Process Biochemistry 44 (2009) 546–555

Contents lists available at ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

Particulate bioprocessing: A novel process strategy for biorefineries

Carolina Botella [a], Ana B. Diaz [b], Ruohang Wang [a], Apostolis Koutinas [a], Colin Webb [a,*]

[a] Satake Centre for Grain Process Engineering, School of Chemical Engineering and Analytical Science, University of Manchester, PO Box 88, Manchester M60 1QD, UK
[b] Department of Chemical Engineering, Food Technology and Environmental Technologies, Faculty of Sciences, University of Cadiz, 11510 Puerto Real, Cadiz, Spain

ARTICLE INFO

Article history:
Received 14 April 2008
Received in revised form 22 January 2009
Accepted 26 January 2009

Keywords:
Particulate bioprocessing
Solid-state fermentation
Wheat-based biorefinery
Bioethanol
Polyhydroxybutyrate (PHB)

ABSTRACT

A novel process strategy based on particulate bioprocessing has been developed for the production of value-added chemicals and biofuels. The process, which involves two main steps, fungal fermentation and discontinuous extraction, leads to the production of generic fermentation feedstocks from cereals. Partially pearled whole wheat grains were used as substrate for the growth of *Aspergillus awamori* in a packed bed bioreactor. Water was trickled through the bed of particles intermittently every 6 h to extract glucose and other nutrients and to maintain moisture and temperature levels. The feedstocks obtained through this system have been used for subsequent fermentations by *Wautersia eutropha* to produce the biodegradable plastic PHB (polyhydroxybutyrate) and by *Saccharomyces cerevisiae* for ethanol production. These preliminary results demonstrate the potential suitability of the novel concept of particulate bioprocessing in the development of biorefineries.

1. Introduction

There is an increasing social and political demand to establish renewable feedstocks for chemical and fuel production that can decrease our dependency on dwindling oil reserves, and reduce greenhouse gas emissions. We depend on petroleum for 90% of our transport, for food and for pharmaceuticals [1], but this most widely used of raw materials is neither sustainable nor environmentally friendly. Global oil reserves were estimated at the end of 2001 to be sufficient for 40.3 years [2]. At the same time, under the Kyoto protocol, industrialised countries must reduce total greenhouse gas emissions by an average 5.2% (by 2008) compared with 1990 levels [3]. The EU commission has invited its members to move forward with a 20% reduction by 2020, suggesting a 20% substitution of traditional road transport fuels by alternatives [4]. So with crude oil prices reaching historically high levels and the need to diversify energy sources, governments and industries are inevitably turning to renewable resources as replacement raw materials.

While renewable energy can come from a variety of sources, such as wind, sun, water and biomass, the production of material substances is fundamentally dependent on biomass, in particularly on plant biomass. Nature produces 200 billion tonnes of biomass per year through photosynthesis and only 3–4% is currently used by man for food or non-food purposes [5]. The most intensive crop plants are those based on starch and, of these, cereals are the most important. The world production of wheat is 617 millions tonnes per year [6] and a large amount is wasted through, for example, poor post-harvest techniques and stockpiling. Moreover grain processing for human consumption leaves approximately 15% of the grain as waste product. So the use of waste cereals and cereal byproducts could play an important role in providing sustainable feedstocks for the production of chemicals and materials.

The range of products from cereals as renewable feedstocks could be as diverse as that from petroleum, i.e. solvents, plastics, fuels, bulk chemicals, fibres, fine chemicals and oils, and hence the concept of a biorefinery is a meaningful one [7]. With the move to renewables, which will necessarily be biological material, will come a move to alternative processing routes, many of which will also be biologically based. In these cases the term biorefinery is doubly relevant, on the one hand because of the biological nature of the raw material and on the other hand because of the increasing biological character of selected treatment and processing methods [8].

Novel bioprocesses based on renewable feedstocks that utilise resources more efficiently, and at the same time produce less waste, can make a significant contribution to sustainable development. However, it is not enough just to develop more environmentally friendly processes; sustainability also requires economic and social justification [9]. Current conventional biotechnological processes, which are more likely to be used in biorefinery systems, generally require the use of relatively expensive raw materials, and large amounts of water and are therefore often uncompetitive. Industrial practices that use cereals

* Corresponding author. Tel.: +44 161 306 4379; fax: +44 161 306 4399.
E-mail address: colin.webb@manchester.ac.uk (C. Webb).

1359-5113/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.procbio.2009.01.012

as a raw material utilise commercial enzymes (glucoamylase and α-amylase) and separate liquefaction and saccharification stages, increasing production costs [10,11]. In the Satake Centre for Grain Process Engineering (SCGPE) we have been working on the development of a novel biorefinery process that leads to the production of generic fermentation feedstocks from cereals, making the processes and the products more competitive with petrochemical products.

The first process for the production of a generic feedstock from wheat was presented in 1996 [12]. The most important unit operations involved in the production are: milling, fermentation, glucose enhancement, and nitrogen enhancement. The process involves continuous submerged fermentation (SmF) of wheat flour with *Aspergillus awamori* for *in-situ* enzyme production and the generation of two liquid streams, one of which is rich in glucose while the other is rich in nitrogen. The generic feedstocks produced by the combination of the two liquid streams have been utilised successfully for the production of ethanol, lactic acid, monascus pigments, enzymes, glycerol, succinic acid and polyhydroxybutyrate (PHB), [13–15]. Theoretical work carried out by Koutinas et al. [16] showed that, if a two-fold increase in world wheat production could be achieved, this would be sufficient to enable the production of the majority of current petrochemical products or their equivalents, via bioprocessing, without any reduction in the food outlets of the wheat. A cost estimation was also carried out, concluding that the operating cost for an industrial plant producing approximately 200 m^3 per day of the generic feedstock was approximately US$ 0.51 kg^{-1} glucose in the feedstock [16]. At this level prices compare favourably with conventional feedstocks and there is no need for additional nutrient materials, such as would be the case with glucose syrups, molasses etc. These characteristics make wheat and the process proposed a feasible solution towards the commercialisation of efficient and cost-competitive renewable feedstocks for chemical production.

1.1. Particulate bioprocessing

In order to overcome some of the challenges of producing the generic fermentation feedstock, we are now developing a novel process strategy for biorefineries (particulate bioprocessing), based on solid-state fermentation (SSF). This novel process addresses two of the current problem areas of conventional bioprocessing. First, in conventional processing milling of the cereal grains is required and this is an energy intense pre-treatment, requiring around 36 kJ kg^{-1} wheat [17]. The use of pearling instead of milling could in principle reduce this by up to 60%. Pearling is a process in which the bran layers of the wheat are removed sequentially by friction and abrasion and is operated in a modified rice polisher or debranner [18]. Second, bioprocessing involves usually submerged fermentation, which requires large amounts of water, in this case 12.5 kg of water per kg of wheat (for an 8% (w/v) flour suspension). The process strategy proposed in this paper for the production of feedstocks based on SSF could reduce water requirement by more than 90% (using whole wheat grains at 0.8 kg water per kg grain solid moisture content). The use of whole grains reduces viscosity problems associated with the use of flour suspensions which is one of the limitations of SmF.

