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Evaluating renewable carbon sources as substrates for single cell oil production by *Cunninghamella echinulata* and *Mortierella isabellina*

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ABSTRACT

The biochemical behavior (biomass production, accumulation of total lipid, substrate uptake, fatty acid composition of fungal oil) of two oleaginous Mucorales strains, namely *Mortierella isabellina* ATHUM 2935 and *Cunninghamella echinulata* ATHUM 4411, was studied when the aforementioned microorganisms were cultivated on xylose, raw glycerol and glucose under nitrogen-limited conditions. Significant differences in the process of lipid accumulation as related to the carbon sources used were observed for both microorganisms. These differences were attributed to the different metabolic pathways involved in the assimilation of the above substrates. Therefore, the various carbon sources were channeled, at different extent, to storage lipid or to lipid-free biomass formation. Although glucose containing media favored the production of mycelial mass (15 g L^{-1} of total biomass in the case of *C. echinulata* and 27 g L^{-1} in the case of *M. isabellina*), the accumulated lipid in dry matter was 46.0% for *C. echinulata* and 44.6% for *M. isabellina*. Lipid accumulation was induced on xylose containing media (*M. isabellina* accumulated 65.5% and *C. echinulata* 57.7% of lipid, wt wt^{-1}, in dry mycelial mass). In these conditions, lipids of *C. echinulata* contained significant quantities of γ-linolenic acid (GLA). This fungus, when cultivated on xylose, produced 6.7 g L^{-1} of single cell oil and 1119 mg L^{-1} of GLA. Finally, the growth of both *C. echinulata* and *M. isabellina* on raw glycerol resulted in lower yields in terms of both biomass and oil produced than the growth on xylose.

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1. Introduction

Microbial oils, known as single cell oils (SCO), have long been considered as alternative oil sources, specifically as regards lipids rarely found in the plant or animal kingdom [i.e. lipids containing rare polyunsaturated fatty acids (PUFAs) or cocoa-butter equivalents] [1–9]. As regards PUFAs produced by microbial means, γ-linolenic acid (GLA) is of great pharmaceutical interest, due to its selective anticancer properties [8]. Another attractive perspective that emerged recently refers to the use of SCO as the starting material for the production of bio-diesel [10,11]. Indeed, this approach represents a very

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interesting alternative to the use of plant oils for bio-diesel production because oleaginous microorganisms (i.e. those microorganisms which can accumulate more than 20% of their dry biomass as oil) can transform a variety of substrates to SCO, which can then be transformed to bio-diesel. However, the production costs of SCO are still high, with serious efforts being made to reduce these costs by using waste materials as substrates, either in submerged or in solid state fermentation systems [4,7,11–13].

Among waste materials, lignocellulose is of great importance because it is produced in enormous quantities annually, as a result of land cultivation [14]. Xylose is the second most abundant sugar of lignocellulosic biomass, being produced during the dilute acid hydrolysis treatment of this biomass [14]. It was suggested that xylose and its polymer xylan could be used for SCO production by yeasts; this oil could then be used as a fuel [4]. Although it was surmised that xylose may be more efficient than glucose in terms of oil yield [15], reports on the efficiency of xylose conversion to SCO are equivocal [6,16–18].

Another attractive substrate is raw glycerol, the major by-product discharged after the bio-diesel manufacturing process. The constantly increasing demand for renewable fuels has substantially increased bio-diesel production [19] and this fact can lead to the accumulation of enormous quantities of glycerol deposit into the market in the near future. It should be noted that with the production of 10 kg of ester fuel from various oils, 1 kg of glycerol becomes available [19]; therefore glycerol valorization should have much to offer to the cost reduction of the overall bio-diesel production process. The main way of valorization of raw-unpurified glycerol by means of fermentation technology is referred to its biotransformation in 1,3-propanediol, using bacterial strains belonging to the genera *Clostridium* [20,21] and *Klebsiella* [22], while recently, alternative ways of raw glycerol valorization leading to its biotransformation to either SCO or citric acid with the aid of the non-conventional polymorphic yeast *Yarrowia lipolytica* have been developed [7,13,20,21,23,24].

Besides their abundance, xylose and raw glycerol may be considered as model substrates for SCO production, since the pathways involved in their assimilation are different from the pathway of glucose assimilation, which is the best-studied substrate for SCO production. The stoichiometry of glucose metabolism is about 1.1 moles of acetyl-CoA per 100 g of glucose utilized [25]. Xylose could be either metabolized through the phosphoketolase reaction, which is the most efficient pathway yielding 1.3 moles of acetyl-CoA per 100 g of xylose utilized, or the pentose phosphate pathway, where 1 mole of acetyl-CoA is formed per 100 g of xylose utilized [25]. About 1 mole of acetyl-CoA is produced per 100 g of glycerol utilized [15].

