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Characterization of the metabolism of the yeast *Yarrowia lipolytica* growing as a biofilm

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Abstract

Yarrowia lipolytica is a well-characterized yeast with remarkable metabolic adaptability. It is capable of producing various products from different carbon sources and easily switching between planktonic and biofilm states. A biofilm represents a natural means of cell immobilization that could support continuous cultivation and production processes, such as perfusion cultivation. However, the metabolic activities of *Y. lipolytica* in biofilms have not yet been studied in detail. Therefore, this study aimed to compare the metabolic activities of *Y. lipolytica* in biofilm and planktonic states. Conventionally, a stirred tank bioreactor was used to cultivate *Y. lipolytica* in a planktonic state. On the other hand, a trickle bed bioreactor system was used for biofilm cultivation. The low pH at 3 was maintained to favor polyol production. The accumulation of citric acid was observed over time only in the biofilm state, which significantly differed from the planktonic state. Although the biofilm cultivation process has lower productivity, it has been observed that the production rate remains constant and the total product yield is comparable to the planktonic state when supplied with 42% oxygen-enriched air. This finding indicates that the biofilm state has the potential for continuous bioprocessing applications and is possibly a feasible option.

Keywords: *Yarrowia lipolytica*; microbial bioconversion; biofilm formation; trickle bed reactor; glycerol conversion; yeast immobilization

Introduction

Yarrowia lipolytica is a yeast that has gained much attention due to its potential biotechnological applications displaying a wide genomic diversity originating from various habitats in different geographic regions. (Rywińska et al. 2013). *Yarrowia lipolytica* has a high number of functional genes coding for unique features such as the metabolism of *n*-alkanes, fats, and fatty acids (Thevenieau et al. 2009). From a physiological standpoint, it has been observed that the majority of isolated *Y. lipolytica* strains are haploid and possess the capability to transition between yeast and hyphal forms. The transition stimuli have been identified as *N*-acetylglucosamine and/or serum (bovine albumin), both of which induce pseudohyphal growth in *Y. lipolytica*, in contrast to glucose, which does not elicit morphological transition. While adjustments to environmental conditions such as pH, temperature, and ammonium sulfate concentration (as the nitrogen source) during the process did not result in an enhanced degree of pseudohyphal transition, it is noteworthy that pretreating the yeast cells with nitrogen starvation and low temperature prior to induction significantly improved the pseudohyphal transition. This underscores the influence of specific stressors on the morphological alterations of *Y. lipolytica*. (Pérez-Campo and Domínguez 2001, Rodriguez and Dominguez 2011). The formation of biofilm can occur regardless of the current morphological state. As described by Dusane et al. (2008), *Y. lipolytica* observed in the biofilm matrix exhibited a mixture of yeast and pseudohyphal forms when glucose was used as the carbon source, and mostly pseudohyphal form when glycerol was used. The inability to induce pseudohyphal growth using glucose as the carbon source was consistent with the literature. It is possible that glycerol or the osmotic pressure from glycerol induces the pseudohyphal growth of *Y. lipolytica*.

The first reports of *Y. lipolytica* application date back to around 1960 when it was used to metabolize hydrocarbons like *n*-alkanes, 1-alkenes, or *n*-paraffins. Additionally, it can convert various carbon sources into products such as α-ketoglutarate, organic acids, alcohols, lipids, and fatty acids (Klug and Markovetz 1967, Tsugawa et al. 1969, Marchal et al. 1977, Papanikolaou et al. 2007, Pawar et al. 2019). *Yarrowia lipolytica* is generally regarded as safe (Groenewald et al. 2014). It has been utilized for the production of various food additives. Additionally, the yeast can be employed for the production of single-cell protein and single-cell oil (Zinjarde 2014). The metabolic activity of *Y. lipolytica* is highly adaptable, as shown in experiments where different strains were grown at varying pH levels. It was found that a pH range of 2.5–3.5 led to the predominant production of polyols, while a pH range of 4.5–7.5 favors the production of citric acid (Egermeier et al. 2017). In addition to its versatility in adapting to different pH levels, *Y. lipolytica* is also capable of adapting to nitrogen and phosphorus limitation, which alters its growth and forces the carbon source to be

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converted into metabolites instead of biomass (Egermeier et al. 2017, Wierzchowska et al. 2021). According to recent studies, *Y. lipolytica* exhibits the ability to shift from planktonic growth, characterized by the free movement of cells within a liquid medium, to an immobilized state as a biofilm upon encountering an appropriate solid surface. The formation of biofilm by *Y. lipolytica* was investigated and emphasized by Dusane et al. (2008). They used *Y. lipolytica* NCIM 3589 and reported that the biofilm can be formed in a wide pH range, from pH 3 to 9. The solid support materials can be both polystyrene plastic (96-well plate) and glass slides. A comparison between glucose and glycerol showed that glycerol improved the biofilm formation and induced the pseudohyphal formation of the cells within the biofilm matrix. In the medical field, yeast biofilm formation is also a topic of study, as several yeast species, including *Y. lipolytica*, exhibit the ability to immobilize themselves on the polyurethane material of catheter tubes (Abbes et al. 2017). In the environmental biotechnology field, *Y. lipolytica* strain NCBI 3589 was applied for the bioleaching of fly ash, and the yeast was observed to form a biofilm on the immobilized fly ash (Bankar et al. 2012). There is not a large body of literature focusing on the metabolic profile using *Y. lipolytica* as a biofilm or investigating the production process of biofilm on a reactor scale. The research at hand could provide a fundamental understanding of these gaps.

The process of biofilm formation is a common response of numerous microorganisms to changes in their environment. This process is characterized by the secretion of extracellular polymeric substances (EPS), which facilitate the attachment of the cells to solid surfaces and among themselves. The EPS normally consists of a glycocalyx or slime that immobilizes the microorganisms (Pan et al. 2016). The response is considered a crucial adaptation that allows microorganisms to survive in various environments. Biofilms are known for their highly dynamic nature (Watnick and Kolter 2000). This characteristic allows for the presence of multiple species within the same matrix, enabling adaptation to the environment (Battin et al. 2007). Interestingly, unicellular organisms can come together to form biofilms and perform coordinated functions as a multicellular community (Štovíček et al. 2012). Although the composition of the EPS varies depending on the microbial species, the primary composition is often made of polysaccharides, which possibly contribute to a major part of the organic carbon of biofilms (Donlan 2002, Zarnowski et al. 2014). In general, EPS is synthesized for both biotic and abiotic adhesion processes (Blankenship and Mitchell 2006, Beauvais et al. 2007) but provides also other benefits. It supports the biosorption of organic and inorganic matter from the environment, which cells can utilize (Pal and Paul 2008, Bankar et al. 2009) or constitutes a physical barrier protecting the cells from harmful substances (Alonso et al. 2023).

For *Y. lipolytica*, the ability to switch to a biofilm state was confirmed using the marine-isolated strain NCIM 3589 (Dusane et al. 2008). Additionally, the application of *Y. lipolytica* in a biofilm state was used for the production of γ-decalactones using an air-lift bioreactor made of methyl-polymethacrylate (Escamilla-García et al. 2014). While biofilms provide a simple way to immobilize cells, allowing for continuous processes, any possible effect of biofilm formation on the metabolism of *Y. lipolytica* has not been studied. This study aims to address this gap and investigate the production pattern of *Y. lipolytica* strain DSM 3286 grown as a biofilm. The production patterns of *Y. lipolytica* strain DSM 3286 in planktonic state in a stirred tank bioreactor is compared to growth as biofilm in a trickle bed bioreactor (TBBR).

