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Potential of *Wickerhamomyces Anomalus* in Glycerol Valorization

Alberto Amaretti[a,*], Benedetta Russo[a], Stefano Raimondi[a], Alan Leonardi[a], Giorgia Foca[a], Adele Mucci[b], Alfonso Zambon[b], Maddalena Rossi[a]

[a] Dipartimento di Scienze della Vita, Università di Modena e Reggio Emilia, via Campi 103, 41125 Modena, Italia
[b] Dipartimento di Scienze Chimiche e Geologiche, Università di Modena e Reggio Emilia, via Campi 103, 41125 Modena, Italia
alberto.amaretti@unimore.it

Five-carbons polyalcohols, such as xylitol and arabitol, and microbial oils are important targets for biotechnological industries. Polyalcohols can find application as low-calories sweeteners and as building block in the synthesis of valuable compounds, while lipids are interesting for both biofuel and food industry. The osmophilic yeast *Wickerhamomyces anomalus* WC 1501 was preliminary known to produce arabitol from glycerol. Production kinetics were investigated in this study. Production was not growth-associated and occurred during a nitrogen-limited stationary phase, in presence of an excess of carbon source. Typical bioreactor batch cultures, carried out with 160 g/L glycerol, yielded 16.0 g/L arabitol in 160 h. A fed-batch process was developed, in which growth is carried out batchwise in a balanced medium containing 20 g/L glycerol, and arabitol production is induced at the entrance into the stationary phase with a pulse of concentrated glycerol to provide the remaining 140 g/L carbon source. At the end of the process 18.0 g/L arabitol were generated. Under these conditions, the yeast also accumulated intracellular triacylglycerols, with fatty acids of 16-18 carbons bearing 0 to 2 unsaturations, reaching up the 23% of biomass dry weight. Therefore, *W. anomalus* WC 1501 is a good candidate for the development of a fermentative process yielding arabitol and has potential also as oleaginous yeast for producing lipids, further improving the interest in this strain for glycerol biorefinery. The utilization of a fed-batch process allows to carry out distinct growth and production phases and thus allows the optimization of both phases separately, in order to achieve the highest concentration of catalytic biomass during growth and the maximum efficiency during production. This strain deserves further investigation to better exploit its biotechnological potential in the valorization of glycerol.

1. Introduction

Glycerol is an unavoidable by-product of the biofuel industry and is becoming an attractive feedstock for biorefinery due to its abundance, low price, and high degree of reduction (Cheng and Liu, 2016). Biological approaches that convert glycerol into more valuable products have been receiving increasing attention and several microbial processes, utilizing wild type or engineered strains, have been described in the last decade. Promising fed-batch processes have already been shown to produce high yields of the most important targets of glycerol valorization, such as ethanol, diols (1,3-propanediol and 2,3-butanediol), organic acids (D-lactate and hydroxypropionate), and triacylglycerols (Wong et al., 2014; Xu et al., 2009; Cho et al., 2015; Chu et al., 2015; Feng et al., 2013; Amaretti et al., 2012). Considerable efforts have also been devoted to the development of a fermentative process for the transformation of glycerol to compounds such as amino acids, polyhydroxyalkanoates (PHA), and polyunsaturated fatty acids (Cheng and Liu, 2016; Kalia et al., 2016).
The strain *Wickerhamomyces anomalus* WC 1501 (former *Hansenula anomala* WC 1501) produces the polyalcohol arabitol in a glycerol-based nitrogen-limited medium and has thus attracted attention as a strain for a potential transformation of glycerol into a value-added chemical (Amaretti et al., 2018). Sugar alcohols such as xylitol and arabitol have been ranked among the top 12 value added chemicals derivable from biomasses (Werpy and Petersen, 2004; Erickson et al., 2013). Arabitol, in particular, is a low-calories anti-cariogenic sweetener and can also serve as substrate or building block in chemical synthesis of several products (Werpy

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and Petersen, 2004). The ability of some fungi to produce arabitol from glucose is well established and efficient processes have been proposed (Kordowska-Wiater, 2015; Saha et al., 2007; Zhu et al., 2010), but only few strains produced arabitol from glycerol, and these with yields still far from those obtained with glucose (Koganti et al., 2011; Yoshikawa et al., 2014). Arabitol production by *W. anomalus* WC 1501 was not growth-associated and occurred during the stationary phase, in presence of an excess of glycerol. In the present study, this strain was used in a fed-batch process, in order to separate the growth and production phases and to allow the optimization of production conditions without affecting the growth performance.
The culture conditions employed to produce arabitol are similar to those used to accumulate lipids in oleaginous yeasts, that also require nitrogen-limiting conditions and carbon excess (Raimondi et al., 2014). Furthermore, another strain of *W. anomalus* accumulated PHA within intracellular bodies (Ojha and Das, 2017; Ojha and Das, 2018), although glycerol was not investigated as carbon source. This study also investigated whether *W. anomalus* WC 1501 accumulates lipids or PHA during arabitol production, since any capability of producing these products from glycerol would further improve the potential of the strain in glycerol biorefinery.

