Developing Confectionery Industry Biorefineries

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ABSTRACT

Food wastes are generated worldwide at significant quantities and could be regarded as a valuable renewable resource for biorefinery development aiming at the production of fuels, chemicals and materials. This study presents novel bioprocessing and biorefinery concepts based on the utilisation of waste streams from confectionery industries that are generated in significant quantities and are enriched in carbohydrates (starch, sucrose, glucose, fructose and lactose), protein and various micro-nutrients. Such waste streams can be utilised as fermentation media for the production of microbial oil using oleaginous microorganisms. Confectionery industry flour-rich waste streams were enzymatically hydrolyzed to produce fermentation media with high glucose and Free Amino Nitrogen (FAN) concentrations. The produced hydrolysates were evaluated as fermentation feedstocks for the production of microbial oil using oleaginous yeasts and their efficiency was compared with synthetic media. Shake flask fermentation carried out with *Rhodosporidium toruloides* produced up to 24.4 g/L dry cell weight with a microbial lipid content of 50 % (w/w). Preliminary bioreactor fermentations with *R. toruloides* carried out in fed-batch mode enhanced significantly yeast growth and microbial oil accumulation. The results obtained indicate that integration of microbial oil production in existing confectionery industries may lead to the production of an important feedstock for the chemical industry

Keywords: biorefinery, confectionery industry waste, enzymatic hydrolysis, microbial oil, novel bioprocess

1 INTRODUCTION

The development of integrated and sustainable biorefinery concepts can be based on the utilisation of waste and by-product streams from current industrial plants for the creation of the bio-economy era where traditional industrial sectors (e.g. food industry) will evolve into cross-sectoral industrial plants (e.g. integration of food and chemical production). The food and chemical industries are among the first three industrial sectors regarding both generated added value and investment in the EU manufacturing sector [1]. Future prospects for these two sectors could be enhanced through integration of chemical production in current food industries.

Microbial oil could be used as a renewable raw material for the production of biodiesel or oleochemicals. Especially in the case of oleochemicals, the high versatility of fatty acid content produced by different oleaginous yeast and fungi could lead to the replacement of vegetable oils as raw material for the production of fatty acids, fatty esters, fatty alcohols and glycerol, currently used as precursor molecules for conversion into lubricants, waxes, cosmetics, plastics and other end-products [2,3]. Furthermore, the waste streams generated by confectionery industries either during processing or as end-of-date products returned from the market (e.g. supermarkets, catering services) can be utilised as microbial feedsock for microbial oil production. This processing concept constitutes a step-change in conventional waste management approaches and will lead to sustainable processing schemes based on industrial synergies.

The aim of this study was to investigate the feasibility of the proposed biorefinery concept. Flour-based waste streams from a confectionery industry were converted into a nutrient-rich fermentation feedstock via enzymatic hydrolysis using crude enzyme extracts from solid state fermentations (SSF). Crude hydrolysates were subsequently evaluated as the sole fermentation media for the production of microbial oil using two oleaginous yeast strains.

2 MATERIALS AND METHODS

2.1 Raw materials and microorganisms

Flour-based industrial waste streams (FBW) were kindly provided by the Greek confectionery industry JOTIS SA. An industrial strain of *Aspergillus awamori* 2B.361 U2/1 was utilized in SSF for the production of crude enzymes [4,5]. Fungal spores were stored in slopes containing 5 % (w/v) wheat bran (WB) and 2 % (w/v) agar, at 4 °C.

Two oleaginous yeast strains were employed in order to evaluate the efficiency of the produced hydrolysate as suitable medium for microbial fermentations. In particular, the strains *Rhodosporidium toruloides* DSM 4444 and *Lipomyces starkeyi* DSM 70296 were maintained on agar slopes containing 10 g L^{-1} yeast extract, 10 g L^{-1} peptone and 10 g L^{-1} glucose. Both yeast strains were preserved at 4

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°C. The same medium composition was used for the preparation of fermentation inocula.

2.2 Solid state fermentation

Solid state fermentations (SSF) of wheat milling by-products using the fungal strain *A. awamori* were conducted in 250 mL Erlenmeyer flasks at 30 °C. A quantity of 5 g of total solid medium was added in each flask that was autoclaved at 121 °C for 20 min. Subsequently, the solids in each flask were inoculated with a fungal spore suspension that was also used to adjust the moisture content of the substrate at 65 % (w/w, on a dry basis). All inoculated flasks were incubated for 48 h.