Solid-state fermentation is defined as the cultivation of microorganisms on moist solid materials, either on inert carriers or on insoluble substrates that can, in addition, be used as carbon and energy source [19]. According to Moo-Young et al. [20] the term solid-state fermentation should be used specifically for those situations in which the spaces between the solid materials are occupied by a continuous gas phase.

The moist solid material used as substrate can either be a continuous or a discrete phase, dividing SSF systems into two subsets (Fig. 1). When the solid is a continuous phase, the microorganism grows in a large surface area such as agar plates or solid cakes and in this case can be classified as Solid Surface Culture. The second subset of SSF involves a discrete solid phase in which the microorganisms grow at the surface of moist discrete particles, inside them and between the particles. The term chosen to describe this kind of SSF system, which involves the use of particles is particulate bioprocessing, a new and more specific term that has been defined and introduced to stress the particulate nature of the substrate.

Nowadays, most of the commercially important solid-state fermentations involve substrates in a solid particulate state. So in this case we can refer to them as particulate bioprocessing systems. The particulate nature, which involves high surface area to volume ratio and the absence of free water, makes these systems quite different to traditional submerged fermentation and confers on particulate bioprocessing many potential benefits such as waste minimisation and process intensification. Particulate bioprocessing is close to the natural environment to which many microorganisms are adapted, allowing higher growth, morphological diversity and differentiation [21]. In particulate bioprocessing systems high substrate concentration due to the absence of free

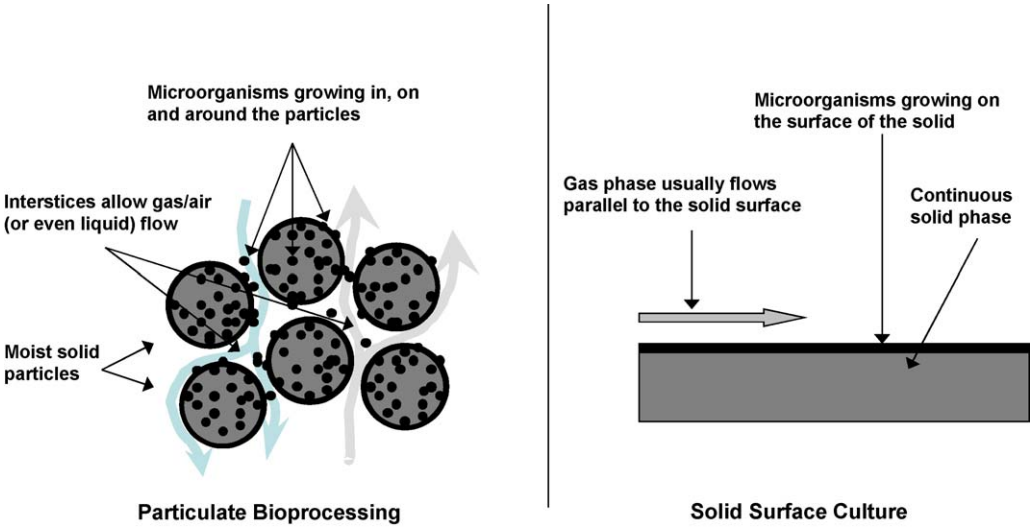

Fig. 1. Illustration of the two subsets of solid-state fermentation systems according to the new definition introduced in the text.

water can enable high biomass and product concentration. This permits the use of smaller reactors and simpler product recovery. Particulate bioprocessing is recognised to be a good system for enzyme production using various raw materials [22–25]. It has been demonstrated by Viniegra-González et al. [26] that the advantages of particulate bioprocessing in enzyme production are due to: high productivity (because of the increased biomass), low proteolysis and large surface area for gas exchange. Another advantage associated with the lack of free water is that the oxygen is taken by the microorganisms more directly from the air by diffusion so the aeration requires less power since pressures are lower [27]. There is therefore less energy requirement, absence of foam build-up and the risk of contamination is smaller since most microorganisms need high water activity to grow. Such advantages would suggest that there is considerable potential for particulate bioprocessing in the development of biorefinery processes.

There are also, of course, disadvantages associated with solid-state fermentation. These include poor mixing, heterogeneity, desiccation, poor heat transfer characteristics and growth rates limited by nutrient diffusion [28,29]. Having the solid in the form of discrete particles (particulate bioprocessing) can help to alleviate some of these problems particularly if an intermittent addition of water is incorporated into the operation of the fermentation.

This paper describes results obtained in the development of a process based on particulate bioprocessing, presenting some experimental results from the major unit operations within the process. This novel process strategy for biorefineries can be summarised using the flowsheet in Fig. 2. The original process to produce the generic fermentation medium based on cereals [12] involved many process steps. Particulate bioprocessing offers the possibility to reduce the complexity of the overall process by combining some of the steps. The proposed process involves two main fermentation steps. In the first a packed bed of partially pearled whole wheat grains is used as a substrate for the fungus *A. awamori* which produces amylolytic enzymes to degrade the starch into glucose. The fungi also produce proteases that convert wheat proteins into free amino nitrogen (FAN). Intermittent trickling of water through the reactor during cultivation helps to maintain moisture and temperature levels, and is also used to extract soluble components.

The second fermentation step is carried out using the extracts obtained from the first step. These have high glucose and free amino nitrogen concentrations and can be used, for example, by *Wautersia eutropha* to produce the biodegradable plastic PHB or by *Sacharomyces cerevisiae* for ethanol production. These two products were chosen to test the fermentation feedstocks because of their environmental benefits over petroleum-based plastics and over transport fossil fuels respectively [30,31].

2. Material and methods

2.1. Microorganisms

Fungal fermentations were carried out using a strain of *A. awamori* 2B. 361 U2/1. Prior to experimental work, *A. awamori* spores were purified and incubated in flasks containing solid medium of 5% (w/w) whole wheat flour and 2% (w/w) agar on a dry basis (db). The resultant spores were suspended in 10% (v/v) glycerol solution and stored in 2 ml cryo-vials at −30 °C.

Bacterial fermentations were carried out using the strain *W. eutropha* NCIMB 11599. A lyophilized culture was reactivated and incubated at 30 °C for 24 h in a growth medium that contained MRS broth (Oxoid) and glucose (0.5%). Bacterial cultures were stored at 4 °C on slopes containing 5 g L^{-1} glucose, 10 g L^{-1} yeast extract and 20 g L^{-1} agar. Fermentation inocula were prepared by transferring cells using a sterile wire loop into liquid medium containing 5 g L^{-1} glucose and 10 g L^{-1} yeast extract.

Yeast fermentations were carried out using *S. cerevisiae* ATCC 22602 for the production of bioethanol. Yeast cells were cultivated in standard MYGP medium (3 g L^{-1} maltose extract, 3 g L^{-1} yeast extract, 50 g L^{-1} glucose, and 5 g L^{-1} peptone) for inoculum preparation.