2. Materials and methods

2.1 Strain and culture conditions

The strain *W. anomalus* WC 1501 was obtained from our laboratory collection. The strain was routinely maintained at 4°C in agar slants of YPD medium (BD Difco, Sparks, MD, USA) and aerobically cultured at 30°C in shake-flasks of YPD broth.
The inoculum for bioreactor experiments was prepared in shake-flasks of MY medium, containing 20 g/L glycerol, 3 g/L yeast extract (BD Difco, Sparks, MD, USA), 2 g/L $(NH_4)_2SO_4$, 3 g/L KH_2PO_4, 1 g/L K_2HPO_4, and 1 g/L $MgSO_4 \cdot 7H_2O$.
All chemicals were purchased from Sigma-Aldrich (Steinheim, Germany), unless otherwise stated.

2.2 Batch and fed-batch experiments and bioreactor operation

Fermentation experiments were performed in laboratory-scale autoclavable bioreactors (500 mL Mini Bio, Applikon Biotechnology, Delft, the Netherlands). Batch arabitol production was carried out in 400 mL of MY medium, containing 160 g/L glycerol. Fed-batch cultures were started batch-wise in MY medium, containing 20 g/L glycerol, and were given a pulse of a solution of 800 g/L glycerol after 24 h of cultivation, in order to achieve the concentration of 140 g/L. The processes were inoculated 5% v/v with a 24-h seed culture.
The cultures were maintained at 30°C and aerated with 0.5 v/v/min filter-sterilized air. Cascade-controlled stirring was applied in the range from 1000 to 1700 rpm, in order to maintain dissolved oxygen at 20%. The pH was prevented from decreasing below 5.0 by automatic addition of 4 M NaOH. A defoaming mixture (1:1, v/v) of Xiameter 1520 (Dow Corning, Midland, MI, USA) and polypropylene-glycol was automatically added to keep foaming under control. Samples were periodically collected, in order to monitor growth, glycerol consumption, and arabitol production. All the fermentations were carried out in triplicate. The mean values are herein reported.

2.3 Chemical analysis

Growth was monitored by measuring the turbidity at 600 nm (OD_{600}). Cell counts were determined in a Bürker chamber. To determine the biomass dry weight (DW), the cells from a known volume were harvested by centrifugation, washed twice with water and brought to constant weight onto a pre-weighed tray in a thermo-balance (MB 64M, VWR, Radnor, PA, USA).
Culture samples were centrifuged (10000 rpm for 5 min at 4°C) and filtered (0.22 μm) to obtain clarified supernatants for glycerol, arabitol, and ammonium analysis. Glycerol and arabitol were quantified by HPLC with refractive index detector (1200 System, Agilent Technologies, Waldbronn, Germany) and ion exclusion column (Aminex HPX-87 H, Bio-Rad, Hercules, CA, USA). Isocratic elution was carried out at 60°C with 0.8 mL/min of 5 mM H_2SO_4 (Raimondi et al., 2014). Product / substrate conversion yield ($Y_{P/S}$) was calculated as the ratio between produced arabitol and consumed glycerol. Ammonium was quantified with an ammonia-selective membrane electrode (Orion, Thermo Fisher Scientific, Waltham, MA, USA).
Cell free extract were prepared to quantify intracellular arabitol. The biomass from 5 mL of culture was harvested by centrifugation, washed twice, resuspended in 5 mL of water, then subjected to mechanical disruption through one passage at 40 Kpsi in OneShot cell disrupter (Constant System, Daventry, UK). The cell free extract was clarified and analyzed by HPLC as above described.

