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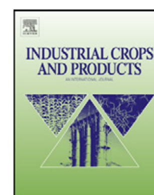

Biotechnological conversions of bio-diesel derived waste glycerol into added-value compounds by higher fungi: production of biomass, single cell oil and oxalic acid

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ABSTRACT

Waste bio-diesel derived glycerol was used as the sole carbon source by higher fungi; two *Lentinula edodes* strains were flask cultured in carbon-limited conditions and displayed satisfactory growth in media presenting weak agitation, pH 4.0 and temperature 25 °C. Maximum biomass of 5.2 g/l was produced. Mycelia were synthesized, containing around 0.1 g of fat per g of biomass, with linoleic acid ($^{\Delta9,12}$C18:2) being the principal cellular fatty acid produced. Two *Aspergillus niger* strains were grown in nitrogen-limited flask cultures with constant nitrogen and two different initial glycerol concentrations into the medium. In 250-ml flask cultures, large-sized pellets were developed, in contrast with the trials performed in 2-l flasks. Nitrogen limitation led to oxalic acid secretion and intra-cellular lipid accumulation; in any case, sequential production of lipid and oxalic acid was observed. Initially, nitrogen limitation led to lipid accumulation. Thereafter, accumulated lipid was re-consumed and oxalic acid, in significant quantities, was secreted into the medium. In large-sized pellets, higher quantities of intra-cellular total lipid and lower quantities of oxalic acid were produced and vice versa. Maximum quantities of oxalic acid up to 20.5–21.5 g/l and lipid up to 3.1–3.5 g/l (corresponding to 0.41–0.57 g of fat per g of biomass) were produced. Lipid was mainly composed of oleic ($^{\Delta9}$C18:1) and linoleic ($^{\Delta9,12}$C18:2) acids.
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1. Introduction

Bio-diesel fuels, defined as principally methyl- and to lesser extent ethyl- or butyl-esters deriving from *trans*-esterification of low value vegetable or animal fats, already constitute an alternative type of fuel for various types of diesel engines and heating systems (Johnson and Taconi, 2007; Papanikolaou, 2008). Due to the potential exhaustion of conventional fuels and the various environmental issues imposed, the application of bio-fuels in a large commercial scale is strongly recommended (e.g. EU directive 2003/30/EC which plans to introduce 5.75%, w/w, of bio-fuel in the conventional fuel by 2010). This fact may have as result the accumulation of tremendous quantities of glycerol into the market in the near future (Johnson and Taconi, 2007). In 2007 only in Europe, an over-capacity of more than 6×10^5 metric tones of glycerol occurred (Papanikolaou, 2008, 2009); to give the magnitude of the imposed problem, it is pointed out that with the production of 10 kg of bio-diesel deriving from *trans*-esterification of various oils, 1 kg of (pure) glycerol is generated (Johnson and Taconi, 2007; Papanikolaou, 2008). Therefore, glycerol over-production and disposal is likely to cause serious environmental problems in the near future (in Germany in 2007, crude glycerol deriving from various bio-diesel plants was already treated as a typical "industrial waste-water"—Papanikolaou, 2008); conversion, thus, of this low- or, even, negative value material to higher added-value products by the means of chemical or fermentation technology currently attracts much interest. Thermo-chemical processes have already appeared in the international literature that deal with conversion of glycerol into propylene glycol, acetol, hydrogen or various other compounds (Cortright et al., 2002; Dasari et al., 2005; Chiu et al., 2006; Alhanash et al., 2008). The biotechnological (fermentative) valorization glycerol has also been substantially developed during

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the last years, and various microbial added-value metabolites (e.g. 1,3-propanediol, organic acids, storage lipids, carotenoids, butanol) have been produced through utilization of this waste stream as microbial substrate (Morgunov et al., 2004a; Johnson and Taconi, 2007; Koutinas et al., 2007, 2008; Mu et al., 2008; Papanikolaou, 2008, 2009; Xiu and Zeng, 2008; Mantzouridou, 2009; Papanikolaou and Aggelis, 2009; Taconi et al., 2009; Wen et al., 2009a,b).

Aim of the current investigation was to demonstrate potential biotechnological ways of valorizing crude glycerol stemming from bio-diesel industries, by using this renewable substrate as carbon source of natural fungal species. Specifically, the present study was focused upon the transformation of crude glycerol into organic acids (principally oxalic acid) and microbial lipid (the so-called "single cell oil—SCO") by using *Aspergillus niger* strains and microbial mass by using *Lentinula edodes* strains. *A. niger* is an ascomycetous mold very often used in food technology and white biotechnology; strains of the above-mentioned microorganism have been successfully employed in bio-processes related with the production of organic acids (such as citric acid and to lesser extent gluconic acid) or enzymes (Soccol et al., 2006; Papagianni, 2007). However, to the best of our knowledge, with an exception of a recent book chapter (Musiał and Rymowicz, 2009) (crude) glycerol has never been used as substrate by strains of this microorganism. Although in some manuscripts production of oxalic acid by *Aspergillus* strains has been reported (Soccol et al., 2006; Papagianni, 2007), in most of the cases carbon substrates other than glycerol (e.g. glucose, crude fatty acids, whey) have been used for this purpose. As far as the microorganism *L. edodes* is concerned, it is a basidomycetous mold known also as Shiitake; this fungus is a medicinally important edible mushroom (Israilides and Philippoussis, 2003; Philippoussis et al., 2007). Only the last years some research has been made using *L. edodes* as a potential biotechnological tool, with studies focused upon the production of anti-tumor and anti-bacterial agents (Hassegawa et al., 2005; Surenjav et al., 2006; Israilides et al., 2008) or enzymes (Cavallazzi et al., 2005). In some cases, due to the capability of strains of this species to produce extra-cellular oxidizing enzymes (e.g. laccases) this microorganism has been successfully used in the simultaneous detoxification (removal of phenolic compounds and color) of phenol-rich residues (i.e. olive-mill waste-waters) and the production of enzymes and biomass (D'Annibale et al., 2004). In any case though, glycerol has never been used as substrate by strains of this mushroom.

