Development of Cereal-Based Biorefineries for the Production of Biodegradable Plastics and Platform Chemicals

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Abstract

In the Satake Centre for Grain Process Engineering we are developing cereal-based biorefining strategies for the production of biofuels, biodegradable plastics and platform chemicals. Cereal grains are complex biological entities and we target the exploitation of all cereal components providing both value-added end-products and precursors for chemical synthesis. Hydrolysis of natural polymers (e.g. starch, protein) contained in cereals requires supply of a range of hydrolytic enzymes (e.g. amylase, protease), which are produced by fungal bioconversions. On-site production of these enzymes would result in the production of a high amount of fungal biomass. Fungal autolysis can be used to bioconvert this low-cost byproduct into a nutrient-rich supplement (fungal extract) for microbial bioconversions. Mixing fungal extracts with cereal hydrolysates results in nutrient-complete microbial feedstocks. Optimising the exploitation of protein in cereal grains would enable the provision of optimum amounts of free amino acids and peptides to subsequent microbial bioconversions and the extraction of the remaining protein as a value-added co-product with various current (food) and potential (biodegradable plastics) market outlets. The use of amino acids and peptides would enhance productivities (e.g. polyhydroxybutyrate, succinic acid) and, in certain cases, production yields (e.g. polyhydroxybutyrate) improving significantly current fermentation practices that exploit only the starch component in cereal grains. In addition, the exploitation of all cereal components and low-cost by-product streams produced in a cereal-based biorefinery will result in waste minimisation and maximisation of carbon as well as other nutrient utilisation from the original cereal grain.

This work will present different feedstock formulation strategies based on the production of wheat hydrolysates and fungal extracts for the microbial production of polyhydroxybutyrate and succinic acid.

1. Introduction

Succinic acid is a dicarboxylic acid produced as an intermediate of the tricarboxylic acid cycle. It has unique properties mainly because of the reactivity of the methylene (CH_2) groups. Succinic acid is currently produced predominantly from petrochemical precursors through reduction of maleic anhydride. The application of succinic acid and its derivatives include surfactants, detergents, electroplating, food, pharmaceutical, antibiotics, amino acids and vitamins. The production of succinic acid from renewable resources would be expected to lower the overall production costs from \$2.20 per kg to \$0.55 per kg. The microorganism *Actinobacillus succinogenes 130Z* was selected because it is a facultative anaerobe, osmophile and can produce high amounts of succinic acid from glucose. In addition, succinic acid production by this microorganism requires CO_2 fixation creating a novel CO_2 sequestration process.

Polyhydroxybutyrate (PHB) belongs to the family of polyhydroxyalkanoates (PHAs). It is accumulated in various bacterial cells as a carbon and energy storage compound. More than 300 microorganisms are known to accumulate PHB under nutrient-limiting conditions in the presence of excess carbon source. Several efforts have been devoted on the industrial implementation of PHB production, but the high production cost has hindered successful operation. Selecting a cheap raw material is an important parameter in improving PHB production economics. Various carbon sources have been used in bacterial PHB fermentations, including glucose, fructose, lactic acid, sucrose and methanol (Choi and Lee, 1997).

A novel cost-competitive wheat-based biorefining strategy (Figure 1) for the production of nutrient-complete feedstocks for microbial fermentations has been proposed (Koutinas et al., 2004; Webb et al., 2004). This upstream processing strategy exploits the hydrolytic capability of fungi and the natural autolysis of fungi to produce two liquid streams, one rich in glucose (wheat hydrolysates) and one rich in supplementing nutrients (fungal autolysate). This study presents preliminary results on the utilisation of various microbial feedstocks produced from wheat via the proposed biorefinery for the production of PHB and succinic acid.