2.4 Biomass extraction and NMR analysis

Cellular lipids were extracted from 1 g of freeze dried biomass, utilizing 50 mL of a chloroform:methanol mixture (2:1, v/v) (Amaretti et al., 2012). To extract polyhydroxy butyrate, 1 g of biomass was treated with 50 mL NaClO for 1h, washed with water, acetone, and ethanol, and extracted three times with boiling chloroform (Law and Slepecky, 1961; Ojha and Das, 2018). Solvents were evaporated and the extracts were quantified gravimetrically.

^{1}HNMR spectra of the extracts in $CDCl_3$ were recorded with a Bruker Avance 400 instrument operating at 400.13 MHz, using standard pulse sequences on 3-10 mg/mL solutions. NMR signals were assigned according to literature data on triacylglycerols and polyhydroxy butyrate (Nieva-Echevarría et al., 2014; Satyarthi et al., 2009; Ojha and Das, 2018).

3. Results and discussion

3.1 Batch and fed-batch fermentations

Figure 1A reports a typical batch culture of *W. anomalus* WC 1501 cultured in in MY medium containing 160 g/L glycerol, in presence of an automatic pH control set at 5.0. The ammonium run out within the first 18 h, during which approx. 20 g/L glycerol were consumed. In this period, arabitol was not produced and cell counts exponentially increased, reaching the stationary level of 1.6×10^9 cells/mL. The nitrogen-limited culture continued to consume the excess of glycerol at a rate that settled to 0.35 g/L/h. Glycerol consumption was associated with arabitol production, which proceeded nearly linearly, with a rate of 0.11 g/L/h and a $Y_{P/S}$ of 0.31, reaching 16 g/L after 160 h of cultivation. The trend herein described is coherent with batch cultures previously reported, although the automatic titration of acidity, preventing the pH to drop at values < 2.5, significantly improved the final arabitol production by two-fold (Amaretti et al., 2018). During the production, the cell counts remained constant (P > 0.05) although the OD increased, especially within the first 40 h, likely because of composition changes in the biomass, with the cells becoming optically denser.

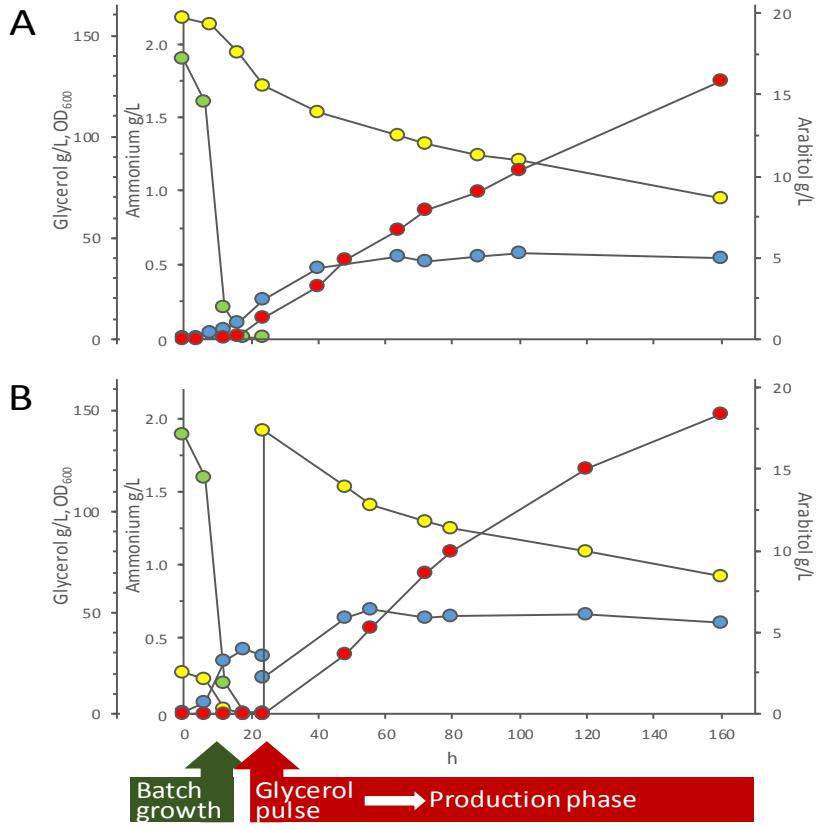

Figure 2: Time-course of OD_{600} (cyan), ammonium (green), glycerol (yellow), and arabitol (red) during batch (A) and fed-batch (B) cultures of W. anomalus *WC 1501. Fed-batch cultures were started batch-wise with 20 g/L glycerol; after 24 h, the culture was given a pulse of concentrated to initiate arabitol production.*