As previously stated, while oxalic acid has rarely been produced through microbial conversion of glycerol (Musiał and Rymowicz, 2009), this substrate has been used as carbon source by a number of eukaryotic microorganisms (principally wild or mutant *Yarrowia lipolytica* strains) and citric acid (Papanikolaou et al., 2002, 2008; Rymowicz et al., 2006, 2008; Imandi et al., 2007; Papanikolaou and Aggelis, 2009; Makri et al., 2010) or puryvic acid (Morgunov et al., 2004a) has been produced. On the other hand, conversion of (waste) glycerol into SCO has been investigated in a number of works, in which oleaginous fungi (Mantzouridou et al., 2008; Papanikolaou et al., 2008; Fakas et al., 2009a,b; Wen et al., 2009b), (heterotrophically growing) algae (Chi et al., 2007; Pyle et al., 2008; Wen et al., 2009b) or yeasts (Meesters et al., 1996; Papanikolaou and Aggelis, 2002, 2009; Makri et al., 2010) have been used. Finally, glycerol has been utilized as substrate by eukaryotic microbial strains in order for biomass to be produced (Kim et al., 2000). The current investigation aimed at assessing potentialities of bio-valorization of waste glycerol by using *L. edodes* and *A. niger* strains in order for the production of microbial mass, organic acids and SCO to be performed. Biochemical and kinetic considerations related with the growth of these microorganisms on glycerol were considered and discussed.

2. Materials and methods

2.1. Microorganisms and media

A. niger strain NRRL 364 was kindly provided by the NRRL culture collection (Peoria, USA). Another newly isolated *A. niger* strain was also used in the present study. This strain was isolated from edible products (fat-rich foods), was identified and characterized in the Laboratory of Food Microbiology and Biotechnology (Department of Food Science and Technology, Agricultural University of Athens, Greece). The culture number given to this newly isolated *A. niger* strain was LFMB 1. *L. edodes* strains AMRL 119 and AMRL 121 were obtained from the culture collection of the Laboratory of Edible Fungi (National Agricultural Research Foundation, IAMC). All strains were maintained on yeast peptone dextrose agar (YPDA) at 4 °C and sub-cultured every 4 months in order to maintain their viability.

All experiments were performed in liquid-submerged flask cultures. The culture medium used contained (in g/l): KH_2PO_4 7.0; Na_2HPO_4 2.5; $MgSO_4{\cdot}7H_2O$ 1.5; $CaCl_2$ 0.15; $FeCl_3{\cdot}6H_2O$ 0.15; $ZnSO_4{\cdot}7H_2O$ 0.02; $MnSO_4{\cdot}H_2O$ 0.06. Ammonium sulphate [$(NH_4)_2SO_4$] and yeast extract were used as nitrogen sources in various concentrations. Crude glycerol provided from the "Hellenic Industry of Glycerin and Fatty Acids SA" [purity ±70%, w/w, impurities composed of potassium and sodium salts (12%, w/w), non-glycerol organic material (1%, w/w), methanol (2%, w/w) and water (14%, w/w)] was used as the sole carbon source in various concentrations. Production of intermediate organic acids (e.g. oxalic acid) or SCO by molds during cultivation on glucose or similarly metabolized compounds (e.g. glycerol) is a biosynthetic activity conducted after nitrogen depletion from the medium, therefore cultures should be carried out in nitrogen-limited conditions (Morgunov et al., 2004b; Soccol et al., 2006; Wynn and Ratledge, 2006; Papagianni, 2007; Papanikolaou and Aggelis, 2009). In contrast, to direct the cellular metabolism towards the formation of microbial mass, cultures should be carried out in rich-nitrogen media. Given that in the case of *L. edodes* strains it was desirable to direct the microbial metabolism towards the synthesis of microbial mass, cultures were carried out in carbon-limited media. Crude glycerol was used as substrate at initial glycerol concentration 20 g/l and initial $(NH_4)_2SO_4$ and yeast extract concentrations at 4.0 and at 2.0 g/l respectively. Concerning the fermentations of *A. niger*, it was desirable to carry out fermentations in nitrogen-limited media in order to direct the microbial metabolism towards the synthesis of extra-cellular organic acids and intra-cellular reserve lipid. For these cultures, initial concentration of both $(NH_4)_2SO_4$ and yeast extract was 0.5 g/l. For *A. niger* experiments, initial glycerol was adjusted to 30 and 60 g/l.

2.2. Culture conditions

All experiments were performed in batch flask cultures in either 250-ml or in 2-l Erlenmeyer flasks. Before inoculation, Erlenmeyer flasks filled with the culture medium were sterilized at 121 °C/20 min. Concerning *L. edodes* cultures, experiments were performed in 250-ml flasks containing 50 ± 1 ml growth medium inoculated with five 7 mm diameter mycelial disks cut from the edge of 7 days old *L. edodes* colonies grown on YPDA. Cultures were performed in a rotary shaker (Lab-Line, IL, USA) for a period of 12 days and the tested parameters were as follows: incubation temperature (T) at 28, 25 and 22 °C; agitation rate at 180, 60 and 0 (no agitation) rpm; initial pH at 5.0, 4.0 and 3.5 (adjustment with a HCl 5 M solution).

For *A. niger*, both strains were kept on YPDA at 4 °C and were re-generated in YPDA slant tubes (incubated at T = 28 °C for 4 days before inoculation). Then, one YPDA slant tube was rinsed 3 times

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