Materials and methods

Yeast strain and cultivation medium

Yarrowia lipolytica strain DSM 3286 was kept frozen at −80°C using yeast extract–peptone–glycerol medium (YPG) supplemented with 20% glycerol. In general, the YPG medium contains (per liter) 18 g soy peptone, 9 g yeast extract, and 20 g glycerol. 20 g agar was added to prepare the YPG plate. The nitrogen limit (N-limit) medium (Jost et al. 2015) was applied with slightly modified into three formulas for flask scale and bioreactor cultivation. The first formula is for flask scale cultivation, referred to as a general formula, contains (per liter) 100 g glycerol, 3.1 g $(NH_4)_2SO_4$, 1.0 g KH_2PO_4, 1.3 g $Na_2HPO_4 \times 2\ H_2O$, 1.0 g $MgSO_4 \times 7\ H_2O$, 0.2 g $CaCl_2 \times 2\ H_2O$, 0.5 g citric acid, 21 mg $FeCl_3$, 1 mg thiamin–HCl, 0.5 mg H_3BO_3, 0.06 mg $CuSO_4 \times 5\ H_2O$, 0.1 mg KI, 0.45 mg $MnSO_4 \times H_2O$, 0.71 mg $ZnSO_4 \times 7\ H_2O$, and 0.23 mg $Na_2MoO_4 \times 2\ H_2O$. The glycerol and $(NH_4)_2SO_4$ concentrations were changed to make the second and third formulas while maintaining the same concentration of other medium components. The second formula for generating the initial biomass in a bioreactor (growth phase), referred to as a growth medium, contains 30 g/l glycerol and 3.1 g/l $(NH_4)_2SO_4$. The third formula for inducing the product formation in a bioreactor (production phase), referred to as a production medium, contains 70 g/l of glycerol without $(NH_4)_2SO_4$. The pH was adjusted to 3 using H_3PO_4 before the cultivation. The pH-adjusted medium was then sterilized using filtration.

Preculture preparation

Yarrowia lipolytica's frozen stock was streaked on a YPG plate to observe its colony morphology. A single colony was passed into a YPG medium to prepare a preculture batch. The working volume for preculture cultivation was typically 10% of the flask's total volume. The cultivation was carried out at a temperature of 30°C for 24 h, with 180 rpm of orbital mixing.

Cultivation of *Y. lipolytica* in the planktonic state using stirred tank bioreactor

The DASGIP bioreactor system (Eppendorf AG in Hamburg, Germany) was used for planktonic state cultivation. The system can operate up to four bioreactor setups independently and automatically control the pH, temperature, and dissolved oxygen content (%DO). Each bioreactor vessel and probe were sterilized using an autoclave at 121°C for 20 min. Then, 400 ml medium was added to each bioreactor vessel. Before inoculation, an antifoam (SB 2121, Schill+Seilacher, Hamburg, Germany) was prepared and added to the cultivation medium. The antifoam was diluted to 5% v/v using distilled water and added to the cultivation medium at a ratio of 1:100 ml (5% antifoam solution: cultivation medium). As a result, the final concentration of antifoam in the medium was 0.005% (v/v).

The preculture was washed with sterile distilled water and used to inoculate the bioreactor for the initial optical density at 600 nm (OD_{600}) of 1. The temperature was maintained at 30°C, and the %DO was controlled at 50% through the automated control of agitation speed and gas mixing. pH was maintained at 3 by adding 8 M NaOH. The cultivation process involves two phases, growth and production phase. During the growth phase, the initial biomass is created using the growth medium. The biomass is then harvested via centrifugation and resuspended in the production medium to induce product formation during the production phase. To prevent nutrient deficit stress, the switch between the two cultivations happened as soon as glycerol was completely

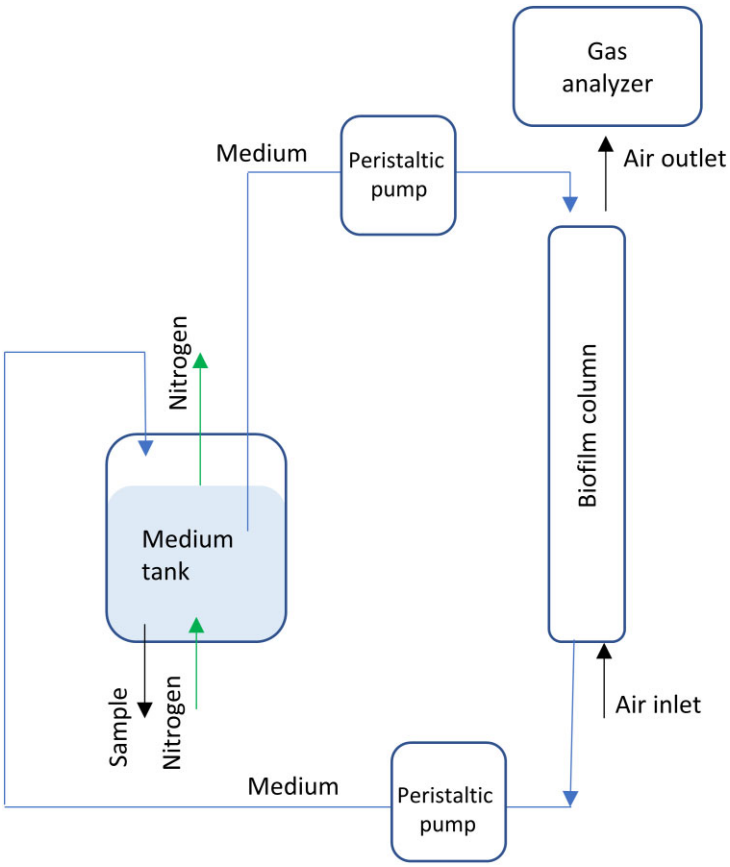

Figure 1. Schematic representation of the TBBR illustrating the flow of liquid circulating between the medium tank and biofilm column, and also the air and nitrogen gas feeding the biofilm column and medium tank, respectively.

consumed. The cultivation process lasted between 0 and 18 h for the growth phase from 18 to 48 h for the production phase.

Cultivation of *Y. lipolytica* in the biofilm state using a flask-scale cultivation

Before inoculation, the *Y. lipolytica* preculture was washed and re-suspended in sterile distilled water. The cultivation process was divided into two phases, growth and production phase. For the growth phase, biofilm was allowed to grow in YPG medium for 3 days before changing the medium to a nitrogen-limited medium (general formula) to start the production phase. The sterile empty flasks were prepared by dry heat at 180°C for 4 h (Thermo Scientific, Heratherm OMH 180, USA). As a biofilm solid support, the sintered glass tubes were sterilized by autoclaving at 121°C for 20 min. The sterile sintered glass tubes and medium were transferred into the sterile empty flask. *Yarrowia lipolytica* DSM 3286 was inoculated at an initial OD_{600} of 1. The flask-scale biofilm cultivation was carried out under static conditions (0 rpm, rotations per minute) to avoid the interruption of the biofilm growth. Cultivation temperature was maintained at 30°C. Samples were taken on the 0th, 1st, 3rd, and 5th day of cultivation for further analysis using high performance liquid chromatography (HPLC).

Cultivation of *Y. lipolytica* in the biofilm state using a TBBR

The TBBR consists of the medium tank and the biofilm column (Fig. 1). The DASGIP bioreactor system prepared the same way as in planktonic cultivation, was used as the medium tank to control the pH, temperature, and %DO. Glass cylinders with a length of 50 cm and an inner diameter of 3.5 cm were used as biofilm columns. These cylinders were randomly packed with sintered glass tubes and then sterilized via autoclaving at 121°C for 20 min. Following sterilization, the biofilm columns were connected to the bioreactor vessel. Peristaltic pumps were connected to the tubing to circulate the cultivation medium between the medium tank and the biofilm column. Nitrogen gas was continuously supplied to the medium tank to maintain an anaerobic condition and maintain low planktonic cell activities. Conversely, compressed air, with either 21% or 42% oxygen, was continuously fed to the biofilm column to promote the growth of biofilm and support the activities of cells in the biofilm state. Similar to the planktonic cell cultivation, the preculture of *Y. lipolytica* was inoculated for the initial OD_{600} 1. Biofilm cultivation also consists of two phases as described in the planktonic state cultivation. The cultivation process lasted between the 0th and the 4th day for the growth phase and the 4th and 16th day for the production phase. The cultivation process was divided into two phases: the growth and production phases. Different batches of cultivation medium were used to ensure that the initial biofilm mass and planktonic cells (used as a reference experiment) were generated under identical conditions. The cultivation medium generally contains trace elements that are not analysed or monitored during the process. To eliminate uncertainty about the consumption rate of these trace elements, fresh medium was supplied in the next cultivation step. Separating the cultivation process also helps reduce the amount of metabolic waste produced during the growth phase, and the production pattern was monitored with a similar starting medium composition.