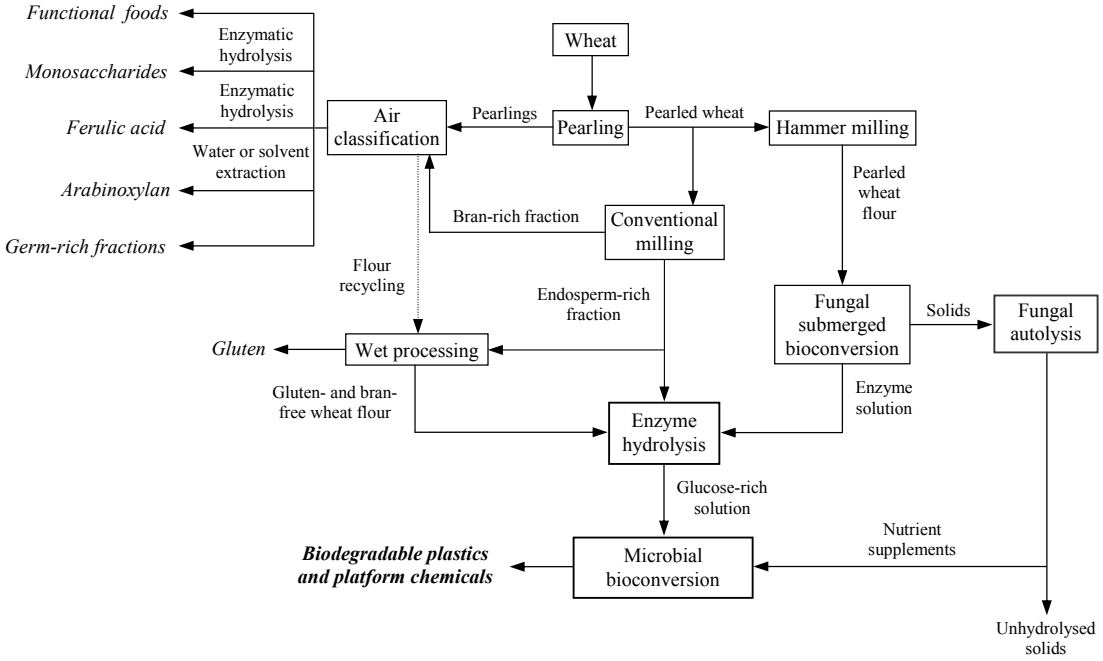

Figure 1 Proposed wheat-based biorefinery for the production of PHB and succinic acid

2. Materials and Methods

2.1 Microorganisms and growth conditions

An industrial strain of *Aspergillus awamori* 2B.361 U2/1 was used in all fungal fermentations for the production of enzyme consortia. Detailed description of fungal fermentations were presented in previous publications (Koutinas *et al*., 2001; Koutinas et al., 2004; Koutinas et al., In press1).

A. succinogenes (ATCC 55618) was obtained from the American Type Culture Collection. The seeds were grown on trypicase soya agar (TSA) plates at 30°C for 48 hours and then transferred to 200 ml Duran bottles containing 50 ml medium, and incubated at 30°C and 120 rpm. Batch fermentations were carried out in a 2 L bench-top bioreactor with 0.8 L working volume, at 37°C, pH 6.6-6.8. About 200 rpm agitation was applied and the CO_2 flow rate was 0.4 l/min. The semi-defined culture medium contained (Guettler *et al*., 1996) (l^{-1}): glucose 10 g; Bacto yeast extract, 5 g; $NaH_2PO_4.H_2O$, 1.16 g; Na_2HPO_4, 0.31 g; NaCl, 1.0 g; $MgCl_2.6H_2O$, 0.2 g; $CaCl_2.2H_2O$, 0.2 g; B_{12}, 10 µg; biotin, folic acid, 200 µg; thiamine, riboflavin, niacin, pantothenate, p-aminobenzoate, and lipoic acid, 500 µg; B_6, 1 µg. In this study, fungal autolysate or flour hydrolysate was used instead of corn steep liquor. For fermentations with semi-defined medium, culture medium was autoclaved for 20 mins at 121°C.

Wautersia eutropha NCIMB 11599 (formerly known as *Ralstonia eutropha* and recently designated as *Cupriavidus necator*) was utilised for the production of PHB. *W. eutropha* was subcultured monthly in liquid medium from the cryopreservation vials. After cultivated for 24 hours at 30ºC on a 200 rpm shaker, two drops of the mature broth were spread onto solid medium of 5 g/l, 10 g/l yeast extract and 20 g/l technical agar. After incubating at 30ºC for 36 hours, the Petri dishes were thereafter stored at 4 ºC. One Petri dish was used as inoculum for each fermentation. Cells were transferred with a wire loop in 50 ml growth medium containing 5 g/l glucose and 10 g/l yeast extract and cultivated for 24 h at 30ºC. The inoculum was then transferred into a 1.5-l fermenter (Electrolab 351 Series) containing 700 ml wheat-derived medium.