2.2. Preparation of fermentation media

A soft wheat variety, *Consort* (harvested in 2003 and supplied by Fisher Seed and Grain Limited, Cranswick, UK) was the principal raw material used in this study. Wheat kernels were pearled for 5 s using a Satake Abrasive Test Mill (model TM05). To adjust the moisture content to the desired level (0.8–0.9 g water g^{-1} grain solid), pearled grains were soaked in an excess of water for 20 h. After vacuum filtration to eliminate the free water, the conditioned grains were sterilised for 3 h at 120 °C in the autoclave.

The feedstock used for the bacterial and yeast fermentations was obtained by intermittent extraction with water during the fungal fermentation. A combination of extracts with a glucose concentration of 19.3 g L^{-1} and FAN of 0.036 g L^{-1} were used to grow *W. eutropha*. Three different media were prepared to study different conditions, the pH of the extracts (C, D and E) was adjusted from pH 2.9 to the optimum (6.8) for *W. eutropha* growth using 10 M NaOH. Yeast extract (7 mg) was added to medium E in order to increase FAN content up to 0.061 g L^{-1}. Media C and E were autoclaved at 120 °C for 20 min, while media D was filter sterilised using a 0.2 μm filter unit (Polycap 36 AS, Fisher).

For ethanol production the feedstock used was different from that used for *W. eutropha*. This feedstock contained significantly higher glucose and FAN concentrations (142.1 g glucose L^{-1}, 0.33 g FAN L^{-1}) due to the inclusion of agitation during extraction. The extracts were filtered before being inoculated with *S. cerevisiae*.

2.3. Experimental set-up

The production of the generic feedstocks was carried out in a 1.5 L aerated, packed bed reactor with a working volume of 1 L. The novelty of the system is the incorporation of a spray nozzle for trickling water during the fermentation. After sterilisation, 800 g of wheat grains with moisture content 0.87 g water g^{-1} grain solid were inoculated with 6 ml of spore suspension to reach a concentration of 1×10^6 spores g^{-1} of grain solids. After the inoculation, the grains were transferred to a previously sterilised reactor (3 h at 120 °C) and the system was assembled as shown in Fig. 3.

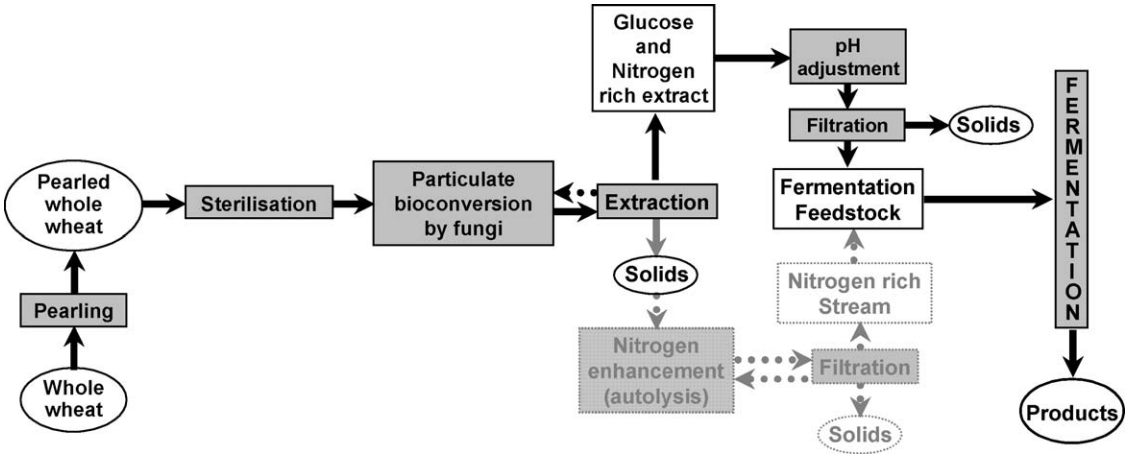

Fig. 2. Proposed process for the production of a generic fermentation feedstock by particulate bioprocessing. The dotted lines indicate optional steps in the process.

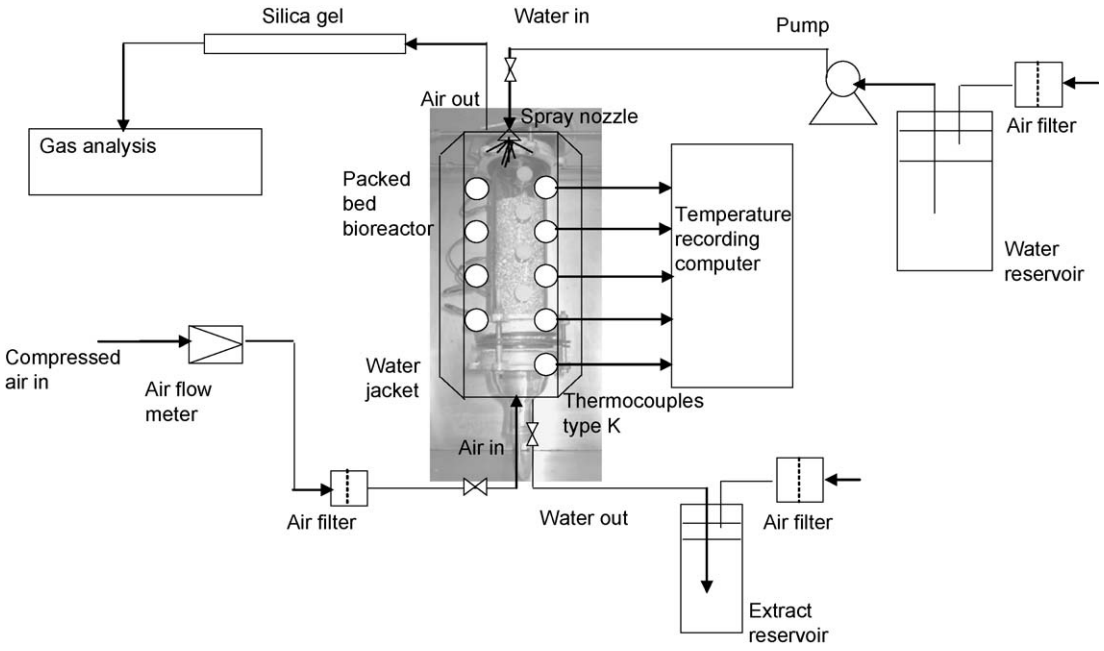

Fig. 3. System for particulate bioprocessing with intermittent addition of water.