Figure 2. Biofilm formation of *Y. lipolytica* strain DSM3286 on the liquid/gas interface using YPG medium while applying different solid materials as a support, including (A) ceramic, (B) glass tube, (C) nylon, and (D) sintered glass.

Analytical methods

The samples were taken out of the bioreactor vessel during the cultivation process of planktonic or biofilm states. The pellet was collected and resuspended into distilled water for dry weight analysis using heat at 105°C for 24 h. The supernatant was collected for HPLC analysis and $(NH_4)_2SO_4$ measurement. HPLC analysis (Shimadzu, Korneuburg, Austria) was done using the method developed to measure polyols (i.e. mannitol, arabitol, and erythitol), glycerol, and citric acid. The HPLC used the Bio-Rad Aminex HPX-87H column (Bio-Rad Laboratories, Inc., USA) and a refractive index detector RID-10A (Shimadzu). The HPLC was operated with 8 mM H_2SO_4 as a mobile phase with a flow rate of 0.6 ml/min at 60°C. $(NH_4)_2SO_4$ was measured using a Hach–Lange ammonium probe (HQ30d portable meter) together with an ammonium selective electrode (Hach Lange GMBH, Düsseldorf, Germany). The experiment was performed in triplicate for flask scale cultivation and quadruplicate for bioreactor cultivation. The data reported represents the average values, and the error bars represent the standard deviations calculated from all replications.

Results and discussion

To explore the metabolic profile of *Y. lipolytica* in the biofilm state, preliminary experiments were conducted to observe biofilm formation. The experiment began with small-scale flask cultivation to monitor the fundamental biofilm growth behaviors. Following this, a bioreactor was utilized with a trickle bed cultivation mode to enhance the regulation of cultivation parameters and improve the liquid/gas interface—which is preferable for biofilm growth. This approach allowed for a more comprehensive understanding of the biofilm formation process and provided valuable insights into the metabolic activities of *Y. lipolytica* in the biofilm state.

Product formation from biofilm state of *Y. lipolytica* in flask-scale cultivation

The first attempt for *Y. lipolytica* biofilm cultivation was made using solid support in flasks, without mixing. This preliminary experiment proved that the chosen strain could grow as a biofilm. The yeast exhibited the potential to form a biofilm on different solid materials, including ceramic, glass, nylon, and sintered glass tubes, the last have been selected for all further experiments. The flask cultivation indicated that a biofilm is exclusively formed at the area of the liquid/gas interface and not submerged in the medium (Fig. 2). As a result, the biofilm morphology could be observed and the first overview over the production pattern of *Y. lipolytica* in a biofilm state was obtained (Fig. 3). The yeast was cultivated on YPG medium to generate the biofilm in the first 3 days before switching to a nitrogen limited medium, containing 100 g/l

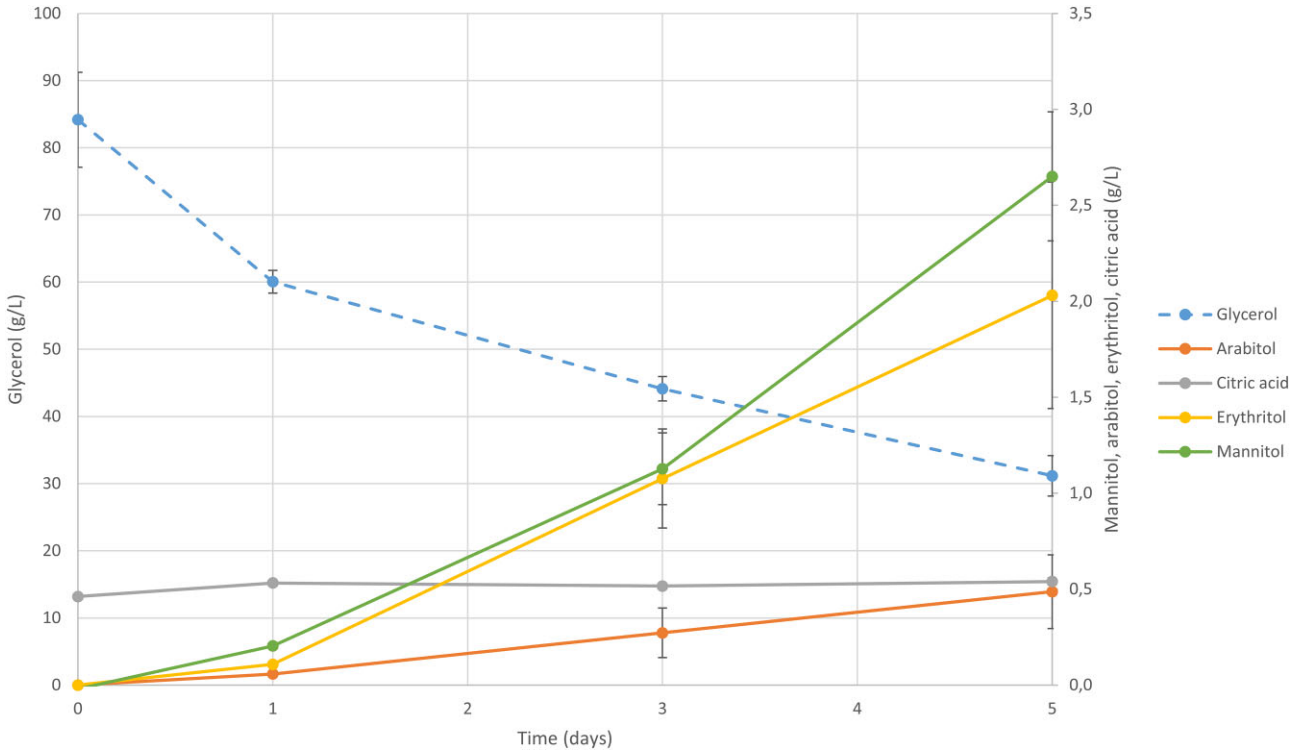

Figure 3. Product and substrate titers (g/l) observed from a flask-scale biofilm cultivation of *Y. lipolytica* strain DSM3286 using the general formula of nitrogen limited medium containing 100 g/l glycerol as carbon source and 3.1 g/l $(NH_4)_2SO_4$ as nitrogen source. The sintered glass tubes were applied as a solid support for biofilm formation. The error bars represent the standard deviation values.

glycerol and 3.1 g/l $(NH_4)_2SO_4$. Mannitol and erythritol production rates were faster than arabitol, and after 5 days of cultivation, mannitol was produced at a titer of 2.7 g/l. Erythritol and arabitol were also produced, with titers of 2 g/l and 0.5 g/l, respectively. Citric acid was not produced. Glycerol was not completely consumed, and ~31 g/l of glycerol remained. The glycerol consumption rate decreased. The product formation pattern was observed to be similar to the planktonic state. Applying a nitrogen limitation medium led to the production of polyols, especially at acidic pH, with mannitol as the main product (Egermeier et al. 2017). The rather inefficient use of the carbon source is caused by a lack of oxygen in the nonshaken cultures. Therefore, for the scale of biofilm cultivation, a TBBR was chosen. Such a setup provides a large area of liquid/gas interface. Sintered glass tubes were used as solid support as their porous nature provides a large surface area, and the tube shape reduces the chance of blockage.