2.3 Production of FBW hydrolysate

After the end of SSF, the fermented solids were suspended in 500 mL sterilized tap water and subsequently macerated using a kitchen blender. Various amounts of fermented solids were used, depending on the initial enzymatic activity required at the beginning of hydrolysis. The resulting suspension was added in 1 L Duran bottles that contained varying quantities of FBW depending on the experiment. All Duran bottles with FBW solids were initially sterilized at 121 °C for 15 min. Duran bottles were placed in a water bath at a constant temperature of 55 °C and hydrolysis was performed until the highest attainable starch hydrolysis was achieved. Samples were collected at random intervals and the solids were separated via centrifugation (10 min, 3,000 g). The supernatant was used for the determination of glucose and free amino nitrogen (FAN) content. After the end of hydrolysis, the pH of FBW hydrolysates was adjusted to the optimum pH range (6.0 – 6.2) for oleaginous yeast cultivation with 5 M KOH.

2.4 Shake flask fermentations

Yeast cultivations were carried out on either FBW hydrolysate or a synthetic medium, which served also as the control experiment. Shake flask fermentations were carried out in 250 mL Erlenmeyer shake flasks containing 50 mL of medium and where inoculated with 1 mL of a 24 h exponential pre-culture (around $1\text{-}3\times10^7$ cells). Flasks were incubated at 28 °C in an orbital shaker (ZHWY-211C Series Floor Model Incubator, PR China) at 180 rpm. The employed synthetic growth medium contained (in g/L): KH_2PO_4, 7.0; Na_2HPO_4, 2.5; $MgSO_4\ 7H_2O$, 1.5; $FeCl_3\ 6H_2O$, 0.15; $ZnSO_4\ 7H_2O$, 0.02; $MnSO_4\ H_2O$, 0.06; $CaCl_2\ 2H_2O$, 0.15. Yeast extract and $(NH_4)_2SO_4$ were used as nitrogen sources at concentrations of 2.0 and 1.0 g/L, respectively. Glucose was used as a carbon source at concentrations of approximately 50 and 100 g/L in both synthetic media and FBW hydrolysates. The fermentation duration in shake flasks was 9 days.

2.5 Bioreactor fermentation

Batch and fed-batch bioreactor trials were performed, by utilising FBW hydrolysates as the sole source of carbon and nutrients, in a 3 L bioreactor (New Brunswick Scientific Co, USA) with a working volume of 1.5 L. The initial pH of the medium was 6.0±0.1 and maintained at the desirable value by automatic addition of 5 M NaOH. Inoculation was conducted with 2% (v/v) of yeast pre-culture. The agitation of the bioreactor was controlled automatically in the range of 150 – 550 rpm and the aeration was maintained at a constant rate of 1.5 vvm. The cultivation temperature was 28 °C. Fed-batch fermentations were initiated in batch mode and feeding started when the glucose concentration was reduced to concentrations lower than 10 g L^{-1}. A 60 % (w/v) glucose solution was employed as feeding medium that was added manually in the bioreactor. Feeding of glucose solution was carried out until glucose consumption was terminated. In both batch and fed-batch cultivations, samples were periodically taken from the bioreactor and were analysed regarding residual glucose concentration, FAN concentration, total dry weight and intracellular lipid accumulation.

2.6 Analytical methods

During enzymatic hydrolysis of FBW, samples of 2 mL were taken at random intervals. They were mixed with equal amount of trichloroacetic acid (TCA, 5% w/v), subsequently processed via centrifugation (9,000 g for 5 min) and the supernatant was stored for measurement of glucose and FAN concentrations. Glucose concentration was determined by High Performance Liquid Chromatography (HPLC, Waters 600E) equipped with an Aminex HPX-87H (300 mm x 7.8 mm, Bio Rad, CA) column, coupled to a differential refractometer (RI Waters 410). Operating conditions were as follows: sample volume 40 μl; mobile phase 0.005 M H_2SO_4; flow rate 0.6 ml min^{-1}; column temperature 65 °C. Before injection samples were diluted to appropriate concentration with deionised water and filtered through a 0.2 μm membrane filter.