Compressed air entered the system at 1 L min^{-1} and was sterilised using an air filter. The air was fed to the bioreactor through an air distributor. The bioreactor consisted of a sterilisable glass cylinder of 8 cm diameter and 30 cm height, closed at both ends with stainless-steel plates. The bottom plate had two ports for air inlet and collection of extracts and the top plate had ports for the exhaust gas and water inlet. Five temperature probes (thermocouples type K) and four sampling points were located through the glass cylinder. The reactor was covered by a silicon tube wrapped around the glass cylinder to act as a water jacket. Water was circulated through the jacket from a water bath controlled at 30 °C and the temperature at the thermocouples was recorded by a Microlab II monitor (Aglicon, UK). To reduce the humidity and impurities of exhaust gases leaving the reactor, the outlet stream was passed through a filter and a tube containing silica gel. A reservoir of sterile water was connected to the spray nozzle through a pump set to distribute water at 180 ml min^{-1}. This flow rate was the minimum required to give good distribution of water using the spray nozzle. The pump was activated every 6 h for 30 s, providing 90–100 ml of water for each extraction.

In order to improve and to study some of the conditions of extraction, several batch extraction experiments were carried out using the solid left at the end of the fungal fermentation. 10 g of the fermented solid was mixed with 10 ml of water in Erlenmeyer flasks and then agitated on a rotary shaker. Different variables concerning the extraction were studied: extraction temperature, duration of extraction, stirring rate, recirculation and number of extraction steps. Every variable was studied separately, maintaining the rest at the standard values: 10 ml of distilled water, 15 min of shaking, 30 °C and 200 rpm. The suspension resulting after every extraction was centrifuged (4000 rpm, 15 min) and the supernatant stored at −20 °C until required for the analysis. Each of the results presented was generated from duplicates.

The temperatures studied were around the optimum fermentation temperature 20, 25, 30 and 35 °C. The stirring rates evaluated were 0, 150, 200 and 250 rpm. The kinetics of extraction were also studied, checking the concentration of products extracted after 0, 0.5, 1, 2, 3, 4, 5, 10, 15 and 20 min of extraction. In order to increase the concentration of glucose and to decrease the amount of water used, an experiment was carried out where the water obtained after the first extraction was recirculated several times using fresh solid. The extract was only recirculated four times because the volume of liquid decreased in each extraction and it was almost exhausted after the 4th cycle. Finally to check if there were still nutrients left in the solid after a single extraction and to calculate the total amount of nutrients and enzyme present in the solid, consecutive extractions were carried out using the initial fermented solid and renewing the solvent. After each extraction step, the contents of the flask were centrifuged to separate the solid from the liquid. Then, a further 10 ml of distilled water were added to the solid for the next extraction. This procedure was repeated eleven times, obtaining eleven extracts.

The evaluation of the feedstock for PHB production was carried out using the three different media described earlier. Bacterial fermentations in 500 ml Erlenmeyer flasks were carried out on a 250 rpm rotary shaker (Infors AG, CH-4103 Bottmingen) at 30 °C. The same volume (50 ml) of fermentation media and the same initial inoculum (1 ml) were used in each flask.

For ethanol production 100 ml of a mixture of the extracts was inoculated with 2 ml of a suspension of *S. cerevisiae* giving a concentration of around 4.5×10^{06} cell ml^{-1}. Yeast fermentations were performed under static and anaerobic conditions at 30 °C in 250 ml Duran flasks fitted with "swan-neck" caps.

2.4. Analytical methods

The different extracts obtained during particulate bioprocessing, after being centrifuged at 4000 rpm for 15 min, were analysed in terms of glucose, FAN and glucoamylase activity. Glucose concentration was analysed using an Analox GL6 glucose analyser. FAN concentration was analysed by the ninhydrin colorimetric method [32]. Glucoamylase activity was assayed using previously gelatinised (>75 °C, 15 min) whole wheat flour suspension 6% (w/v, db) as a substrate. To 2 ml of wheat flour suspension, 2 ml of appropriately diluted enzyme was added. The resulting solution was incubated at 60 °C for 10 min. At time 0, 5 and 10 min, 0.5 ml of the solution was transferred to a micro centrifuge tube containing 0.5 ml of frozen Tricloroacetic acid (TCA) (5%, v/v) to stop the reaction. The glucose concentration was then determined by the Analox GL6 glucose analyser. One enzyme activity unit was defined as the amount of enzyme that releases 1 μmol of glucose per minute under the assay conditions.

Individual wheat kernels were taken during fungal fermentation and observed under an Environmental Scanning Electron Microscope (ESEM Quanta 200 Scanning Electron microscope). Photomicrographs with magnifications from 50 to 12,000 were taken.

During PHB production experiments, samples of 1 ml were taken from the bacterial fermentations at varied reaction intervals to measure total dry weight, glucose, FAN concentrations and PHB. Each sample was centrifuged at 13,000 rpm for 10 min and solids were washed with distilled water and centrifuged twice consecutively. The solids were re-suspended in acetone and transferred into universal bottles. Dry weight measurements were carried out by drying the solids at 50 °C and cooling in a desiccator to constant weight.

PHB was measured by gas chromatography using the protocol proposed by Riis and Mai [33]. A GC analyser (Hewlett Packard 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 and 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.

During the yeast fermentation, 1 ml samples were taken at different intervals and ethanol concentration was measured using the same GC, column and carrier gas as for PHB. The samples were also used for glucose analysis and cell counting using an Improved Neubauer Counting Chamber (Assistant-Germany; BDH).

3. Results and discussion

3.1. Production of the feedstock by particulate bioprocessing

Preliminary experiments to study the effect of raw material conditioning revealed that 5 s pearling using the Satake debranner