Cultivation of *Y. lipolytica* in planktonic state using a stirred tank bioreactor

In this experiment, *Y. lipolytica* was cultivated in a stirred tank bioreactor to achieve a planktonic state. Since biofilm cultivation requires two phases—the growth phase and the production phase, the same method was used for cultivating the planktonic state—deviating from traditional batch or fed-batch cultures as published before. The purpose of this part was to provide a reference for comparing the production of cells in a biofilm state in a TBBR. The titers (g/l) of both substrate (e.g. glycerol and ammonium sulfate), products (e.g. mannitol, arabitol, erythritol, and citric acid), and cell dried mass (g/l) are shown in Fig. 4. The cultivation of *Y. lipolytica* showed a slight lag phase during the first 8 h. This was followed by a rapid consumption of glycerol and ammonium sulfate. During the growth phase, 30 g/l of glycerol and 3.1 g/l of ammonium sulfate were consumed within 18 h and 12 h, respectively. In the production phase, a larger amount of glycerol at 70 g/l (without ammonium sulfate) was used, which took 30 h to be completely consumed. The entire cultivation process took 48 h. The overall production rate (g/l/h) was calculated using the final titer of each product with the total time of the cultivation process.

During the growth phase, the biomass (dry cell weight) was not detectable until after the first 8 h. It reached 10.4 g/l by the end of the growth phase with an overall biomass production rate of ~0.6 g/l/h. Mannitol had the highest final titer of 3.7 g/l among the polyols produced in the growth phase. Mannitol production did not occur until the ammonium sulfate was completely consumed, which underlines the effect of nitrogen limitation on inducing the product formation. Other polyols were produced in much lower quantities, with arabitol and erythritol reaching final titers of only 0.5 g/l and 0.9 g/l, respectively in the growth phase. The production of citric acid was not detected.

During the production phase, mannitol remained the most significant product, with a final titer of 24.9 g/l (corresponding to the rate of 0.8 g/l/h). Other polyols, such as erythritol and arabitol, reached final titers of 3.7 g/l (0.1 g/l/h) and 3.3 g/l (0.1 g/l/h), respectively. The growth rate of biomass was slower during the production phase, resulting in a final titer of 7.6 g/l after 30 h, with an overall rate of 0.2 g/l/h. The nitrogen limitation induces the glycerol conversion into products, which can be observed from the calculated yield shown in Fig. 5. During the growth phase, biomass yield was higher than mannitol yield. The highest biomass yield was 0.5 $g/g_{glycerol}$, while the mannitol yield was only 0.1 $g/g_{glycerol}$. In the production phase without ammonium sulfate, mannitol yield increased and surpassed biomass yield. The highest mannitol yield was 0.4 $g/g_{glycerol}$, while the biomass

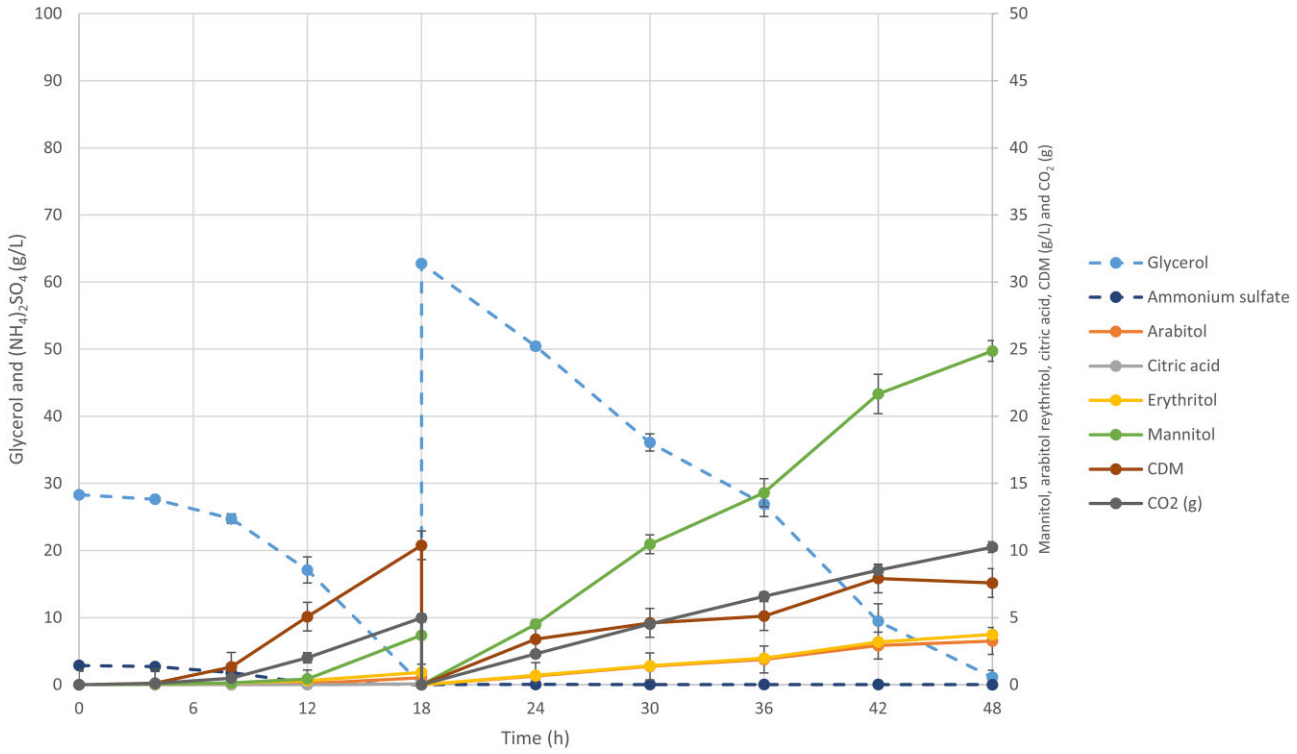

Figure 4. Product titers (g/l), substrate titers (g/l), and absolute CO_2 mass (g) obtained from bioreactor cultivation of *Y. lipolytica* strain DSM 3286 in planktonic state. The growth and production phases were separated at 18 h of the cultivation process. The error bars represent the standard deviation values.

