Lipid production by *Rhodosporidium toruloides* growing on media presenting composition similarities with the spent sulfite liquor in batch and fed-batch cultures

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Abstract
Aim of this study is to explore the effect of sodium lignosulfonate (SL), a paper industry by-product, on cell growth and lipid production by the yeast *Rhodosporidium toruloides*, cultivated on xylose-based media, that mimic the principal waste-stream originated from paper production facilities (viz. the spent-sulfite liquor). Yeast lipids present increasing interest as alternative non-food feedstocks for biodiesel production. Strains DSM 4444 and NRRL Y-27012 were shake-flask cultured under nitrogen-limiting conditions using xylose at 50 g/L, and SL was added at varying concentrations. Finally, a fed-batch bioreactor trial of the strain NRRL Y-27012 with optimum SL addition was carried out.
In the strain DSM 4444, maximum lipid production was obtained in media supplemented with 20 g/L SL, where lipid of 4.8 g/L occurred. In NRRL Y-27012 strain, maximum lipid production was seen with the addition of 10 g/L SL (lipid =5.3 g/L). In fed-batch bioreactor experiments carried out with the strain NRRL Y-27012, lipid =17.0 g/L (corresponding dry biomass =29.7 g/L) was achieved. The yield of lipid produced per unit of xylose consumed was ≈0.19 g/g. Lipids containing increased concentrations of oleic acid, constituting thus perfect materials amenable to be converted into "2nd generation" biodiesel were synthesized.
Keywords: 2nd generation biodiesel, oleaginous micro-organisms, microbial lipid, spent sulfite liquor, Rhodosporidium toruloides

1. Introduction

Lignocellulosic materials represent the largest and the most attractive biomass resources worldwide that can serve as cheap feedstock of monosaccharides in a variety of microbial fermentations. Lignocellulosic residues from wood, grass, agricultural and forestry wastes, municipal solid wastes and wastewaters deriving from several agro-industrial facilities are particularly abundant in nature and have a potential for bioconversion (Koutinas et al 2014). Xylose is the principal C-5 sugar found in lignocellulosic biomass hydrolysates and in various lignocellulose wastewaters like the spent sulfite liquor (Koutinas et al 2014). The capability of oleaginous microorganisms to utilize C-5 sugars as carbon source is highly desired in order to increase the efficiency of lipid production from lignocellulosic materials (Papanikolaou and Aggelis 2019; Sarris and Papanikolaou 2016).
Single cell oils (SCOs) have received large attention for a sustainable production of oleochemicals, replacements of high-added value fatty materials (e.g. substitutes of the very costly cocoa-butter) and 2nd or 3rd generation biofuels (Papanikolaou and Aggelis 2010; 2011a; 2011b; 2019). In the current investigation, strains of the oleaginous yeast *R. toruloides* were cultured on xylose-based media that mimic the spent sulfite liquor, the principal waste stream deriving from paper-production units, in order for SCOs that could further be transformed into biodiesel to be created.

2. Materials and Methods

In the experiments conducted, two strains of R. toruloides, namely DSM 444 and NRRL Y-27012, were used. All cultures contained xylose (initial concentration of ≈50 g/L) as nominal source of carbon and nitrogen-limiting conditions prevailed. Sodium lignosulfonate was provided by the company LignoTech Iberica (initial concentration of 10-40 g/L). In all cases the nitrogen source used were yeast extract (1.0 g/L) and peptone (2.0 g/L). The composition of mineral salts in the media (in g/L) was: KH_2PO_4 7.0, Na_2HPO_4 2.5, $MgSO_4*7H_2O$ 1.5, $FeCl_3*6H_2O$ 0.15, $CaCl_2*2H_2O$ 0.15, $MnSO_4*H_2O$ 0.06 $ZnSO_4*7H_2O$ 0.02.
Submerged fermentations were conducted in Erlenmeyer flasks (250 mL) filled with 50±1 mL liquid medium, and inoculated with 1 mL of exponential pre-culture. Flasks were incubated in an orbital shaker (180±5 rpm, 28±1 ºC). Fed-batch fermentation was carried out in a 3-L bioreactor, with working volume of 1.5 L (agitation 300 rpm, aeration 1.5 vvm, incubation temperature T=28±1 ºC). The pH was maintained at 6.0.
Biomass was harvested by centrifugation (9000 rpm, T=4 ºC, 10 min), washed twice with distilled water and centrifuged again. Biomass concentration (X, g/L) was determined through its dry cell weight. Lipids concentration (L, g/L) was determined gravimetrically

after extraction of cellular lipids using a mixture of solvents chloroform/methanol 2/1 (v/v). Xylose (Xyl, g/L) was determined by 3,5-dinitrosalicylic acid (DNS) assay. Lipids analyzed in gas chromatograph-flame ionization detector.