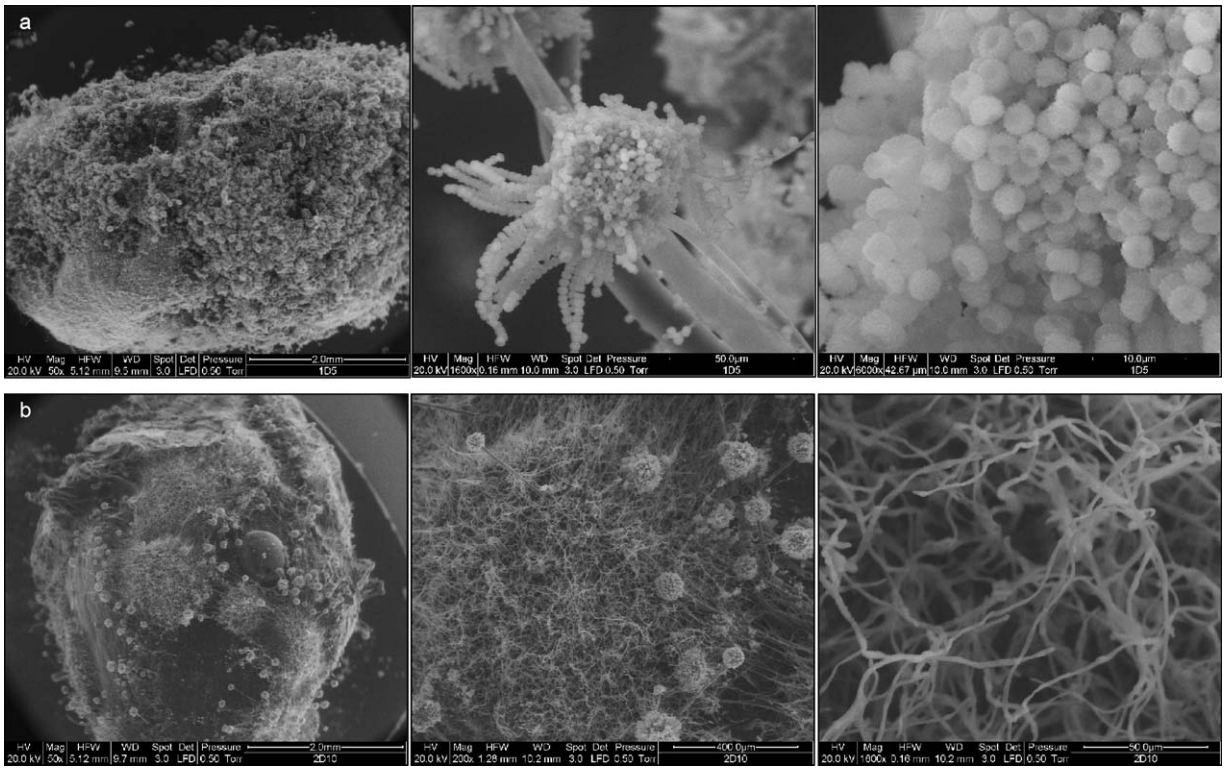

Fig. 4. ESEM pictures of pearled wheat kernels after 120 h of fermentation with *Aspergillus awamori*, (a) without addition of water, showing a grain covered by spores and (b) with intermittent trickling of water, showing hyphal filaments covering the surface of the grain.

and initial moisture content in the range 0.8–0.9 g water g^{-1} grain solid were the most suitable conditions for fungal growth and the production of the feedstock.

The fermentations in the 1 L reactor with intermittent trickling of water were conducted to confirm the suitability of whole wheat grains for the growth of *A. awamori*, to study the effect of the intermittent trickling of water and extraction during the fermentation on growth and on enzyme, glucose and FAN production and to determine if it is possible to obtain a generic fermentation feedstock with the system proposed.

The intermittent tricking of water was not started until 10 h after inoculation to allow germination and to avoid the washing out of the spores. Observation of single kernels under the microscope confirmed that partially pearled whole wheat grains were suitable for fungal growth and the excess of water did not have negative effects. The tricking of water had immediate consequences on the condition of the fermentation, avoiding the drying of the bed and keeping the temperature levels within the range 30–34 °C during the whole process. Modifications of traditional SSF reactors with intermittent addition of water (in order to maintain moisture and temperature levels) have been reported previously [34–36]. The positive effect of the control of moisture content on growth was also studied by Von Mein and Mitchell [37] who showed that not adding water resulted in drying of the bed and deceleration in growth. Increased lag phase at low water content and related reduction in specific growth rates have also been reported [38].

In our study, the specific growth rate was not calculated because it was not possible to recover the biomass from the fermented solid, but it was observed that the intermittent trickling of water resulted in reduced production of spores and increased production of yellow aerial hyphae which covered the whole kernel after 3 days of fermentation. Usually, mycelial growth allows rapid colonisation of the substrate's surface and penetration into the particle, allowing access to higher nutrient concentrations and achieving higher growth. The presence of water influenced significantly the morphology of the fungus, which exhibited physical characteristics that were not observed in previous experiments when no water was fed to the bioreactor. When the grains were observed under the ESEM the differences could be appreciated even better. The pictures of the sample without the addition of water show spherical spores of around 4 μm with a rough textured surface (Fig. 4a), while the pictures taken of the samples with addition of water show the long branching filaments of the aerial hyphae (Fig. 4b). Previous studies have shown that production rates for biomass and α-amylase as well as overall yields were higher in cultures of *Aspergillus oryzae* with aerial mycelia [39]. It might therefore be expected that if the intermittent trickling of water increases aerial hyphae in *A. awamori* then this will also favour enzyme production rate.

The water introduced during the fermentation more than compensates for moisture losses (6.86 g water g^{-1} grain solid) but can also have negative effects, as high moisture levels result in decreased substrate porosity that in turn can reduce oxygen penetration [40]. It can also increase the risk of microbial contamination [41]. At laboratory scale, oxygen limitations due to the water were not observed, but may need to be taken into account in the scale-up of the process.

During trickling, the extraction of nutrients could also affect fungal growth but the rate of glucose production in such systems is usually much higher than the rate of consumption [42]. Extraction can also be beneficial because of removal of possible inhibitory metabolites. The challenge in this system was to set up the frequency of the intermittent trickling to obtain extracts with high glucose and FAN concentrations. After some preliminary investigations, a frequency of once every 6 h was chosen as sufficient to allow good levels of hydrolysis of starch but without excessive consumption of the glucose. Fig. 5 shows the net production of glucose at the end of each cycle. It can be seen clearly that from the 2nd day of fermentation glucose was produced very rapidly,

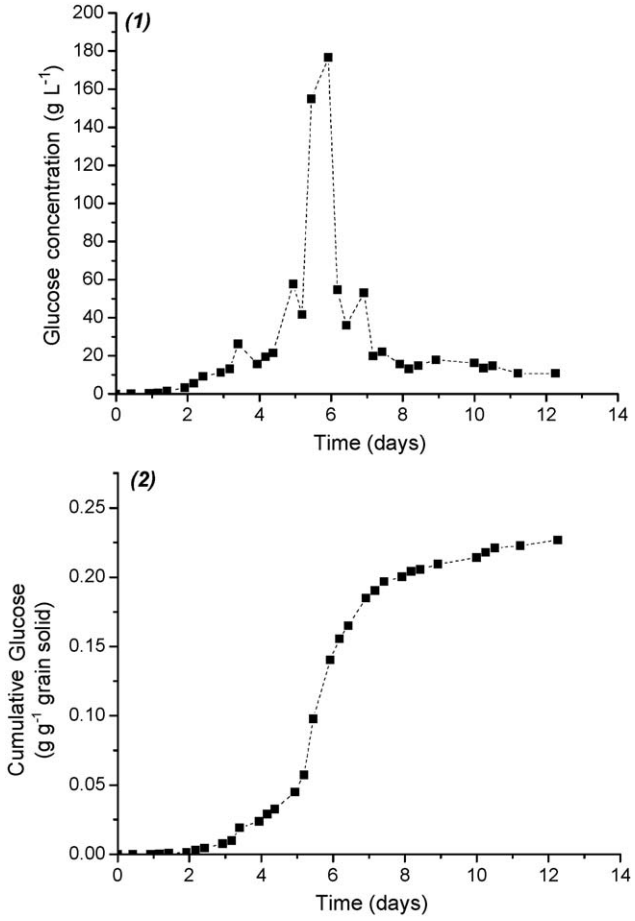

Fig. 5. Glucose concentration (1) and cumulative glucose (2) measured in extracts obtained during fermentation of wheat grains in a packed bed with intermittent trickling of water.

reaching a maximum of 177 g L^{-1} on the 6th day (Fig. 5(1)). After that point the concentration in the extract decreased in equally dramatic fashion and by the end of the 6th day there was very little further net production of glucose (Fig. 5(2)).