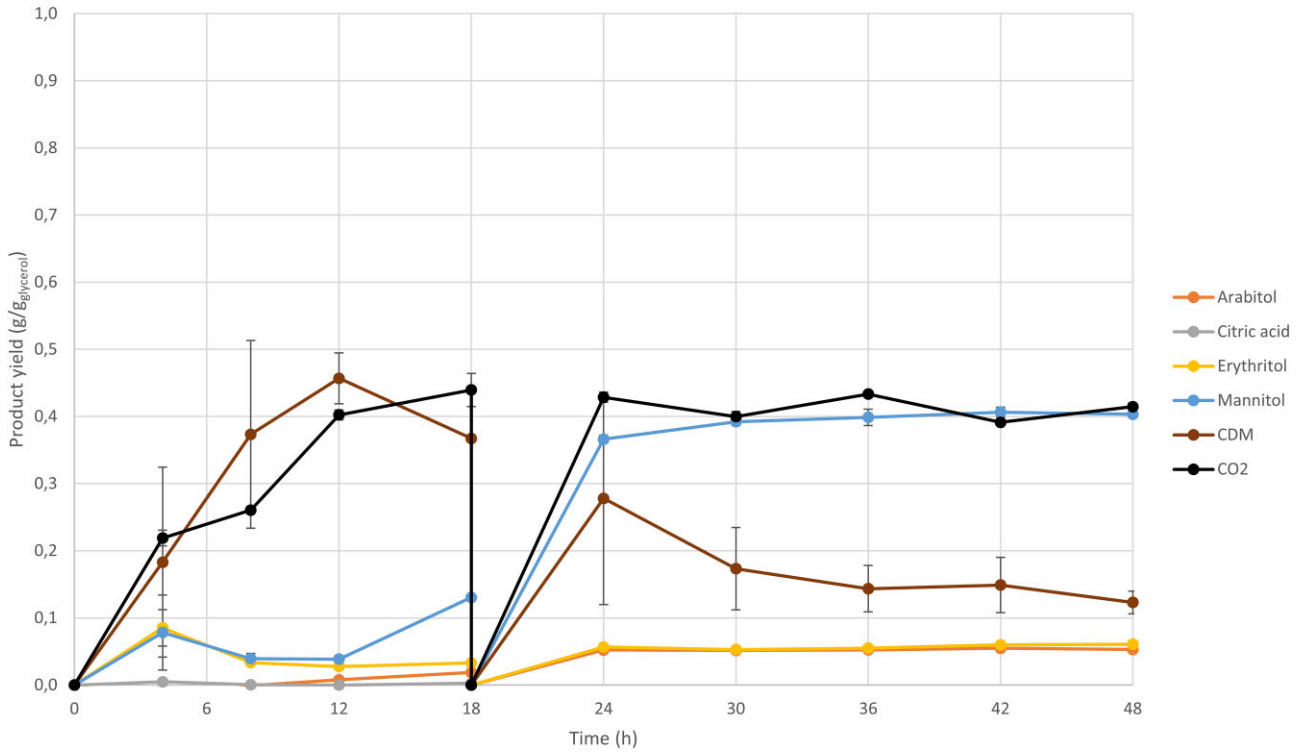

Figure 5. Product yields ($g/g_{glycerol}$) obtained from bioreactor cultivation of *Y. lipolytica* strain DSM 3286 in the planktonic state. The growth and production phases were separated at 18 h of the cultivation process. The error bars represent the standard deviation values.

yield was 0.3 $g/g_{glycerol}$. Previous experiments of single step cultivations on nitrogen limited medium showed similar results. 14.1 g/l of biomass was generated and 0.4 $g/g_{glycerol}$ of total polyol yield, which is similar to the 2-phase cultivation experiment (16.9 g/l of total biomass and 0.5 $g/g_{glycerol}$ of total polyol yield). These results show that splitting the cultivation process did not negatively affect the behavior of the planktonic state of *Y. lipolytica* and this experiment can be used as a reference to compare with biofilm cultivation.

Cultivation of *Y. lipolytica* in biofilm state using a TBBR

Yarrowia lipolytica biofilm requires a different approach than stirred tank bioreactors as it only grows at the liquid–gas interface. A TBBR was selected for biofilm cultivation due to its large liquid–air interface area. The study of biofilm was carried out using either air (21% oxygen) or oxygen enriched air (42% oxygen), which was directly fed into the biofilm column. The rate of product formation (g/l/d) was calculated and represents the current rate at each sampling point. The overall product formation rate was calculated using the final titer and total cultivation time, similar to that reported earlier in the planktonic state cultivation part. The image of *Y. lipolytica* DSM3286 inside the biofilm column can be found in Supplementary Fig. 1.

During the growth phase with air (Fig. 6), it was observed that most of the glycerol was consumed after the first 4 days, with only 2.1 g/l of glycerol remaining. The ammonium sulfate was completely utilized within 2 days. However, in the production phase, a higher amount of glycerol could not be fully utilized even after a long cultivation process of up to 12 days. Only around 72% of the total glycerol was consumed during the production phase. Mannitol was the main product, with a higher titer than erythritol and arabitol. In the growth phase, mannitol production reached 1.4 g/l, while erythritol and arabitol were produced at 0.9 g/l and 0.4 g/l, respectively. The pattern of polyol production remained the same in the production phase, with a final titer of 5.58 g/l for mannitol, compared to 1.9 g/l for erythritol and 0.8 g/l for arabitol. Citric acid was also produced and accumulated up to 2.4 g/l from the initial concentration of 0.5 g/l as a medium component.

Oxygen enriched air with 42% oxygen improved product formation compared to regular air. The production phase showed an increase in the final titer of mannitol, erythritol, and citric acid. The titer of mannitol and erythritol improved from 5.6 g/l (21% O_2) to 8.7 g/l (42% O_2) and from 1.9 g/l (21% O_2) to 2.9 g/l (42% O_2), respectively. Citric acid accumulation increased to 7.2 g/l using enriched air, while only 2.4 g/l was obtained using regular air (Fig. 8). Citric acid production at low pH occurs only in the biofilm state and is influenced by oxygen concentration in the biofilm column. Enriched air improved the glycerol consumption rate to a limited degree, which can be maintained at a higher level of ~8 g/l/d in the growth phase and 4 g/l/d in the production phase, as shown in Fig. 10. The limited diffusion capability due to the EPS of biofilm may lead to the accumulation of products or metabolic waste within the biofilm (Behbahani et al. 2022). This phenomenon may cause the pH in the biofilm environment to be less affected by the pH of the cultivation medium fed into the biofilm column. Considering the optimal pH for citric acid production, which is ~pH 5, it is possible that some accumulation of products or metabolic waste within the biofilm could increase the pH of the biofilm environment. It has been observed several times that at the very end of the growth phase, the pH of the system started to increase as the glycerol was exhausted. This effect of low glycerol concentration could occur in the deeper layer of the biofilm as well due to limited diffusion and cause the pH within the biofilm to rise. Another possibility could be that the production pathway of citric acid has a higher oxidation state than the production pathway of the polyols. This could be demonstrated by the higher titer and yield of citric acid using enriched air with 42% oxygen.

The metabolite productivity is increased significantly by increasing the oxygen provision (Fig. 11). This is interesting as it points to the metabolic potential of the biofilm, which cannot be realized under normal air condition. Under normal air, the major amount of glycerol is going into maintenance and respiration. Under increased oxygen conditions, the glycerol uptake increases to some extent, but the fraction going into respiration is decreased and more carbon is shuttled into metabolite accumulation. The glycerol consumption rate is always significantly lower in the absence of ammonium sulfate under both air conditions—this corresponds also to previous results with planktonic cells. The productivity of all metabolites from both planktonic and biofilm states was concluded in Supplementary Tables 1–3. Also, biofilm-dried mass from both oxygen concentrations can be found in Supplementary Tables 4 and 5.

Oxygen enriched air improved the yield of all polyols and citric acid, especially for mannitol and citric acid. Mannitol yield was 0.2 $g/g_{glycerol}$ (42% O_2) and 0.1 $g/g_{glycerol}$ (21% O_2). The citric acid yield was 0.06 g/g (21% O_2) and increased to 0.1 g/g (42% O_2), as shown in Figs 7 and 9. Enriched air allowed for faster production, resulting in the highest yield obtained 24 h earlier than regular air.

Total product yield was calculated using polyols and citric acid yield. The total product yield improved in the production phase. For the planktonic state, it remained around 0.5 $g/g_{glycerol}$. Biofilm growth with regular or enriched air showed lower total product yield compared to the planktonic state. Total product yield was maintained at around 0.4 $g/g_{glycerol}$ using enriched air and 0.2 $g/g_{glycerol}$ using regular air in the production phase (Fig. 11). However, due to glycerol exhaustion, the product yield from biofilm cultivation with enriched air decreased to almost zero on the last day of the growth phase. As a strict aerobic microorganism, oxygen distribution and transfer are essential for maintaining *Y. lipolytica* activities. Improvement from applying an enriched air suggested that oxygen transfer in the biofilm could still be improved to counteract the biofilm thickness and anoxic medium that trickled into the biofilm column. The TBBR increases the surface area of the liquid/gas interface, improving the efficiency of biofilm formation. This makes the platform more suitable for biofilm investigation. The higher concentration of oxygen gas from enriched-air (42% oxygen) has a positive effect, emphasizing the importance of oxygen transfer into the biofilm. As the biofilm thickens over time, it's possible that oxygen levels are low in the deeper layers of the biofilm. The principle of the membrane aerated biofilm reactor (MABR) could help address this issue. MABR operates by aerating from within the membrane structure. This can enhance our biofilm cultivation platform by providing oxygen to the cells in the deeper layers of the biofilm. Although the oxygen level may decrease at the surface of the biofilm, this can be addressed by aerating the biofilm column, suggesting the possibility of integrating MABR with the TBBR principle. Compared to the TBBR, MABR may demonstrate a higher level of complexity, particularly in the aeration regime, requiring high maintenance, but it also offers better oxygen transfer efficiency, especially in the deeper layers of the biofilm. Both cultivation platforms can utilize various materials as support for biofilm attachment. Therefore, the likelihood of clogging and effectiveness in