Previous studies describe arabitol production by ascomycetous yeasts as triggered by various stress conditions, including the osmotic one (Koganti et al., 2011; Kumar and Gummadi, 2009; Sánchez-Fresneda et al., 2013). In the present study, the mechanical disruption of the cells did not release any additional arabitol, that resulted essentially extracellular and could not act as an osmoprotectant to cope with osmotic stress. At the matter of fact, whatever the mechanism underlying arabitol production, high glycerol concentration and nitrogen depletion were the key conditions triggering production in *W. anomalus* WC 1501. Based on these observations, a fed-batch process was developed, the time course of which is reported in Fig. 2B. Growth was carried out in a balanced medium containing 20 g/L glycerol and proceeded through a trend comparable to that of the batch culture with the yield of 1.6×10^9 cells/mL. Glycerol and ammonium were utilized simultaneously and got both exhausted within 18 h. At 24 h, once the culture had entered the stationary phase, a pulse of concentrated glycerol, supplying the culture with 140 g/L glycerol, was applied to start production. During the production phase, glycerol was consumed at the rate of 0.34 g/L/h and 0.13 g/L/h of arabitol accumulated with a $Y_{P/S}$ of 0.38, reaching 16 g/L after 160 h of cultivation. The successful development of a process in which growth and production are distinct and occur in succession is encouraging, since the conditions of both the phases may be target of separate optimizations. The objective of growth optimization will be to maximize the amount of 'catalytic' biomass, which in turn will directly affect the rate of production in the next phase. On the other hand, the optimization of the production phase will identify the best process conditions for the conversion of glycerol into arabitol. Some process variables affecting arabitol production, such as the concentration of glycerol to be applied during the production phase and the pH, have been identified in the present study and in the preliminary characterization of the strain (Amaretti et al., 2018). Temperature is also worth to be taken into account, as it is expected to affect the kinetics of conversion of glycerol to arabitol.

3.2 Biomass extraction and analysis

Throughout the arabitol production phase of both batch and fed-batch processes, the cells of *W. anomalus* WC 1501 changed morphology and became denser. This change was evidenced by the measurement of the OD_{600}, which increased without any corresponding increase in the cell counts (Figure 1A, 1B), but was also observed in phase contrast microscopy, with the formation and widening of light-opaque intracellular granules (Figure 2) similar to those of lipid or PHA-accumulating fungi (Raimondi et al., 2014; Ojha and Das, 2018).

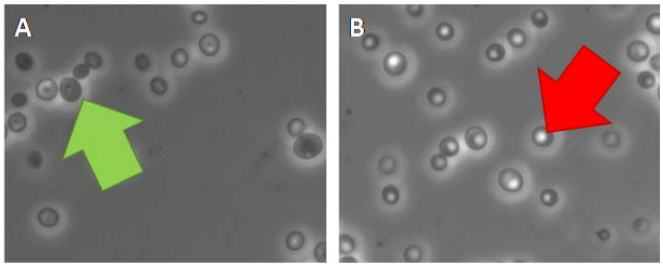

Figure 2: Phase-contrast microscopy (1000 X) image of the cells W. anomalus *WC 1501 at the beginning (A) and the end (B) end of the arabitol-production phase, at 24 and 160 h of cultivation, respectively. Arrows indicate the absence or presence of light-opaque intracellular bodies.*

To determine the nature of the intracellular granules, the biomass was collected at the beginning and the end of the production phase (at 24 and 160 h, respectively) of a fed-batch process and was extracted to recover PHA or lipids, then the extracts were subjected to NMR analysis. The NMR spectra ruled out the presence of PHA at both the time-points, thus *W. anomalus* WC 1501 seems to behave differently from another strain of the same species (Ojha and Das, 2018). This can result from the different carbon sources employed or from strain differences. On the other hand, the presence of fatty acids was already recognizable in the extract of 24 h, while the extract at 160 h consisted mainly of triacylglycerols (Figure 3). Careful integration of the characteristic ^{1}HNMR peaks show that triacylglycerols contained C18 fatty acids, with the saturated, mono-unsaturated, and di-unsaturated forms representing the 35, 40, and 25% respectively of the total.

