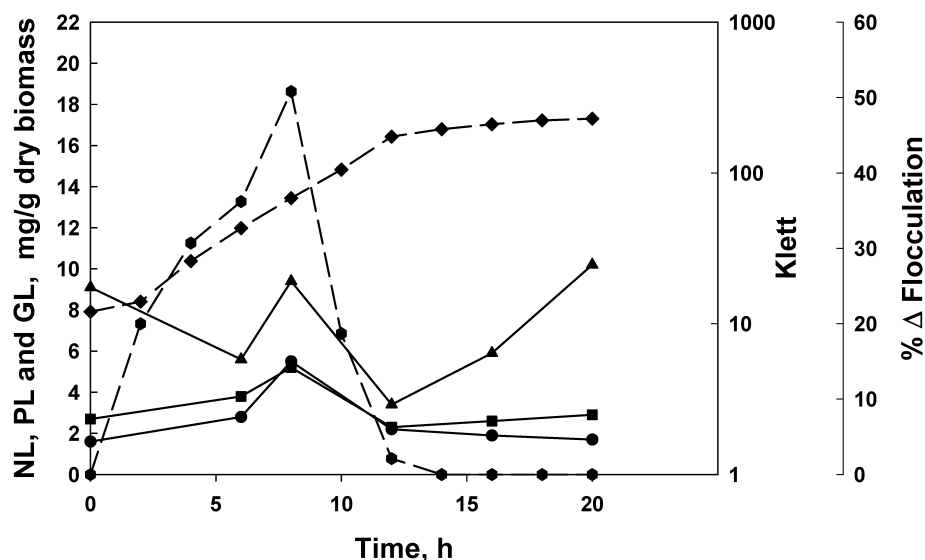
Contrary to this pattern, the PL content increased for the first 8 h, i.e. during active growth, where after it decreased i.e. as cells reach stationary phase (from 5.2 to 2.9 mg/g dry biomass). This may be ascribed to the demand

for PLs needed for membrane development in active growing cells<sup>12</sup>. The GL fraction remained more or less the same throughout the growth cycle. When this experiment was repeated, similar patterns were observed.

Similar patterns were reported by various workers on the lipid turnover (i.e. NL and PL fractions) of *Achlya*<sup>14</sup>, *Blastocladiella emersonii*<sup>16</sup>, *Dipodascopsis uninucleata*<sup>12</sup> and *D. tothii*<sup>10</sup>.



**Fig. 4.** Changes in growth (Klett), %  $\Delta$  flocculation and total lipid content over the growth cycle of *Saccharomyces cerevisiae* UOFS Y-2330 when cultivated in a chemically defined medium (Flo1 behaviour). (--- $\blacklozenge$ ---) Optical density; (--- $\bullet$ ---) %  $\Delta$  flocculation; (— $\circ$ —) Total lipids (TL). This experiment was repeated and produced an SE < 5%.



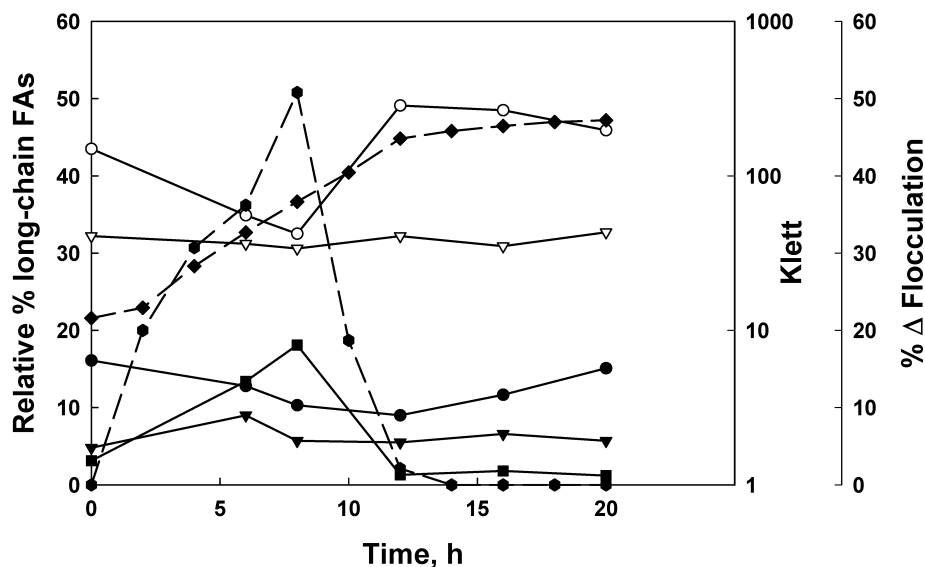
**Fig. 5.** Changes in growth (Klett), %  $\Delta$  flocculation and total lipid content over the growth cycle of *Saccharomyces cerevisiae* UOFS Y-2330 when cultivated in a chemically defined medium (Flo1 behaviour). (--- $\blacklozenge$ ---) Optical density; (--- $\bullet$ ---) %  $\Delta$  flocculation; (— $\blacktriangle$ —) Neutral lipids (NL); (— $\blacksquare$ —) Phospholipids (PL); (— $\bullet$ —) Glycolipids (GL). This experiment was repeated and produced an SE < 5%.