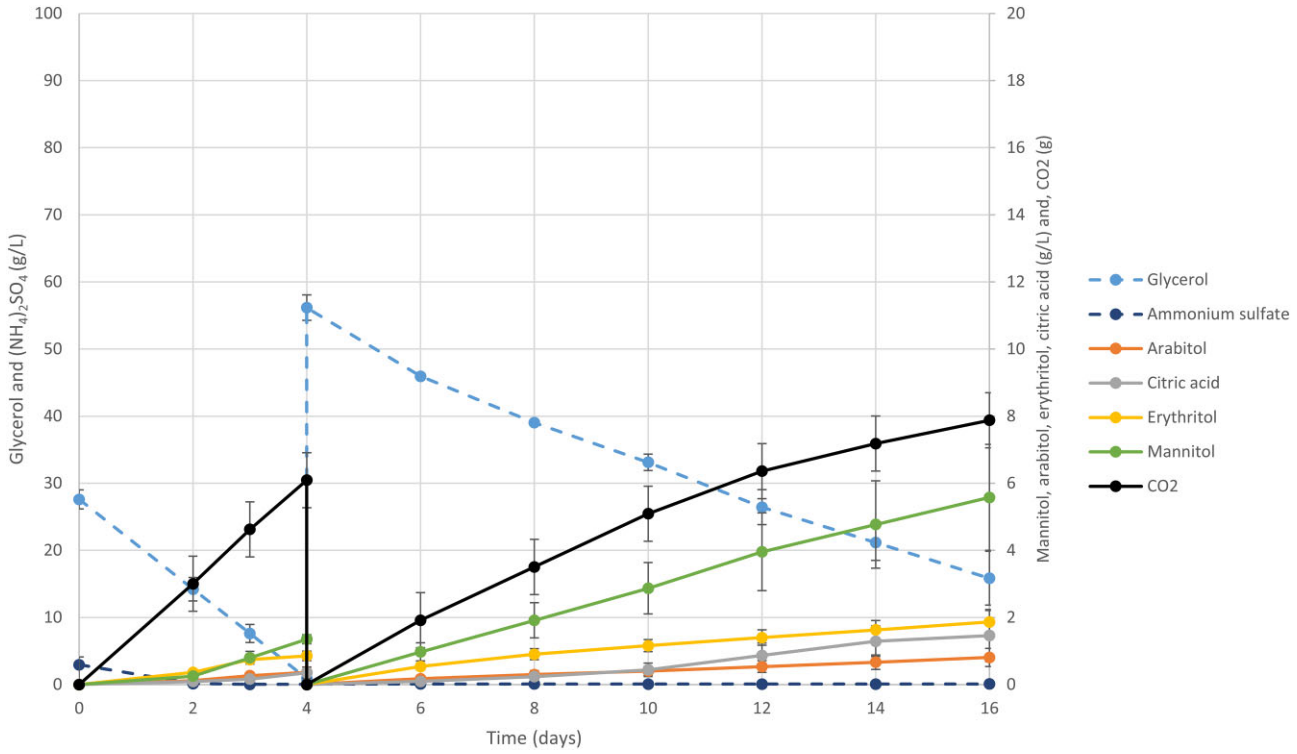

Figure 6. Product titers (g/l), substrate titers (g/l), and absolute CO_2 mass (g) obtained from bioreactor cultivation of *Y. lipolytica* strain DSM 3286 in biofilm state using sintered glass tube as solid support under 21% O_2 air. The growth and production phases were separated at 4 days of cultivation process. The error bars represent the standard deviation values.

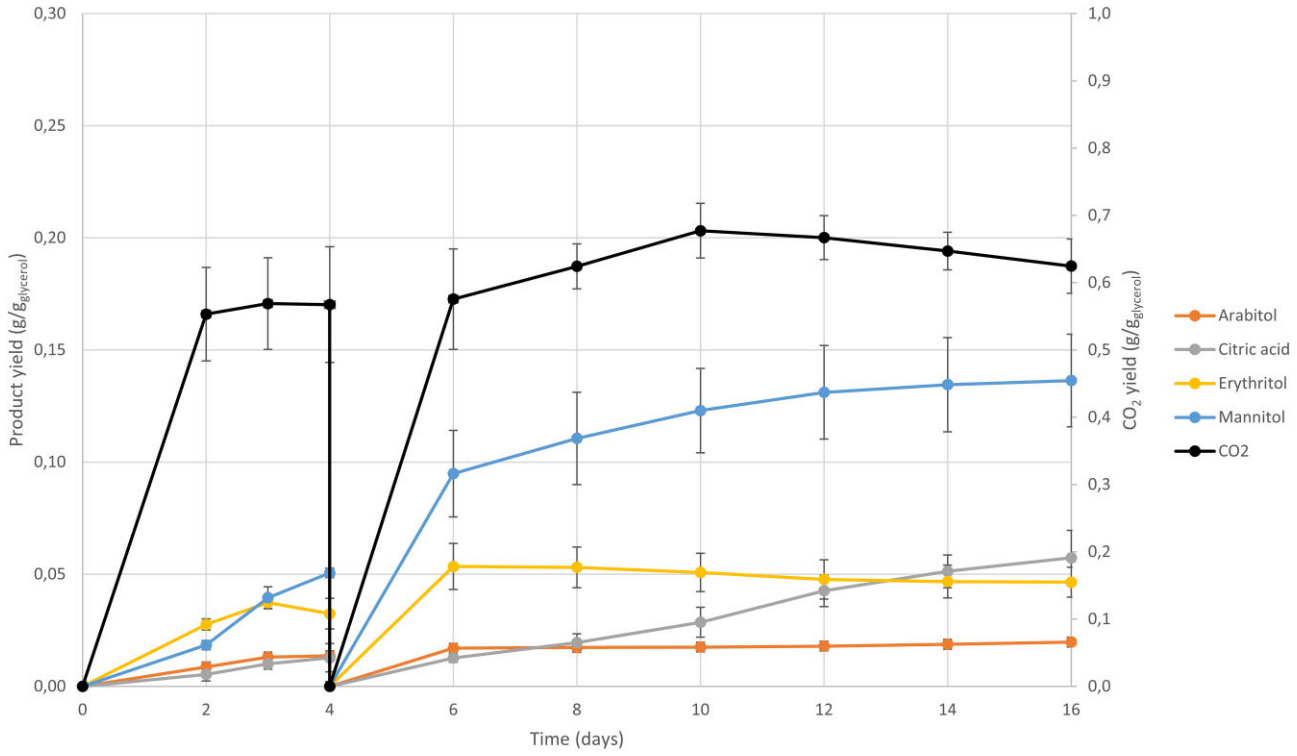

Figure 7. Product yields ($g/g_{glycerol}$) obtained from bioreactor cultivation of *Y. lipolytica* strain DSM 3286 in biofilm state using sintered glass tube as solid support under 21% O_2 air. The growth and production phases were separated at 4 days of cultivation process. The error bars represent the standard deviation values.