The wheat-derived feedstock used in bacterial fermentations was a mixture of wheat flour hydrolysates (FH) and fungal autolysates (FA). Detailed description for the production of such fermentation media were described in previous publications (Koutinas *et al*., 2004; Webb et al., 2004; Koutinas et al., in press1; Koutinas et al., in press2). Both FH and FA were filter sterilised using a 0.2 µm filter (Whatman, POLYCAP 36 AS).

2.2 Analytical Methods

Samples (0.5 - 3 mL) from bacterial fermentations were taken at varied reaction intervals to measure total dry weight, organic acids, PHB, glucose and FAN concentration. Each sample was centrifuged at 3,000 × g for 10 min. Solids from PHB fermentation were washed with distilled water and centrifuged two consecutive times. Then, they were re-suspended in acetone and transferred into universal bottles. Dry weight measurements were carried out by drying the solids at 50ºC and cooling in a desiccator to constant weight. Cell growth of *A. succinogenes* was monitored by measuring the absorbance at 660 nm (OD_{660}) using a spectrophotometer (Cecil CE1020 1000 Series).

PHB was measured by gas chromatography by the protocol proposed by Riis and Mai (1988). A gas chromatographic analyser (Hewlett Packand 5890, series II) with autosampler 7673 was used. The software was Chemistation Version 6.03. The column was poraplot Q-HT 10 m × 0.32 mm. Carrier gas was helium. Injection temperature was 230°C, detection temperature was 200°C and initial temperature was 120°C. Flame ionisation detector (FID) was used for determination.

Glucose concentration was analysed by a GL6 analyser (Analox Instruments, UK). FAN concentration in liquid samples was analysed by the ninhydrin colorimetric method (Lie, 1973). Concentration of fermentation products in succinic acid fermentations were determined by high-performance liquid chromatography (Star Varian Chromatography

Workstation, Prostar 330 PDA UV detector). A monodisperse, sulfonated styrene/divinylbenzene copolymer analytical column (Polymer Laboratory Hi-Plex H 8 µm 300 X 7.7 mm, USA) preceded by a guard column (Polymer Laboratory Hi-Plex H guard column 50 X 7.7 mm, USA). The analysis was carried out under following conditions: sample volume 20 µl; flow phase 0.1% trifluoroacetic acid (TFA); flow rate 0.6 ml min^{-1}; column temperature 60^{o}C.

3. Results and Discussion

3.1 PHB production

Many researchers have studied the effect of nitrogen source on bacterial growth and PHB production. Optimum supply of nitrogen during the growth phase will maximise accumulation of bacterial biomass, which will subsequently lead to higher accumulation of PHB (Hahn et al., 1995).

Four batch fermentations were conducted to study cell growth and PHB production in the medium produced by FH and FA at varying glucose (11, 19, 34, 66) and FAN (80, 107, 190, 560) concentrations. Most of FAN was consumed in the growth phase, while significant glucose consumption started after the initiation of PHB accumulation. Figure 2 presents nutrient consumption, cell growth and PHB production in batch fermentation containing 66 g/l glucose and 560 g/l FAN concentration at the beginning of the fermentation. In the first 20 hours, FAN consumption was contributed to cell growth. Glucose concentration started to decrease when the FAN concentration reached an approximately constant value. At this point, PHB concentration started to increase and continued until complete glucose consumption. A total dry cell weight of 42 g/l and PHB concentration of 23 g/l were achieved at the end of the fermentation.