Free amino nitrogen (FAN) concentration in hydrolysis and fermentation samples was determined according to the ninhydrin colorimetric method [6] promulgated in the European Brewery Convention.

Glucoamylase activity was assayed by measuring the producing glucose from the hydrolysis of 20 g L^{-1} (db) pure starch in phosphate buffer (0.2 M) at pH 6.0 and 55 °C within 15 min. A 5% TCA (trichloroacetic acid) solution was used to stop enzymatic activity (ratio 1:1). One unit (U) of glucoamylase activity was defined as the amount of enzyme that releases 1 mg glucose in 1 min under the assay conditions. Protease activity was measured according to [7]. One unit (U) of proteolytic activity was defined as the amount of enzyme that releases 1 μg FAN in 1 min under the reaction conditions.

During yeast fermentations, biomass concentration (X, total dry weight, g/L) was determined by drying each sample at 105 °C until constant weight. Total cellular lipid (L, g/L) was extracted from the dried yeast cells with a mixture of chloroform/methanol 2:1 (v/v) and weighted after evaporation of the solvent in a rotary evaporator [8].

3 RESULTS-DISCUSSION

3.1 FBW Hydrolysis

The production of fermentation media from FBW was mainly focused on the evaluation of the efficiency of starch and protein hydrolysis. The main goal of FBW hydrolysis was the production of hydrolysates with high glucose concentration and adequate content of nitrogen sources (e.g. amino acids and peptides) that could be used as sufficient fermentation media for microbial oil production. Initial hydrolysis experiments focussed on the optimisation of process temperature, pH and initial FBW concentration. Various enzyme activities produced via SSF of *A. awamori* were used in order to maximise the hydrolysis of high FBW concentrations.

Figure 1 presents the production of glucose during hydrolysis of 205 g/L FBW concentrations using three different glucoamylase activities produced via SSF. The highest glucose concentration achieved in all cases was 156 g/L that corresponds to a starch to glucose conversion yield higher than 85 % (w/w). It is worthnoting that the same final hydrolysis yield was achieved in all cases, but increasing glucoamylase activities resulted in significantly improved reaction rate.

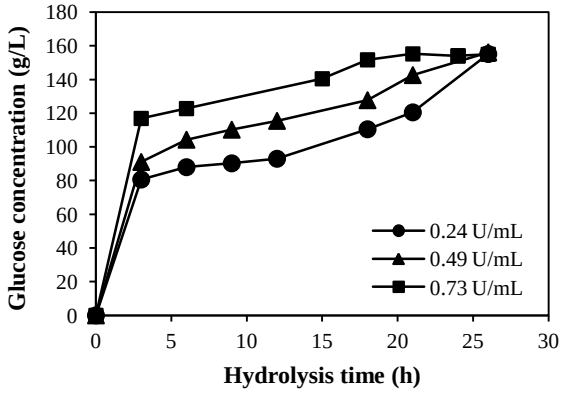

Figure 1: Glucose production during enzymatic hydrolysis using 205 g/L initial FBW concentration and three different initial enzyme activities

3.2 Shake flask fermentations

The produced FBW hydrolysates were evaluated as fermentation feedstocks for the production of microbial oil using the oleaginous yeasts *Rhodosporidium toruloides* DSM 4444 and *Lipomyces starkeyi* DSM 70296. Fermentations were also carried out with glucose-based synthetic (commercial) media in order to evaluate the efficiency of crude FBW hydrolysates. Fermentations were initially carried out using 50 g/L glucose and 160 g/L initial FAN concentrations (Table 1). Fermentation results showed that the use of FBW hydrolysates enhanced microbial oil production. More specifically, in the case of *R. toruloides*, the lipid accumulation achieved with synthetic media and FBW hydrolysates was 1.6 and 2.8 g/L, respectively. Likewise, lipid production by *L strakeyi* was increased to 5.9 g/L when FBW hydrolysate was used. The effect was more pronounced, when higher initial concentrations of glucose were used.

Yeast strain	Media	Time (h)	Biomass (g/L)	Lipids (g/L)	$Y_{L/X}$ (%,wt,wt)
R. toruloides	1	123	16.5	1.6	9.8
	2	123	19.5	2.8	14.3
L. starkeyi	1	164	18.1	3.9	21.7
	2	142	25.2	5.9	23.3

(1) Synthetic media; (2) hydrolysed FBW

Table 1: Fermentation results achieved during cultivation of *R. toruloides* and *L. starkeyi* using two fermentation media

Figure 2 presents fermentation results of *R. toruloides* cultivated on FBW hydrolysates with an initial glucose concentration of 95 g/L. The highest microbial oil concentration produced was 12.2 g/L with a respective microbial oil content of 49.8% (w/w).

