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Valorisation of side streams from wheat milling and confectionery industries for consolidated production and extraction of microbial lipids

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ABSTRACT

Crude enzymes produced via solid state fermentation (SSF) using wheat milling by-products have been employed for both fermentation media production using flour-rich waste (FRW) streams and lysis of *Rhodosporidium toruloides* yeast cells. Filter sterilization of crude hydrolysates was more beneficial than heat sterilization regarding yeast growth and microbial oil production. The initial carbon to free amino nitrogen ratio of crude hydrolysates was optimised (80.2 g/g) in fed-batch cultures of *R. toruloides* leading to a total dry weight of 61.2 g/L with microbial oil content of 61.8% (w/w). Employing a feeding strategy where the glucose concentration was maintained in the range of 12.2–17.6 g/L led to the highest productivity (0.32 g/L · h). The crude enzymes produced by SSF were utilised for yeast cell treatment leading to simultaneous release of around 80% of total lipids in the broth and production of a hydrolysate suitable as yeast extract replacement.

1. Introduction

Waste and by-product streams are generated along any food supply chain starting from agricultural production up to consumption of food products. Flour-rich waste (FRW) streams are produced by various industrial sectors, including the categories *manufacture of bread, fresh pastry goods and cakes* (PRODCOM code 10.71), *manufacture of rusks, biscuits and preserved pastry goods and cakes* (PRODCOM code 10.72) and *food preparations for infants* (PRODCOM 10.86.10.60 and 10.86.10.70) as have been classified by the PRODCOM List 2013 (Anonymous, 2014). Bran-rich wheat milling by-products are produced mainly by the category *manufacture of grain mill products* (PRODCOM code 10.61). To illustrate the approximate quantities of annual waste capacities produced in Europe, it is mentioned that the losses and wastes generated along the wheat and rye supply chains in Europe are: (a) 1.45×10^6 t at agricultural production, (b) 2.56×10^6 t at postharvest handling and storage, (c) 7.45×10^6 t during industrial bread baking, (d) 1.34×10^6 t during distribution, and (e) 16.43×10^6 t at the consumption stage (Gustavsson, Cederberg, Sonesson, & Emanuelsson, 2013). The current trend of policy development for future waste management in Europe focus on the classification of preventable and non-preventable wastes along the food supply chains. Reduction of food losses will focus on the preventable fraction, while the non-preventable fraction could be employed as feedstock to support the growth of bio-economy sectors, including the production of bio-based chemicals, such as microbial oil. The lipids produced by oleaginous yeasts have a similar fatty acid composition to vegetable oils and therefore can be used for the production of various derivatives including fatty acids, glycerol, fatty acid esters and fatty alcohols. Therefore, microbial lipids could be used as a potential alternative to vegetable oils for the production of various oleochemicals including lubricants, wax esters, surfactants, polymers and plastics (Naik, Goud, Rout, & Dalai, 2010).

Wheat milling by-products could be used as the sole substrate for the production of amylolytic and proteolytic enzymes by *Aspergillus awamori* via solid state fermentation (SSF) (Tsakona et al., 2014). These enzymes can be subsequently used for the production of nutrient-rich hydrolysates from FRW streams that were sufficient for microbial oil production by the oleaginous yeast *Lipomyces starkeyi* (Tsakona et al., 2014). It was justified that FRW hydrolysates constitute a nutrient complete fermentation medium that does not require any supplementation with commercial nutrient sources (e.g. yeast extract, inorganic salts). The utilisation

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of yeast extract as nitrogen source increases the cost of microbial oil production by 6% at a production cost of approximately $3.4 per kg that was estimated at 10,000 t of annual production capacity, negligible cost of glucose, bioreactor productivity of 0.54 g/(L · h), total dry weight (TDW) of 106.5 g/L and microbial oil content of 67.5% (w/w) (Koutinas, Chatzifragkou, Kopsahelis, Papanikolaou, & Kookos, 2014). Therefore, utilisation of FRW hydrolysates could eliminate this expenditure.

Besides *L. starkeyi*, *Rhodosporidium toruloides* is another promising strain for the production of microbial lipids. This strain has been mainly studied with commercial or crude carbon sources supplemented with commercial nutrient sources such as yeast extract. The potential of *R. toruloides* to produce microbial oil using entirely crude renewable resources as nutrient-complete fermentation media has been reported in limited studies. Yang et al. (2015) reported the production of 16.6, 14.6 and 12.9 g/L of microbial lipids in three successive fermentations carried out with *R. toruloides* Y4 cultivated on recycled yeast cell mass hydrolysates and 70 g/L of initial glucose concentration. The utilisation of spent yeast from breweries has been investigated as a crude source of nutrients for the cultivation of *Cryptococcus curvatus* leading to the production of 50.4 g/L of TDW and 37.7% (w/w) of microbial oil content (Ryu et al., 2013). Thiru, Sankh, and Rangaswamy (2011) reported the production of 69.2 g/L of TDW with an intracellular microbial oil content of 48% (w/w) by replacing baker's yeast autolysate and malt extract with autolysates of de-oiled yeast cells combined with crude glycerol and corn steep liquor.