The glucose levels achieved (177 g L^{-1}) during this experiment demonstrate the potential of particulate bioprocessing to produce high product concentrations. In previously reported submerged fermentations for the same system the highest glucose concentrations achievable in a single step were only around 50 g L^{-1} [43]. This is because the practical limit for medium concentration is around 8% w/v original flour, due primarily to the very high viscosity of gelatinised wheat flour suspensions. Similar limitations were also faced in another system using SmF for the production of a generic feedstock in which glucose concentrations up to just 37 g L^{-1} were reported for gelatinised sago starch [44]. The advantage of using particulate bioprocessing is simply that we can make use of whole wheat grains with starch content between 65% and 85%.

Viesturs et al. [45] proposed an innovative bioreactor system combining a submerged fermentation with a solid-state fermentation for the conversion of lignocellulosic material to ethanol. Such a system, first described elsewhere [46], could be very attractive for our wheat-based particulate bioprocessing, given the high concentrations of glucose demonstrated in Fig. 5(1). It could be possible, for example, to include a reservoir below the bed of particles through which the trickled solution could be recirculated and within which a second fermentation could be carried out simultaneously with the fungal fermentation.

Viesturs et al. [45] were able to show that recirculation with a simultaneous yeast fermentation being carried out in the liquid phase was not only successful but also beneficial to both fermentations. Key limitations of their system include the wheat straw substrate used, which required addition of both extra nutrients and the pre-adsorption of cellulose to increase substrate conversion, as well as the frequency at which the intermittent recirculation was carried out, once every 24 h, meaning most of the glucose was consumed by the filamentous fungus. By using wheat grains and trickling every 6 h, these limitations are avoided in our system. Despite the high concentrations reached in the extracts, the cumulative yield of glucose extracted per gram of initial solid grain was not very high, only 0.23 g glucose g^{-1} solid grain (Fig. 5(2)), equivalent to 0.35 g glucose g^{-1} initial total starch. This is partly because some glucose was consumed by the fungus during growth but also because the extraction was not complete. The results discussed so far came from simple percolation of water through the bed of grains. Agitation and recirculation can improve this yield significantly, as is discussed in the next section.

The shape of the curve of cumulative glucose yield clearly indicates that after a lag phase there is a rapid production of glucose. This is because enzyme production is related to growth so its activity increases exponentially during the early stages of the fermentation. It is important also to keep in mind that in this system the glucose is constantly being removed by extraction, so there is a natural pressure for the fungus to continue to produce enzymes. The rate of glucose accumulation decreases after the 6th day of fermentation (Fig. 5(2)), probably because of depletion of wheat nutrients due to the extraction process and fungal consumption.

Another important nutrient source in the feedstock is free amino nitrogen. The measurements of FAN in the extracts (Fig. 6(1)1) followed an irregular trend during the first 4 days of fermentation, with concentrations oscillating between 4 and 24 mg L^{-1}. After the 4th day the values obtained started to increase, reaching a maximum of 67 mg L^{-1} on the 6th day. Although a bit irregular, this trend was similar to the one for glucose, probably because FAN values are also the results of a combination of enzyme activity (in this case proteases), consumption by the fungus and the extraction process. There was, however, one major difference between the two profiles; on the 9th day of fermentation FAN started to increase again, which might be an indication of the natural autolysis of the fungus.

The FAN concentrations obtained directly from the fermentation were quite low; not even reaching the 100 mg L^{-1} obtained previously using liquid fermentation for the same system [12]. If the concentration of FAN in the feedstock is not enough it could be a limiting factor for the growth of any microorganism used in a subsequent fermentation. Although the cumulative FAN collected increased almost linearly through the fermentation, the overall yield was only 0.24 mg FAN g^{-1} initial solid grain (Fig. 6(2)). To improve extraction, mixing or recirculation could be carried out. Another approach to increase FAN content in the feedstock is to integrate an autolysis step into the process [47]. Wang and Webb showed that oxygen limitation and high temperatures enable the autolysis of the fungus, reaching FAN concentrations of up to 450 mg L^{-1} with a single batch or as much as 1580 mg L^{-1} after seven recirculations. This latter value is equivalent to the FAN in 31 g yeast extract L^{-1}.

Glucoamylase activity in the extract was also measured (Fig. 7). Fortunately, it seems that glucoamylase was not fully extracted. During the period from day 2 to day 3 the increase in glucoamylase activity was exponential and after this the activity continued to increase, but in a linear fashion. Through the entire process the overall cumulative yield reached was only 68 U g^{-1} initial solid grain, and this represents a much lower level of activity than

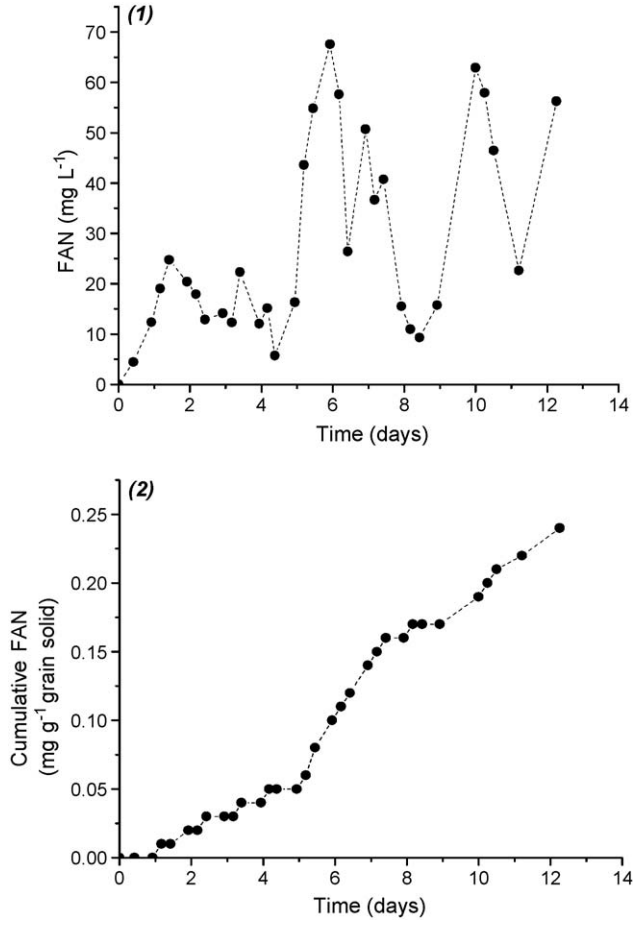

Fig. 6. FAN concentration (1) and cumulative FAN (2) measured in extracts obtained during fermentation of wheat grains in a packed bed with intermittent trickling of water.

should be required for the amount of glucose produced. This was probably because of the low efficiency of enzyme extraction due to the large size of the enzyme molecules and the short residence time of the extract water in the bed. Unlike other substrates, such as wheat bran, the whole wheat grains used in our system created a very open porous bed through which the extract water passed rapidly. A system that used a packed bed of wheat bran with intermittent extraction for protease production was reported to absorb the water added in the first addition and required around 10 h to collect the water after the second addition, increasing the amount of enzyme extracted [48].