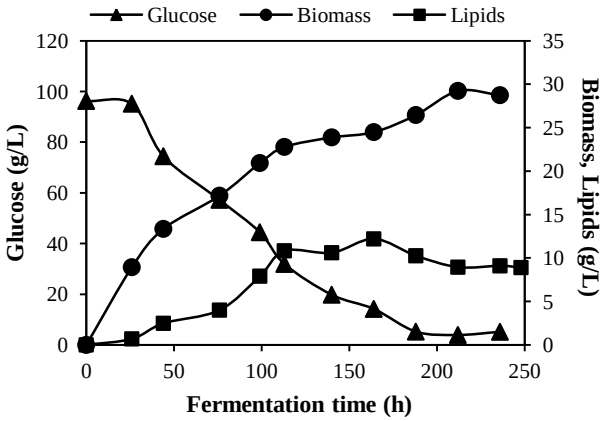

Figure 2: Production of total dry weight (biomass) and microbial oil (lipids) and glucose consumption during shake flask fermentation of *R. toruloides* on FBW hydrolysates

The results obtained in shake flask fermentations when FBW hydrolysate are used as fermentation feedstock are

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quite promising regarding microbial oil production [2,9]. Shake flask fermentations were also carried out with *L. starkeyi* and promising results regarding microbial oil production were also observed.

3.3 Bioreactor fermentations

Preliminary bioreactor fermentations were carried out in order to enhance yeast growth and microbial oil accumulation. Fermentations were carried out with FBW hydrolysates. Figure 3 presents the results from a batch fermentation carried out in a bioreactor where *R. toruloides* was cultivated on FBW hydrolysate with approximately 100 g/L initial glucose concentration. The FBW hydrolysate employed in the bioreactor fermentation was similar to the one used in the shake flask fermentation presented in Figure 2.

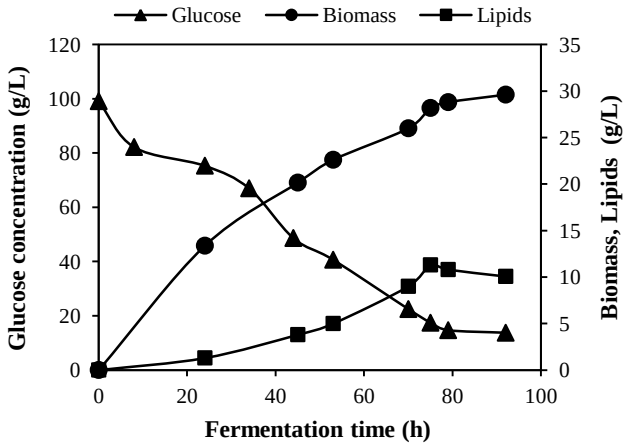

Figure 3: Production of total dry weight (biomass) and microbial oil (lipids) and glucose consumption during bioreactor fermentation of *R. toruloides* on FBW hydrolysates

Comparing the two fermentations carried out in shake flasks and the bioreactor, it can be observed that similar microbial oil accumulation was achieved in both cases. Nevertheless, the productivity of microbial oil achieved in the bioreactor was significantly higher than in the case of shake flask fermentation. It is noted that for various carbon and energy sources, values between 0.18 and 0.22 g of lipid synthesized per gram of carbon substrate consumed can be achieved by efficient oleaginous microorganisms [10, 11]. In the fermentations presented above a glucose to microbial oil conversion yield of approximately 0.13 g/g was achieved. It is expected that fermentation results will be improved in optimised fed-batch fermentations. For instance, preliminary fed-batch fermentations carried out with *R. toruloides* resulted in the production of significantly higher microbial oil concentrations and yields.

4 CONCLUSION

This study presents that confectionery industry waste streams could be used for efficient production of microbial oil. Crude hydrolysates produced from crude flour-based waste streams enhance microbial oil production as compared to synthetic (commercial) media. Further bioprocess optimisation will result in improved production of microbial oil.

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