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Formulation of fermentation media from flour-rich waste streams for microbial lipid production by *Lipomyces starkeyi*

Sofia Tsakona [a], Nikolaos Kopsahelis [a], Afroditi Chatzifragkou [b], Seraphim Papanikolaou [a], Ioannis K. Kookos [c], Apostolis A. Koutinas [a,*]

[a] *Department of Food Science and Human Nutrition, Agricultural University of Athens, 75 Iera Odos, 11855 Athens, Greece*
[b] *Department of Food and Nutritional Sciences, University of Reading, Whiteknights, Reading RG6 6AD, UK*
[c] *Department of Chemical Engineering, University of Patras, 26504 Patras, Rio, Greece*

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ABSTRACT

Flour-rich waste (FRW) and by-product streams generated by bakery, confectionery and wheat milling plants could be employed as the sole raw materials for generic fermentation media production, suitable for microbial oil synthesis. Wheat milling by-products were used in solid state fermentations (SSF) of *Aspergillus awamori* for the production of crude enzymes, mainly glucoamylase and protease. Enzyme-rich SSF solids were subsequently employed for hydrolysis of FRW streams into nutrient-rich fermentation media. Batch hydrolytic experiments using FRW concentrations up to 205 g/L resulted in higher than 90% (w/w) starch to glucose conversion yields and 40% (w/w) total Kjeldahl nitrogen to free amino nitrogen conversion yields. Starch to glucose conversion yields of 98.2, 86.1 and 73.4% (w/w) were achieved when initial FRW concentrations of 235, 300 and 350 g/L were employed in fed-batch hydrolytic experiments, respectively. Crude hydrolysates were used as fermentation media in shake flask cultures with the oleaginous yeast *Lipomyces starkeyi* DSM 70296 reaching a total dry weight of 30.5 g/L with a microbial oil content of 40.4% (w/w), higher than that achieved in synthetic media. Fed-batch bioreactor cultures led to a total dry weight of 109.8 g/L with a microbial oil content of 57.8% (w/w) and productivity of 0.4 g/L/h.

1. Introduction

The development of sustainable processes could be achieved through valorisation of waste and by-product streams generated by current industrial processes (Koutinas et al., 2014a,b). Food supply chain waste and by-product streams are generated along any supply chain of food industrial sectors and sub-sectors. Around 89 million t of food waste is generated annually in the EU-27 through food manufacturing (39%), households (42%), food catering services (14%) and retail/wholesale of food products (Monier et al., 2010). Reports published by FAO estimate more than 1.3 billion t of annual food supply chain waste corresponding to 50% of food lost or wasted along the supply chain from the agricultural field to the consumer (Parfitt et al., 2010). Flour-rich waste (FRW) and by-product streams are generated by many industrial food sectors belonging mainly to the following categories as have been classified by the PRODCOM List 2013 (Anonymous, 2014a):

- *manufacture of grain mill products* (PRODCOM code 10.61) that constitute the 4th (56.9×10^6 t in EU-27 in 2012) most important food sector in terms of production capacity;
- *manufacture of bread, fresh pastry goods and cakes* (PRODCOM code 10.71) that constituted the 7th (26.4×10^6 t in EU-27 in 2012) most important food sector in terms of production capacity;
- *manufacture of rusks, biscuits and preserved pastry goods and cakes* (PRODCOM code 10.72);
- various types of confectionery products and food for infants.

Flour-rich waste streams are mainly generated during the manufacturing process, disposed by consumers and catering services or are returned from the market as end-of-date products. To provide an estimate of the capacity of such waste streams, it could be mentioned that around 800,000 t of bakery waste is produced annually in the UK (Anonymous, 2011). FRW contain significant quantities of starch and protein as well as various micro-nutrients that could

* Corresponding author at: Laboratory of Food Process Engineering, Processing and Preservation of Agricultural Products, Department of Food Science and Human Nutrition, Agricultural University of Athens, Greece. Tel.: +30 210 5294729; fax: +30 210 5294729.
E-mail address: akoutinas@aua.gr (A.A. Koutinas).

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be used for the production of generic fermentation feedstocks. The main by-product stream generated by wheat flour millers is produced via milling and sifting of wheat and contains mainly bran and varying quantities of endosperm depending on the type of wheat flour produced. This by-product stream is mainly used as animal feed, but surplus quantities are treated as wastes. Wheat milling by-products (WMB) can be used for enzyme production via solid state fermentations (SSF) using appropriate fungal strains. Thus, FRW and by-product streams could be employed as the sole renewable resources for bioprocess development employed for chemical production (Lin et al., 2013; Koutinas et al., 2014a).

Microbial lipid production represents a significant field of research during the last decades. The increasing interest towards alternative pathways for cost-effective production of biofuels from renewable resources has set the ground for intensive search on heterotrophic (yeast and fungi) or phototrophic (algae) organisms, capable of accumulating high oil contents, accompanied by high bioconversion yields (Papanikolaou and Aggelis, 2010). Microbial lipids are mainly composed of triacylglycerols (TAGs), together with quantities of free-fatty acids and to a lesser extent sterols and polar fractions (Papanikolaou and Aggelis, 2011). Depending on the oleaginous strain and the applied cultivation method, microbial lipids may present a diversified fatty acid composition attributing varying properties to the produced lipid bodies. The composition of microbial lipids is similar to vegetable oils, comprised of long chain saturated and unsaturated fatty acids, a fact that justifies their general acceptance as suitable starting material for biodiesel production (Vincente et al., 2009). Besides fatty acids methyl esters, other microbial oil derivatives such as fatty acids, glycerol and hydrogenated products of fatty acid methyl esters and fatty alcohols constitute base materials for a broad spectrum of oleochemical products such as surfactants, lubricants, polymers and plastics (Naik et al., 2010). The need to replace petrochemically derived products with bio-based ones has paved the way for research and development towards vegetable oil utilisation for the production of oleochemicals due to their non-toxic and readily biodegradable nature (Böttcher et al., 2009; Buchholz and Bornscheuer, 2005; Metzger and Bornshcheuer, 2006). Moreover, worldwide demand for natural fatty acids and glycerol, nowadays available as a crude by-product stream from biodiesel production processes using natural fats and oils, is expected to grow about 9.8% annually, from the current manufacturing value of $7.7 billion in 2011 to $13.5 billion through 2017, based on the expected continuous increase of prices of key vegetable oils and animal fats (Anonymous, 2014a,b). The utilisation of microbial lipids as precursors for oleochemical synthesis can be considered as a promising path, especially within a biorefinery concept.