The fact that in our system glucose can be extracted without extracting the enzymes represents a clear process advantage. The enzymes retained in the bed are more like those in an immobilised enzyme reactor and can continue hydrolysing starch, producing more glucose.

3.2. Improving the extraction of the feedstock

The extraction process during the solid-state fermentation is one of the key steps in the strategy proposed in this paper. Results have shown that simple percolation of water through the bed of particles during fungal fermentation can extract the nutrients required for the feedstock. Clearly, the conditions under which the extraction is carried out will influence the amount and concentration of products obtained.

To study some of these conditions, several batch extraction experiments were carried out using the solid left in the reactor at the end of the fermentation. These experiments confirmed that varying temperatures around the optimum for fermentation (30 °C) did not have a significant impact on the extraction process. Extraction experiments also showed that for a static bed the maximum extract concentration was reached within 3–5 min, showing that simple percolation through the bed should be sufficient for the extraction of nutrients. Agitation, on the other hand, resulted in an increase of 40% in the extraction of glucose and FAN.

In order to decrease the amount of extract water used and increase glucose concentration, an experiment in which the water was reused to extract further batches of solid was carried out (Fig. 8). With four such reuses, the concentration of glucose was increased by a factor of 3.3 (from 13.09 to 43.15 g L^{-1}), suggesting that extract liquor could be reused throughout the fungal fermentation, successively extracting glucose produced since the last extraction. Filtration and refrigeration of the extracts would be necessary in order to prevent microbial contamination.

Finally, to get an idea of the actual amount of products present in the solid, a set of eleven extractions was carried out using the same fermented solid and fresh liquid. Fig. 9 shows the concentrations measured in the extracts after each successive extraction. After the first extraction, more than half of the total

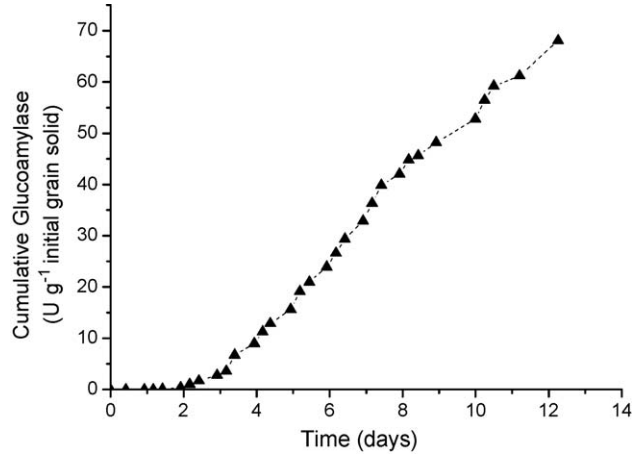

Fig. 7. Cumulative glucoamylase measured in extracts obtained during fermentation of wheat grains in a packed bed with intermittent trickling of water.

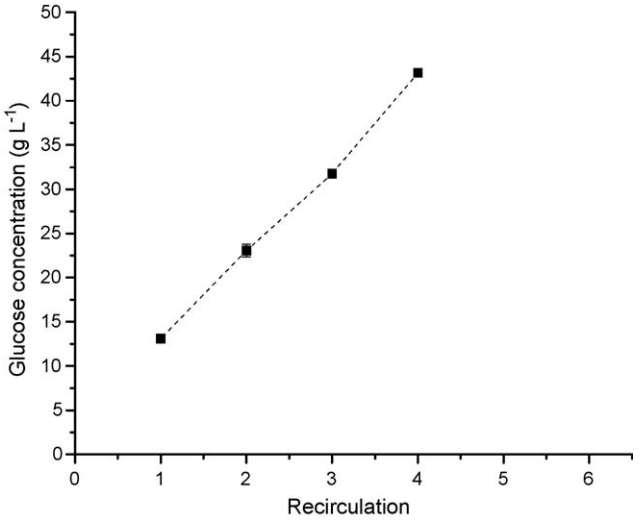

Fig. 8. Glucose concentration in the extract after several reuses of the same extract liquid using fresh batches of solid.

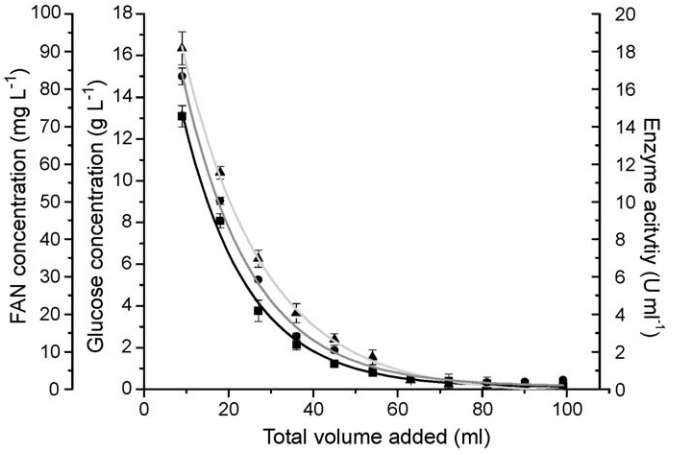

Fig. 9. Glucose (■), glucoamylase (▲) and FAN (●) concentration obtained from successive extractions using the same solid and fresh solvent. The lines were generated using an exponential decay equation.

amount of glucose remained in the solid. After six extractions, the concentrations of glucose, FAN and glucoamylase in the extract were almost zero.

The results were used to calculate the total amount of products extractable from the solid using a model presented previously [49]. As was discussed by Diaz et al. [49], the extraction of enzymes in solid-state fermentation is a process of dissolution and leaching in a heterogeneous liquid–solid system and the process kinetics is expected to follow an exponential form. In our system the results can be fitted with an exponential decay equation of the form $C = Ae^{-kV}$ as demonstrated in Fig. 9. In this expression C is the concentration extracted, V the total volume of water used, A is related with the quantity extracted in the first drop and k is a parameter related to the conditions of the extraction.

3.3. Evaluation of the feedstock obtained by particulate bioprocessing

The feedstocks obtained from the fermented wheat grains were evaluated in terms of their nutritional significance for subsequent microbial bioprocesses. Two different bioconversions, one with a bacterium and one with yeast, were used to test the suitability of the feedstocks for the production of the biodegradable plastic polyhydroxybutyrate and ethanol, respectively.

PHB fermentations, using *W. eutropha*, were monitored in terms dry weight, glucose and FAN measurement. Three media with different conditions of, sterilisation and addition of FAN were used to test the feedstock and some of the conditions that affect bacterial growth and the production of the biodegradable plastic.