The initial ratio of carbon to nitrogen and the feeding strategy employed play a crucial role on microbial oil production. Wiebe, Koivuranta, Penttilä, and Ruohonen (2012) reported the production of TDW in the range of 35–47 g/L with intracellular lipid content of 50–75% (w/w) in fed-batch cultures of *R. toruloides* using different feeding strategies including either provision of nitrogen source at the beginning of fermentation and no supply of nitrogen source during feeding or maintaining the same C/N ratios of 65 or 80 during batch fermentation and feeding stages. The highest intracellular lipid content was achieved in cultures where no nitrogen source was supplied during feeding, while higher TDW were achieved when a constant C/N ratio of 65 or 80 was maintained throughout fermentation. The strain *R. toruloides* Y4 has been cultivated on fed-batch mode using commercial glucose and nutrient supplements under pulsed feeding of nitrogen-free media leading to the production of 71.8 g/L of intracellular lipids with a productivity of 0.54 g/(L · h) (Li, Zhao, & Bai, 2007). Zhao, Hu, Wu, Shen, and Zhao (2011) reported that the highest DCW (127.5 g/L), lipid concentration (78.8 g/L) and productivity (0.57 g/L · h) were achieved when a constant glucose concentration of around 5 g/L was maintained during the feeding stage. The effect of carbon to free amino nitrogen (FAN) ratio and the application of different feeding strategies on microbial oil production have not been studied in the case of crude hydrolysates derived from renewable resources.

Downstream separation of microbial lipids is another crucial issue hindering the implementation of large-scale processes. The yeast cells should be initially separated from the fermentation broth via filtration-based unit operations. Cell disruption combined with solvent extraction is the conventional methodology followed for separation and purification of microbial lipids. Cell disruption can be achieved by various methods including high pressure homogenisation, bead milling, swelling by osmotic pressure, and acidic or alkaline treatment (Koutinas et al., 2014; Lee, Yoo, Jun, Ahn, & Oh, 2010; Li et al., 2007). Jin, Yang, Hu, Shen, and Zhao (2012) reported a 96.6% separation of microbial lipids from *R. toruloides* cells at room temperature and atmospheric pressure directly after fermentation without filtration of yeast cells via pre-treatment with microwave followed by enzymatic hydrolysis with the recombinant β-1,3-glucomannanase plMAN5C and extraction with ethyl acetate. Crude enzymes produced via solid state fermentation of renewable resources have never been used for yeast cell disruption and removal of microbial lipids.

This study focuses on the optimisation of the initial carbon to free amino nitrogen ratio in fed-batch cultures using the oleaginous yeast *R. toruloides* cultivated on crude FRW hydrolysates. Various feeding strategies have been employed at the optimum carbon to free amino nitrogen ratio. A consolidated bioprocess is proposed where the crude enzymes produced by SSF of wheat milling by-products could be employed for the production of FRW hydrolysates and the disruption of yeast cells leading to the release of microbial lipids in the aqueous suspension.

2. Materials and methods

2.1. Microorganisms

The SSF employed for the production of crude enzyme consortia were conducted with the fungal strain *A. awamori* 2B.361 U2/1 that was originally obtained from ABM Chemicals, Ltd. (Woodley, Cheshire, UK) and was kindly provided by Professor Colin Webb (University of Manchester, UK). The purification and sporulation of *A. awamori* spores have been described by Koutinas et al. (2001). Storage of fungal spores was carried out in slopes at 4 °C containing 5% (w/v) wheat bran and 2% (w/v) agar.

Submerged fermentations for the production of microbial oil were carried out with the oleaginous yeast strain *R. toruloides* DSM 4444. Agar slopes containing glucose (10 g/L), yeast extract (10 g/L), peptone (10 g/L) and agar (2%, w/v) were used for maintenance of this strain at 4 °C. The same composition of nutrients was used for the preparation of fermentation inocula (50 mL) in 250 mL Erlenmeyer flasks.

2.2. Raw materials and fermentation media

The solid substrate used in the SSF carried out with the fungal strain *A. awamori* was wheat milling by-products that contained 12% starch, 20% protein, 1.1% phosphorus and 9.7% moisture (w/w). The FRW streams used for the production of fermentation media using the crude enzyme consortia produced by SSF were supplied by Jotis S.A., a Greek confectionery industry. The FRW streams produced during the manufacturing process of food for infants contained 84.8% starch, 7.3% protein and 5% moisture (w/w).

The production of enzyme consortia via SSF and FRW hydrolysates was carried out as was described by Tsakona et al. (2014). SSF of *A. awamori* was carried out in 250 mL Erlenmeyer flasks at 30 °C using 5 g of wheat milling by-products as substrate. Sterilisation of each flask was carried out at 121 °C for 20 min prior to inoculation that was carried out with a fungal spore suspension of 2×10^6 spores per mL. After 3 days, SSF solids were suspended in 500 mL sterilised tap water and were subsequently macerated using a kitchen blender. After centrifugation (3000*g* for 10 min) of the aqueous suspension, the supernatant was transferred in 1 L Duran bottles that contained varying FRW concentrations in order to achieve the desired carbon to FAN ratio. Magnetic stirrers were used for mixing of the suspension. Hydrolysis of FRW was carried out at 55 °C and uncontrolled pH conditions. The initial glucoamylase and protease activities were around 0.5 and 8 U/mL, respectively.

Upon completion of enzymatic hydrolysis, solids were separated via centrifugation (3000*g* for 10 min). The supernatant was filtered (Whatman No1) to remove any remaining insoluble materials. FRW hydrolysates used as fermentation media were either autoclaved (121 °C for 15 min) or filter-sterilised using a 0.2 μm filter unit (Polycap TM AS, Whatman Ltd.). The pH of the