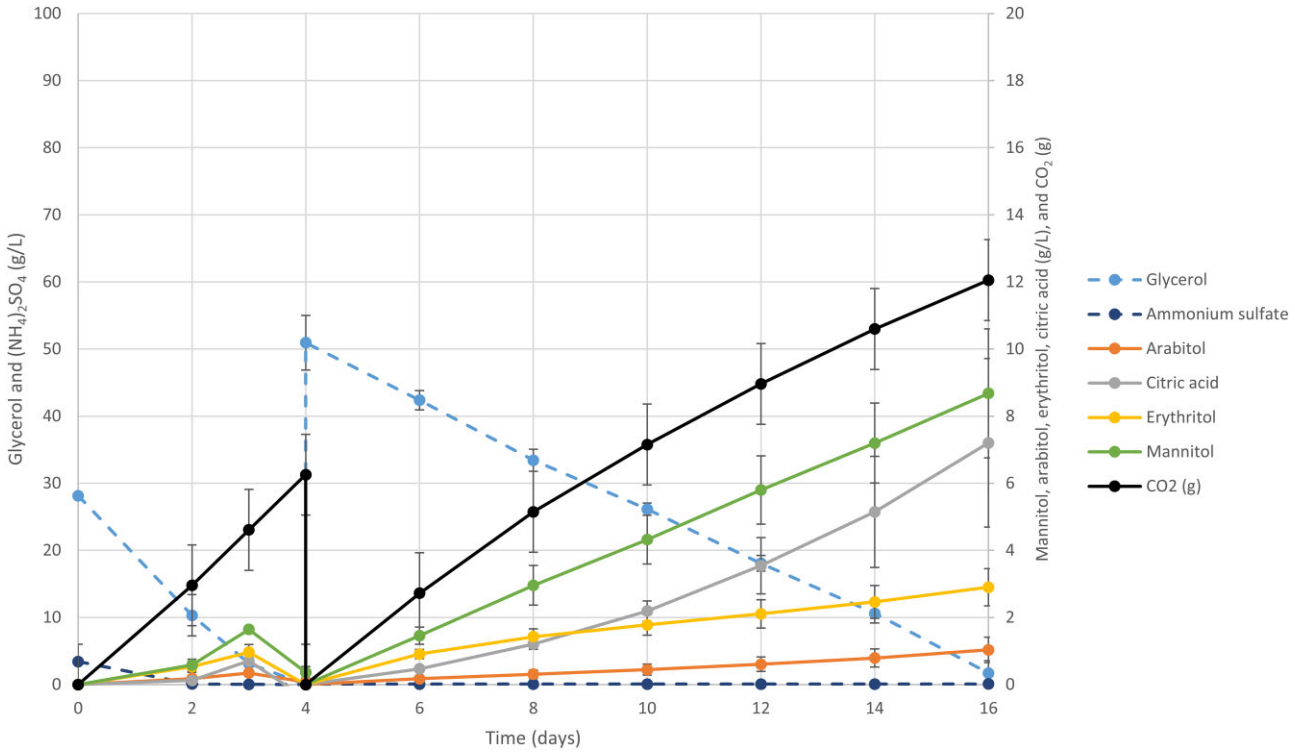

Figure 8. Product titers (g/l), substrate titers (g/l), and absolute CO_2 mass (g) obtained from bioreactor cultivation of *Y. lipolytica* strain DSM 3286 in biofilm state using sintered glass tube as solid support under 42% O_2 air. The growth and production phases were separated at 4 days of cultivation process. The error bars represent the standard deviation values.

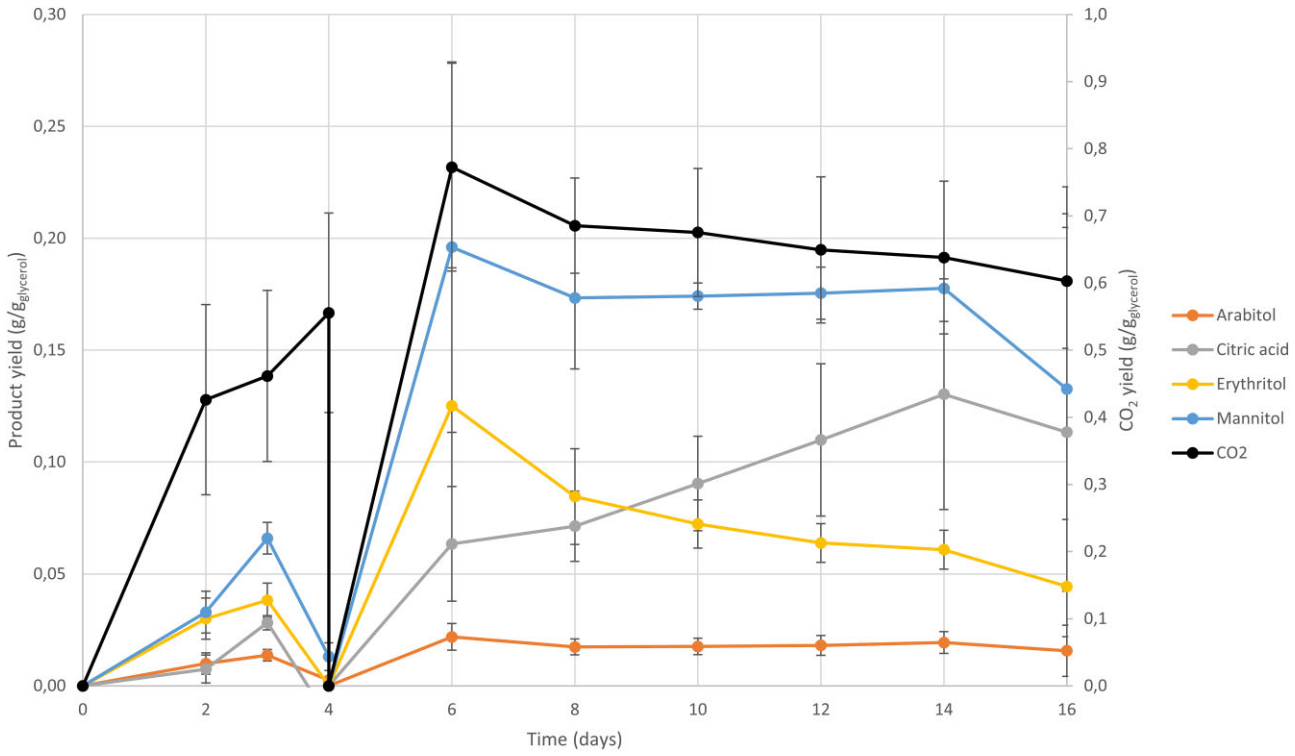

Figure 9. Products yield ($g/g_{glycerol}$) obtained from bioreactor cultivation of *Y. lipolytica* strain DSM 3286 in biofilm state using sintered glass tube as solid support under 42% O_2 air. The growth and production phases were separated at 4 days of cultivation process. The error bars represent the standard deviation values.