Recent research has focused on the improvement of microbial oil production efficiency (Koutinas and Papanikolaou, 2011; Leiva-Candia et al., 2014; Papanikolaou et al., 2013; Shen et al., 2013) and evaluation of its cost-competitiveness (Koutinas et al., 2014b). Utilising crude renewable resources rather than commercial and purified carbon sources and nutrient supplements are of paramount importance towards the development of viable microbial oil production processes. The production cost of purified microbial oil at an annual production capacity of 10,000 t, a bioreactor productivity of 0.54 g/L/h, a TDW of 106.5 g/L and microbial oil content of 67.5% (w/w) has been estimated at $3.4 per kg when the cost of glucose is negligible (Koutinas et al., 2014b). The unitary cost of purified microbial oil production at negligible expenditure for the carbon source could be closer to that of vegetable oils at bioreactor productivities higher than 2.5 g/L/h (Koutinas et al., 2014b). Increasing the price of glucose to $400 per kg corresponds to a microbial oil production cost of $5.5 per kg and therefore higher bioreactor productivities should be achieved in order to create a cost-competitive process. The strain *Lipomyces starkeyi* has recently demonstrated the potential to satisfy the required criteria to achieve industrial implementation. Lin et al. (2011) reported the production of a total dry weight (TDW) of 104.6 g/L with a microbial oil content of 64.9% (w/w) at a productivity of 1.2 g/L/h via fed-batch bioreactor cultures of *L. starkeyi* AS 2.1560 using glucose-based mineral medium. Tapia et al. (2012) carried out fed-batch bioreactor cultures of a mutant strain of *L. starkeyi* DSM 70296 cultivated on mixtures of glucose and xylose to achieve a TDW of 88.7 g/L with a microbial oil content of 55.2% (w/w) at a productivity of 0.29 g/L/h.

This study focuses on the production of a generic fermentation feedstock from WMB and FRW streams that could be used for microbial oil production. WMB were used in solid state fermentations of *Aspergillus awamori* for the production of all the enzymes required to convert starch into glucose, protein into directly assimilable amino acids and peptides, and generate an inorganic source of phosphorus via phytic acid hydrolysis. Enzyme-rich SSF solids were mixed with FRW suspensions for the production of fermentation feedstocks that were subsequently evaluated for microbial oil production using the oleaginous yeast strain *L. starkeyi* DSM 70296. The obtained results are among the highest reported in the literature regarding microbial oil production from glucose-based media with the strain *L. starkeyi*. Multi-enzyme production and FRW hydrolysis were optimised in order to achieve highly concentrated hydrolysates similar to those produced in traditional liquefaction and saccharification processes of starch and cereal flours. The FRW used in this study were produced by a confectionery industry line producing food for infants and flour-based confectionery products. Therefore, starch was the carbohydrate contained in this waste stream. This study was focused on the optimisation of flour hydrolysis and evaluation of microbial oil production using this homogeneous FRW stream. In a forthcoming publication mixed confectionery waste streams containing various carbohydrates (i.e. starch, lactose, sucrose and fructose) as well as proteins and lipids will be used for fermentative microbial oil production in order to demonstrate the efficiency of microbial oil production using heterogeneous FRW streams.

2. Materials and methods

2.1. Microorganisms and media

SSF were carried out with the fungal strain *A. awamori* 2B.361 U 2/1 that was kindly provided by Professor Colin Webb (University of Manchester, UK). The origin, purification, sporulation and storage methods have been described in previous publications (Koutinas et al., 2005; Wang et al., 2009). Fungal spores were stored at 4 °C in slopes containing 5% (w/v) wheat bran (WB) and 2% (w/v) agar.

The oleaginous yeast strain *L. starkeyi* DSM 70296 was employed in fermentations for microbial oil production. It was maintained at 4 °C on agar slopes containing glucose (10 g/L), yeast extract (10 g/L), peptone (10 g/L) and agar (2%, w/v). A liquid medium of the same composition in glucose, yeast extract and peptone was used for the preparation of fermentation inocula. During experimental work, it was observed that efficient microbial oil production in bioreactor cultures by *L. starkeyi* is highly dependent on the production of a dense inoculum. To achieve this in fed-batch bioreactor fermentations, pre-cultures were produced in FRW hydrolysates that led to improved fermentation efficiency.

Fermentations were carried out using either FRW hydrolysates or synthetic media. In the latter case the composition of the medium was (in g/L): glucose, 105.0; yeast extract, 2.0; $(NH_4)_2SO_4$, 1.0; KH_2PO_4, 7.0; Na_2HPO_4, 2.5; $MgSO_4{\cdot}7H_2O$, 1.5; $FeCl_3{\cdot}6H_2O$, 0.15; $ZnSO_4{\cdot}7H_2O$, 0.02; $MnSO_4{\cdot}H_2O$, 0.06; $CaCl_2{\cdot}2H_2O$, 0.15.