In the present investigation, we evaluated the potential of *Cunninghamella echinulata* and *Mortierella isabellina*, for growth and lipid production on two renewable carbon sources, namely xylose and raw glycerol. These molds were chosen since during their cultivation on glucose they presented different patterns for SCO biosynthesis as regards their isocitrate dehydrogenase (ICDH) activity [26]. These differences were thought responsible for the better oil yields achieved with *M. isabellina* [26]. Therefore, it would be of interest to elucidate the biochemical response of these strains cultivated on carbon sources metabolized by different catabolic pathways. Growth on glucose was used, as a comparison basis and biochemical interpretations concerning the distinct growth kinetics on each carbon source were considered and discussed.

2. Material and methods

2.1. Microorganisms and culture conditions

Cunninghamella. echinulata ATHUM 4411 and *M. isabellina* ATHUM 2935 were maintained on potato dextrose agar (PDA – Plasmatec, Dorset, UK) at $6 \pm 1\,^{\circ}C$. The growth medium comprised of ($g\,L^{-1}$): $(NH_4)_2SO_4$ (Merck, Darmstadt, Germany), 0.5; KH_2PO_4 (Panreac, Barcelona, Spain), 7.0; Na_2HPO_4 (Panreac), 2.0; $MgSO_4{\cdot}7H_2O$ (Fluka, Tokyo, Japan), 1.5; $CaCl_2{\cdot}2H_2O$ (Merck), 0.1; $FeCl_3{\cdot}6H_2O$ (Panreac), 0.08; $ZnSO_4{\cdot}7H_2O$ (Panreac), 0.001; $CuSO_4{\cdot}5H_2O$ (Merck), 0.0001; $Co(NO_3)_3{\cdot}H_2O$ (Merck), 0.0001 and $MnSO_4{\cdot}5H_2O$ (Panreac), 0.0001, yeast extract (Panreac), 0.5, supplemented with the appropriate amount of carbon source. The initial pH value of the medium was 6.0 ± 0.1. The carbon sources used were xylose (Fluka), raw glycerol, waste discharged after the bio-diesel manufacturing process [Hellenic Industry of Glycerin and Fatty Acids, Athens, Greece, purity 80%, $wt\,wt^{-1}$, with impurities composed mainly of potassium and sodium salts (4%, $wt\,wt^{-1}$), methanol (1%, $wt\,wt^{-1}$), heavy metals and lignin (1%, $wt\,wt^{-1}$), non-glycerol organic materials (0.5%, $wt\,wt^{-1}$) and water (13.5%, $wt\,wt^{-1}$)], pure glycerol (Merck, purity 95%, $wt\,wt^{-1}$) and glucose (Panreac). Medium pH after sterilization ($121\,^{\circ}C$/20 min) was 6.0 ± 0.1. All cultures were performed in 250-ml Erlenmeyer flasks that contained 50 ± 1 ml of the above medium. The flasks were incubated in a rotary shaker at 180 rpm and $28\,^{\circ}C$.

2.2. Quantitative determinations and chemical analyses

Determination of biomass, ammonium nitrogen, microbial oil, and GC analysis were performed as previously described [27]. Glucose and xylose in the growth medium were measured by the DNS method [28]. Glycerol in the growth medium was measured by HPLC (Waters 400) with an Aminex HPX-87H ($300\,mm \times 7.8\,mm$, Bio-Rad, California, USA) column coupled to a differential refractometer (RI Waters 410). The conditions for HPLC analysis were as follows: sample volume 20 μl, mobile phase 0.005 M H_2SO_4, flow rate $0.8\,ml\,min^{-1}$ and column temperature $65\,^{\circ}C$. The dissolved oxygen concentration was measured as described elsewhere [26]. The dissolved oxygen concentration value was higher than 60% ($v\,v^{-1}$) of the saturation value during all growth phases. The value of pH of the culture medium was measured at all experimental points and all trials, and the measurement was conducted by using a selective pH-meter (Jenway 3020 apparatus). The pH value of the fermentation medium did not significantly change regardless of the carbon source or the microorganism used (final pH in all cases 5.3 ± 0.1, pH value maintained within the range 5.2–6.0 during all fermentation steps of all cultures carried out). Therefore, the fermentations carried out on agitated flasks were neither accompanied by problems related

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