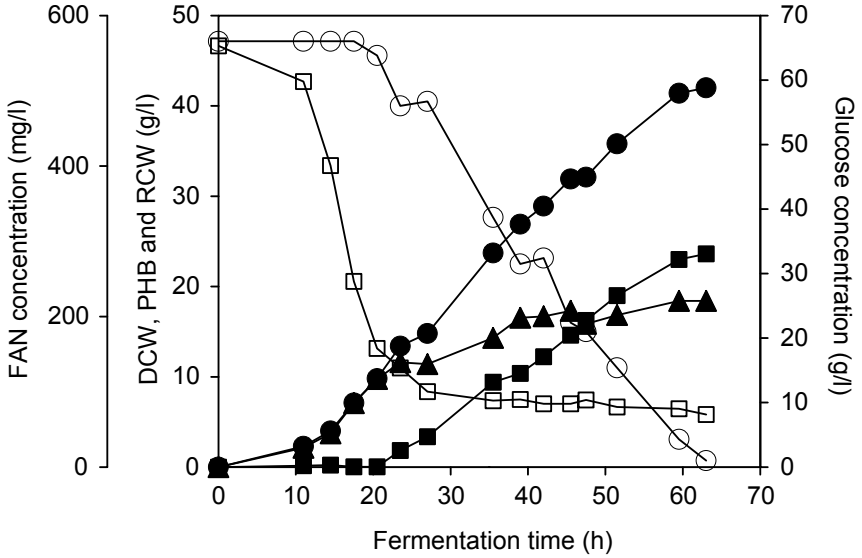

Figure 2 Time profiles of dry cell weight (DCW), PHB, recidual cell weight (RCW), glucose and FAN during batch fermentation of *W. eutropha* on wheat-derived feedstock. (●), DCW; (■), PHB; (▲), RCW; (○), Glucose; (□), FAN.

Batch experiments indicated that the higher the initial FAN concentration in the feedstock the higher recidual cell weight (non-PHB biomass) will be produced. This will eventually lead to increased PHB concentration at the end of the fermentation. To test this observation we carried out several fed-batch experiments with gradual increase in FAN concentration. When the glucose concentration was reduced to values lower than 20 g/l in the bioreactor, feeding of a glucose-rich solution (500 g/l) was initiated in order to increase PHB accumulation by *W. eutropha*. Figure 3 presents nutrient consumption, bacterial growth and PHB accumulation in a fed-batch fermentation containing initial glucose and FAN concentrations of 95 g/l and 960 mg/l, respectively.

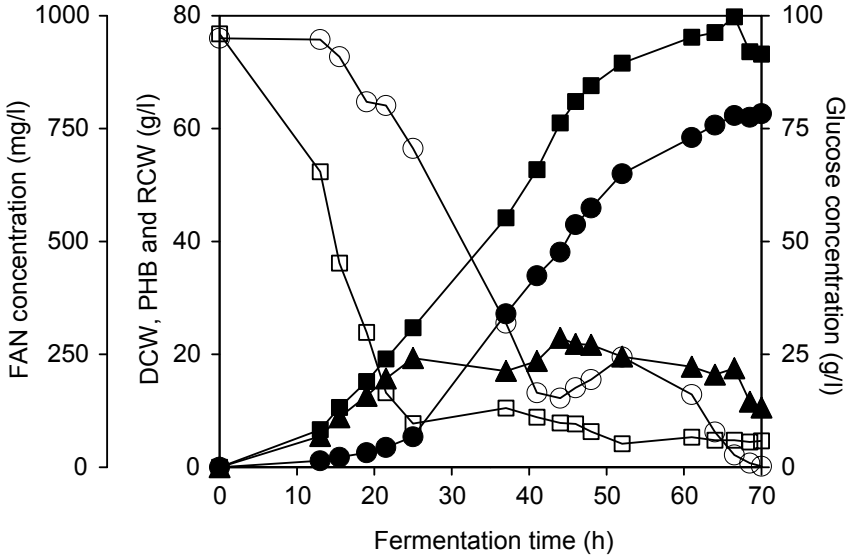

Figure 3 Time profiles of dry cell weight (DCW), PHB, recidual cell weight (RCW), glucose and FAN during fed-batch fermentation of *W. eutropha* on wheat-derived feedstock. (●), DCW; (■), PHB; (▲), RCW; (○), Glucose; (□), FAN.

PHB concentration reached 62.4 g/l at the end of fermentation with a content of 78%. PHB concentration remained the same in the last four hours of the fermentation due to depletion of glucose. During this period, DCW weight was gradually reduced. This should be attributed to cell autolysis due to nutrient depletion. PHB was accumulated until the exhaustion of glucose. These results indicate that higher FAN concentration would increase microbial growth leading to enhanced PHB production.

Future research will concentrate on maximising PHB accumulation by *W. eutopha* fermentation on wheat-derived feedstocks. Initial FAN concentration will be increased further in order to produce even higher residual cell weight that will lead to higher PHB production. Continuous two-stage fermentations will also be developed.