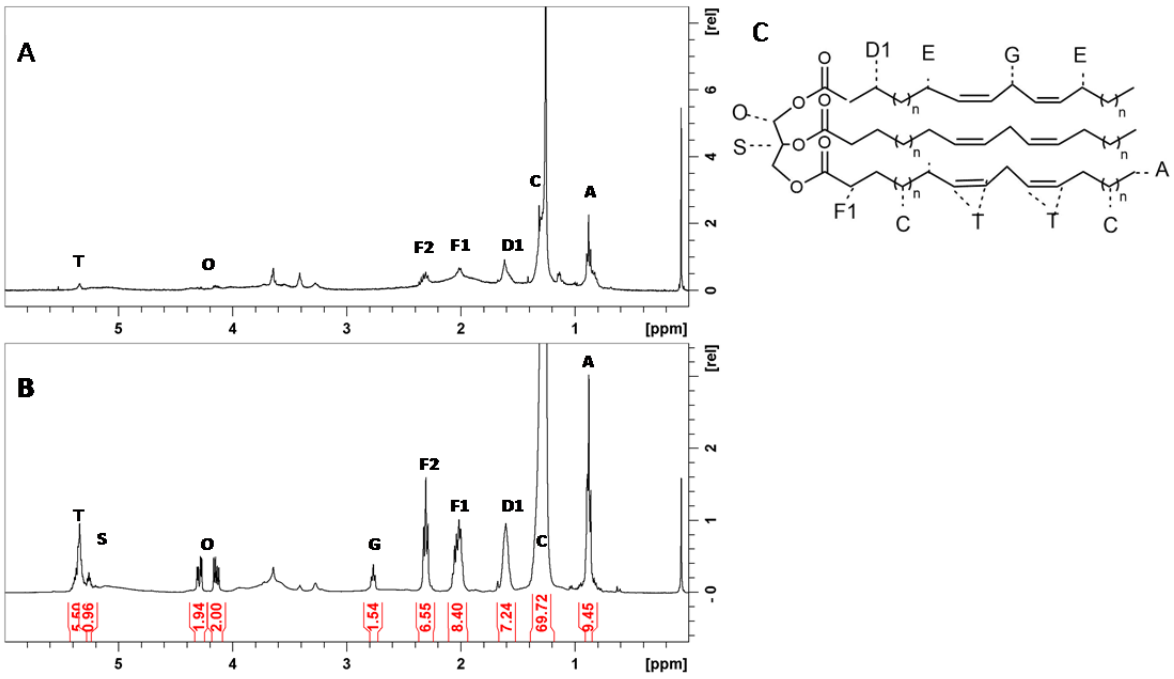

Figure 3: NMR spectra of the lipids extracted from the biomass of W. anomalus WC 1501 at 24 (A) and 160 h (B) of fed-batch cultivation. Signals were assigned (C) according to Nieva-Echevarría et al., (2014).

The gravimetric quantification of the lipids revealed that fatty acids accounted for the 5% of the biomass dry weight at 24 h and were likely utilized by the cells grown in the balanced medium as storage of building blocks for membrane biosynthesis (Table 1). During the arabitol production phase, lipids progressively accumulated within the biomass in the form of triacylglycerols and reached 23% of the dry weight after 160 h. In oleaginous fungi under conditions that are not permissive for growth due to nitrogen limitation, the accumulation of intracellular triacylglycerol is a strategy of carbon and energy storage. The same behavior likely occurs in *W. anomalus* WC 1501, even though the amount of lipids accumulated is lower than in other yeasts, some of which reach the 60% of the dry weight under optimized conditions (Amaretti et al., 2010). It remains to be elucidated whether the simultaneous production of arabitol and lipids is due to metabolic pathways in mutual relationship between each other (e.g. for the need to generate reduced cofactors, necessary for the biosynthesis of fatty acids) or to independent pathways competing for the carbon flow. In the latter case, further interventions in the culture conditions and / or the metabolic engineering of the strain may favor one product at the expense of the other.

Table 1: Biomass DW and lipid content at different time-points during the production phase.

Time	DW g/L	Lipids g/L	Lipids %
24 h	7.8	0.4	5
160 h	9.8	2.2	23

4. Conclusions

A fed-batch process for arabitol production from glycerol was developed with ascomycetous yeast *W. anomalus* WC 1501. The separation of growth and production phases is encouraging, since the conditions of both the phases may be target of separate optimizations, that are expected to improve the rate and yield of arabitol production. This yeast did not accumulate PHA during the process but stored intracellular triacylglycerols. The oleaginous properties of the strain would further improve the potential of the strain in glycerol biorefinery.

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