3. Results and Discussion

In the case of submerged fermentations carried out with the strain DSM 4444, the highest biomass production was observed in the fermentation by adding 40 g/L SL, reaching 18.6 g/L of total dry cell weight (TDCW). The maximum lipid production was identified in the fermentation by the addition of 20 g/L SL, where SCO =4.8 g/L was produced, while the maximum quantity of intracellular polysaccharides formed per g of biomass formed ($Y_{IPS/X}$) was observed in the fermentation in which there was no addition of SL and had a value of 0.31 g/g. In the case of fermentations of the strain NNRL Y-27012, the maximum SCO production was detected in the fermentation with the addition of 20 g/L SL, which reached 5.3 g/L. The maximum biomass value was observed in the fermentation by the addition of 40 g/L SL, which reached 15.2 g/L, and the maximum $Y_{IPS/X}$ was observed in the control fermentation and had a value of 0.30 g/g. In the case of fed-batch fermentation, maximum SCO production obtained was 17.0 g/L, the respective TDCW production was 29.7 g/L and the maximum $Y_{IPS/X}$ was 15.23% w/w (Fig. 1).

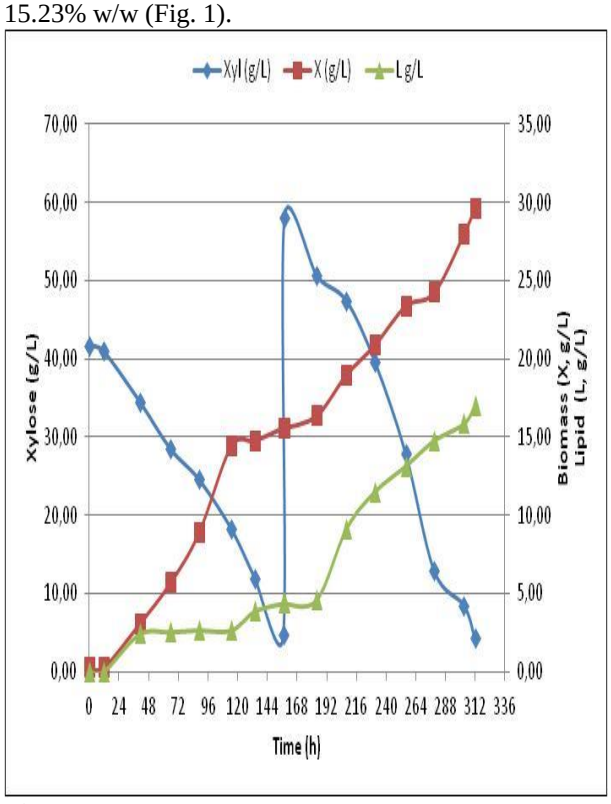

Fig. 1
Kinetics of biomass (X, g/L) production, xylose assimilation (Xyl, g/L) and lipid production (L, g/L) by *Rhodosporidium toruloides* NRRL Y-27012 during growth on xylose-based media presenting composition similarities with the spent sulfite liquor, in fed-batch bioreactor experiment under nitrogen-limited conditions. Culture conditions as in "Materials and Methods". Each point is the mean value of two independent measurements (SE<15%).

SCO produced by both DSM 4444 and NNRL Y-27012 strains, consisted of mainly palmitic acid (C16:0), stearic acid (C18:0), oleic acid (Δ9C18:1), linoleic acid (Δ9,12C18:2) and α-linolenic (Δ9,12,15C18:3) acids, with the dominant fatty acid in all the fermentations being oleic acid (Δ9C18:1).

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