3.2 Succinic acid production

3.2.1 Succinic acid production from wheat flour hydrolysates

Preliminary experiments for the production of succinic acid by *A. succinogenes* focused on proving whether the wheat-derived feedstock could support microbial growth and induce succinic acid formation. Figure 4 and 5 present fermentation results when a complex medium containing wheat flour hydrolysate and a semi-defined medium containing commercial glucose were used, respectively. The defined medium was formulated according to Guettler et al. (1996). It was obvious that succinic acid could be successfully produced without any inhibition of uncertain compounds in the flour hydrolysate. These two experiments resulted in the same yield of 0.65. Moreover, the one using hydrolysate resulted in higher productivity.

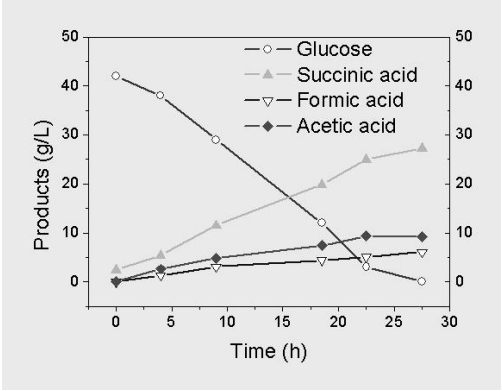

Figure 4 Profile of glucose and end product concentrations of succinic acid fermentation using flour hydrolysate as major carbon source.

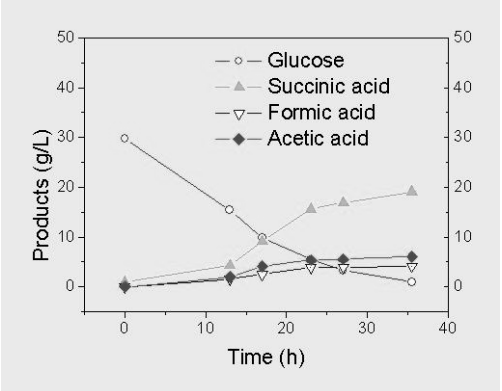

Figure 5 Profile of glucose and end product concentrations of succinic acid fermentation on semi-defined medium.

The main by-products of succinic acid fermentation with flour hydrolysate are formic acid and acetic acid, which is the same as the fermentation with the semi-defined medium. The yields of formic acid and acetic acid of these two fermentations are almost identical. This indicates that there is no significant difference in *A. succinogenes* metabolism when those two media were used.

3.2.2 Succinic acid production from wheat flour hydrolysate and fungal autolysate

Fungal autolysate is a nutrient-rich supplement that can be used as a substitute for yeast extract. It can be produced on-site via fungal autolysis. Fungal autolysates may contain up to 1.6 g/L FAN, 5.3 g/L TKN, and 0.5 g/L phosphorus (Koutinas *et al.*, 2005). Fermentations with fungal autolysate were carried out in order to replace the nutrients of semi-defined medium, such as yeast extract, in the succinic acid fermentation (Figure 6). *A. succinogenes* grew well in the wheat-derived complex medium and around 17.1 g/L succinic acid was produced in 66 hours. This experiment demonstrated that the wheat-derived

feedstock contain essential nutrients for *A. succinogenes* growth and succinic acid production.

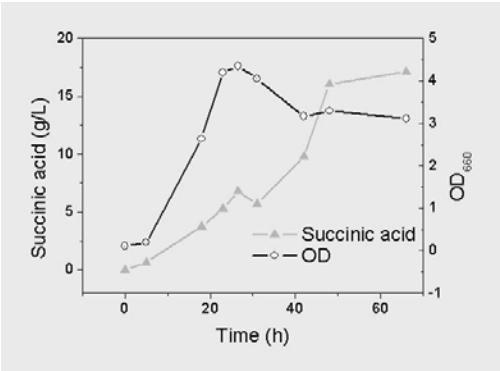

Figure 6 Profiles of succinic acid and optical density of succinic acid fermentation carried out with wheat flour hydrolysate and fungal autolysate.

Future research will concentrate on maximising the production of succinic acid by optimising feedstock composition and selecting the appropriate bioreactor design. Succinic acid will be purified and subsequently used for the production of various chemicals via green processing developed at the Green Chemistry Centre of Excellence (Department of Chemistry, York, UK).

A case-specific biorefining concept will be developed for both succinic acid and PHB. Feedstock composition will be optimised and the process presented in Figure 1 will be optimised so as to produce tailor-made fermentation media for each product. In addition, wheat components that are not necessary for the production of succinic acid and PHB, such as pearlings and excess gluten, will be used as co-products and potential end-uses will be identified.

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