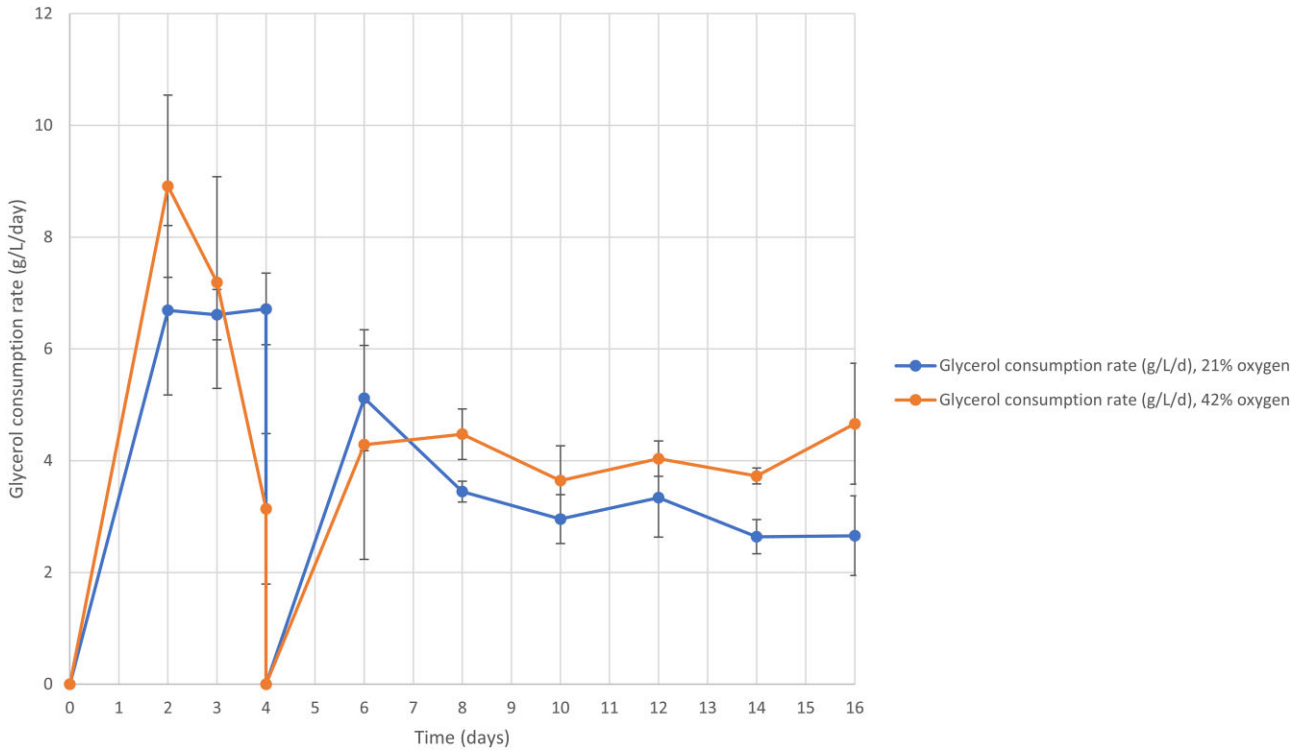

Figure 10. Glycerol consumption rate (g/l/d) obtained from bioreactor cultivation of *Y. lipolytica* strain DSM 3286 in biofilm state using sintered glass tube as solid support. The growth and production phases were separated at 4 days of cultivation process. The error bars represent the standard deviation values.

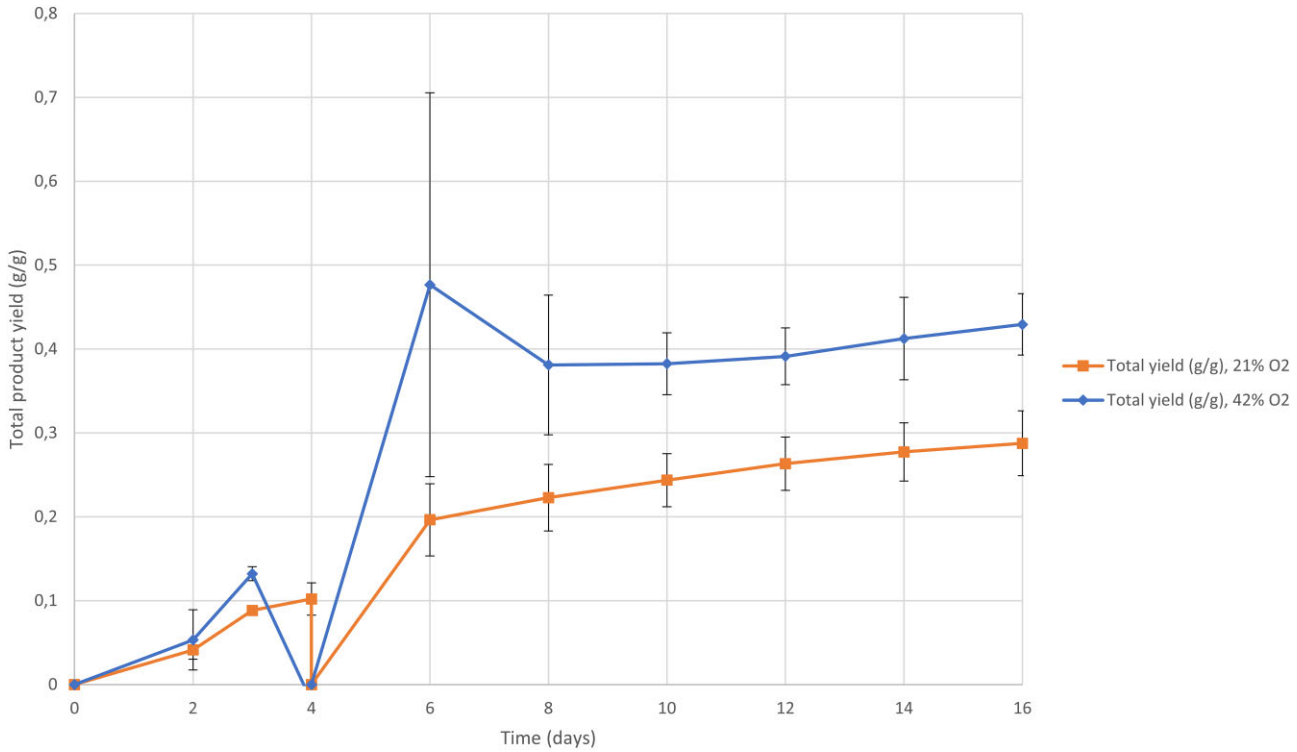

Figure 11. Total yield ($g/g_{glycerol}$) obtained from bioreactor cultivation of *Y. lipolytica* strain DSM 3286 in biofilm state using sintered glass tube as solid support. The growth and production phases were separated at 4 days of cultivation process. The error bars represent the standard deviation values.

nutrient distribution depends on the shape and material of the solid support, which could be a topic for process optimization. The fine structure (i.e. the filler of the cooling tower) can effectively increase both the retention time of the liquid medium within the biofilm column and the surface area for biofilm formation, which are both beneficial for the cultivation platform. However, it is important to carefully consider the geometry of the material, as the fine structure of the material could potentially cause the biofilm column to clot later in the process.

Conclusions

Yarrowia lipolytica is a well-known microorganism with great biotechnological potential due to its flexible metabolic activity. We could prove that this yeast species has the capability for biofilm formation on various solid support materials and retain productivity. Biofilm formation exclusively occurs at the gas–liquid phase boundary, which is important to consider and in fact a significant challenge for upscaling. Nitrogen limitation induces product formation in both biofilm and planktonic states. The pattern of metabolites produced is similar for both growth modes under the conditions analysed (pH 3), but not identical as the cells accumulate some citric acid when grown as biofilm, which is in contrast to planktonic cells of this specific yeast strain, which do not accumulate any citric acid at low pH.

Oxygen transfer into the biofilm turned out to be a major limiting factor for productivity. As a measure for improvement, oxygen enriched air (42% oxygen) was applied to the biofilm column instead of 21% oxygen air, resulting in increased productivity and significantly improved yield of metabolites. The total product yield from biofilm cultivation with enriched air (excluding biomass and CO_2) was not much lower than that observed in planktonic cultivation. However, the productivity was significantly lower, resulting in very long cultivations. A constant product formation rate was obtained with the biofilm in the trickle bed reactor. This is interesting and quite promising as it points to a stable operation in an envisioned continuous process, provided the productivity can be improved. A number of approaches, including optimizing oxygen provision, nutrient distribution, and the retention time of the medium on the biofilm could help to this end.

A challenge arising from the obtained results is the availability of oxygen to all cells—also deep in the biofilm. This is an essential factor for optimal productivity of the biomass. If oxygen does not reach the cells deep down in the biofilm they will not be productive. Oxygenation with enriched air was a successful approach, to increase oxygen diffusion into the biofilm, but it has hardly any industrial relevance for cost reasons. Some innovation will be required here. Another parameter is the medium retention time in the column, which must be increased to allow maximum contact time for cells to convert the substrate and improve the nutrient availability and productivity from the cells inside the biofilm. This could be achieved by changing the column dimension or changing the manner of packing the solid support inside the biofilm column. Lastly, to achieve an ideal process setup, the feeding rate should be adapted to match the conversion rate of the system. The final aim would be to remove the medium circulation need. This would simplify the setup but most importantly improve productivity of the continuous process significantly. The substrate should ideally be fed continuously into the system, and the product can be harvested at the end of the column without accumulated substrate.

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Supplementary data

Supplementary data is available at *FEMSMC Journal* online.

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