**Fatty acyl composition.** Changes observed in the major lipid classes over the growth cycle prompted an investigation into changes in their fatty acyl composition. All fractions contained 16:0 (palmitic acid), 16:1 (palmitoleic acid), 18:0 (stearic acid), 18:1 (oleic acid) and 18:2 (linoleic acid), while only 16:1 and 18:1 showed significant changes during the growth cycle (Fig. 3). In the NLs the percentage 16:1 decreased to a minimum value as this yeast approached the stationary phase (i.e. after 12 h), where after it started to increase. The opposite is true for

18:1. This phenomenon cannot be explained at present. Similar FA patterns were observed over the growth cycle in the other fractions (GL and PL) studied. When this experiment was repeated, similar patterns were observed.

#### Lipid turnover in Flo1 phenotype behaviour

**Changes in total lipid (TL) content.** Here, the first 8 h of growth (flocculent phase) is characterised by actively growing and flocculent yeast cells (Fig. 4). After 8 h (start of non-flocculent phase) the still actively growing cells (in



**Fig. 6.** Changes in growth (Klett), %  $\Delta$  flocculation and % long-chain fatty acids of NLs over the growth cycle of *Saccharomyces cerevisiae* UOFS Y-2330 when cultivated in a chemically defined medium (Flo1 behaviour). (---◆---) Optical density; (---●---) %  $\Delta$  flocculation; (---●---) 16:0 (palmitic acid); (---○---) 16:1 (palmitoleic acid); (---▼---) 18:0 (stearic acid); (---▽---) 18:1 (oleic acid) and (---■---) 18:2 (linoleic acid). This experiment was repeated and produced an SE < 5%.

exponential phase) almost completely deflocculated in a 4 h period (from 8 h to 12 h), where after they entered the stationary phase after 12 h of growth.

Changes in the intracellular TL content over the growth cycle are depicted in Fig. 4. During the first 6 h of the flocculent phase, the TL content decreased from 14.6 to 12.9 mg/g dry biomass, where after it increased markedly to a maximum of 21.0 mg/g dry biomass during the last two hours of flocculation. Strikingly, the rapid deflocculating period (8 h to 12 h) was accompanied by a concomitant decrease in the TL content from 21.0 mg/g dry biomass to a minimum value of 9.9 mg/g dry biomass after 12 h. It was furthermore evident that as the cells entered the late exponential phase (i.e. after 12 h), another increase in the TL occurred from 9.9 to 16.1 mg/g dry biomass. When this experiment was repeated, similar patterns were observed.

**Composition of total lipids (TLs) over the growth cycle.** When the TLs extracted from the cells during different stages of growth were fractionated into three lipid classes by column chromatography, the results depicted in Fig. 5 were obtained. Changes in the TLs as well as NL and PL fractions followed similar patterns. The gradual decrease in NLs during the first 6 h of growth (from 9.1 to 5.6 mg/g dry biomass) may be ascribed to the fact that it was used as an energy source for growth through oxidation of stored FAs<sup>23</sup> and/or PL synthesis via diacylglycerol (DAG)<sup>6</sup>. Surprisingly, after 6 h, the cells started to accumulate NLs (i.e. from 5.6 to 9.4 mg/g dry biomass) after which (8 h) it decreased again to reach a minimum (3.9 mg/g dry biomass) after 12 h. Increase in NLs is probably a result of flocculation, which can be regarded as a survival mechanism or prelude to sexual reproduction<sup>18</sup>.

As cells entered stationary growth phase, (from 12 h) the NLs increased from 3.9 to 10.2 mg/g dry biomass

after 20 h. This may be ascribed to the tendency of yeasts to accumulate NLs during stationary growth phase as an endogenous energy source for later utilisation<sup>23</sup>.

The PL patterns observed were similar over the growth cycle as reported for NewFlo phenotype behaviour. The increased PL content during the first 6 h of flocculation (exponential growth phase) may be due to a bigger demand for membranes required for cell growth<sup>12</sup>. Similar patterns to that observed for the PL fraction were also evident for the GL fraction. This cannot be explained at present. When this experiment was repeated, similar patterns were observed for all lipid fractions.

**Fatty acyl composition.** When looking at the changes in FA composition of the different fractions over the growth cycle, interesting results were obtained. All the fractions contained 16:0, 16:1, 18:0, 18:1 and 18:2, but only 16:1 and 18:2 showed significant changes during the course of growth (Fig. 6). In the NLs, the percentage 16:1 decreased to a minimum value during the flocculent phase, where after it started to increase. The opposite trend was observed for the percentage 18:2, which increased to a maximum value during the first 8 h of flocculation, and then gradually decreased. We conclude that during the first 8 h of growth, 16:1 is probably converted to 18:1 via an elongase enzyme, which is then further desaturated to 18:2 via a  $\Delta^{12}$  desaturase enzyme<sup>5</sup>. Furthermore, the demand for polyunsaturated FAs (PUFAs) seems to be higher during the exponential growth phase probably necessary to keep membranes fluid during active cell growth<sup>12</sup>. The FA patterns were similar in the GL and PL fractions studied. Similar patterns were observed when this experiment was repeated.

It is interesting to note that according to previously reported research<sup>7</sup> *Saccharomyces cerevisiae* cannot produce 18:2. The strain used in our study does not only de-

viate from other *Saccharomyces cerevisiae* strains by showing both Flo1 and NewFlo behaviours, but also in regard to its lipid metabolism.

#### ACKNOWLEDGEMENTS

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