The results represented in Fig. 10 show the effect of media sterilisation. Observing the differences in glucose consumption profiles (Fig. 10(2)) between autoclaved medium (C) and filtered medium (D), it can be concluded that filtration was a better form of sterilisation. Consumption of glucose was faster and the production of PHB was three times higher in the filtered medium D (0.78 g L^{-1}). This is probably because of damage caused by high temperatures and pressures during autoclaving and the possible production of Maillard products. These problems are avoided in the filtration process.

A further medium, supplemented with an additional nitrogen source (yeast extract) was also tested, as the feedstock generated for these particular experiments contained a relatively low FAN concentration and the results (for media C and D) showed some nitrogen limitation. The FAN content was increased from 35 to 61 mg L^{-1} in medium E and this resulted in higher bacterial growth, higher glucose consumption (Fig. 10) and greater PHB

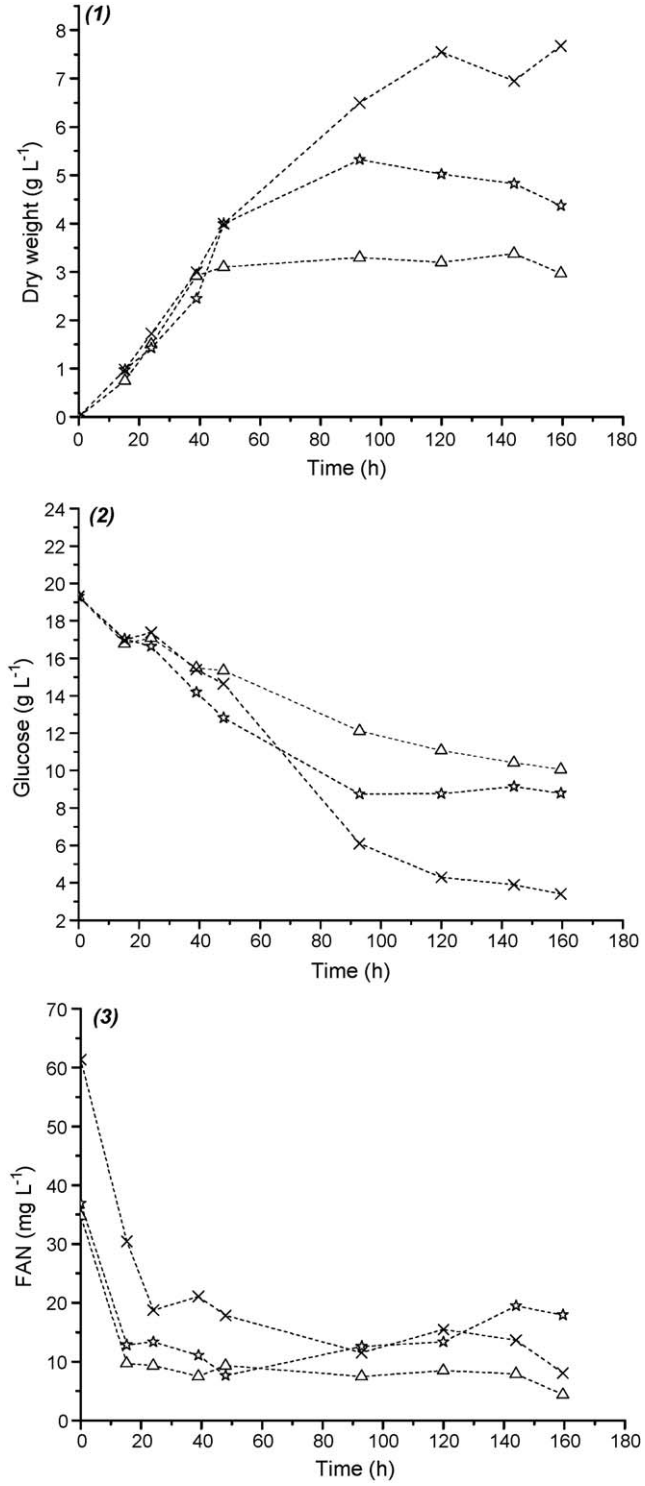

Fig. 10. Dry weight, glucose, and FAN measured during the growth of *Wautersia eutropha* in the feedstock obtained using particulate bioprocessing. (Media C (△), media D (☆), media E (×))

production (3.96 g L^{-1}). These results agree with findings reported previously, which concluded that higher FAN content increases PHB productivity [14]. Therefore, to obtain higher yields without the need to supplement the nitrogen source, it will be necessary to improve FAN extraction by, for example, adopting the measures discussed in the previous section.

The nutritional value of different extracts, with higher FAN and glucose concentrations, was validated by growing yeast *S.*

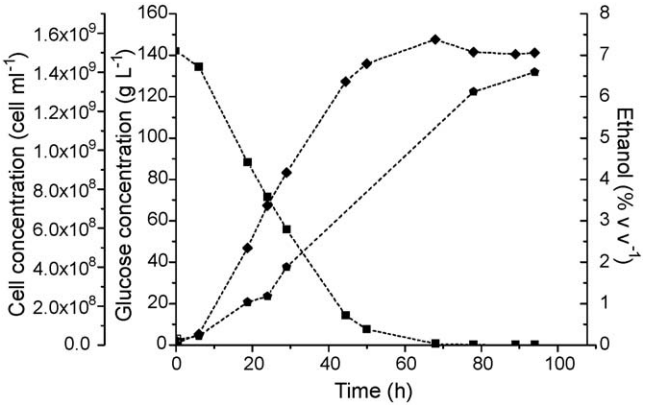

Fig. 11. Cell concentration (⬟), glucose consumption (■) and ethanol production (◆) measured during the growth of *Saccharomyces cerevisiae* in the feedstock obtained using particulate bioprocessing.

cerevisiae anaerobically for ethanol production. The results demonstrated that the feedstock can be used for the growth of *S. cerevisiae*. Glucose was consumed after 4 days of fermentation and the ethanol produced reached a concentration of 7% (v/v) (Fig. 11). The glucose to ethanol yield reached 0.41 g g^{-1}, equivalent to 80% of the theoretical value, proving that our feedstock and the system proposed is suitable for biofuel production.

The results of these studies confirm that the feedstocks obtained by particulate bioprocessing do not present any severe inhibition for the organisms tested. Therefore this system could be used in a whole grain biorefinery for the production of PHB and bioethanol.

4. Conclusions

A novel process strategy for biorefineries based on particulate bioprocessing has been developed. Experiments using a packed bed reactor have confirmed the suitability of partially pearled whole wheat grains for the growth of *A. awamori*. Intermittent tricking of water every 6 h during fungal cultivation was shown to improve mycelial growth and to increase glucose and FAN production. The addition of water helps to maintain moisture and temperature levels, and has also been used to extract soluble components, obtaining the generic fermentation feedstock. The extraction can be improved by agitation and recirculation of the extract, which can increase glucose concentration by more than three times. Experiments with repeated extraction have shown that less than half of the glucose in the solid is extracted in a single pass but the total amount present can be easily estimated. The extracts obtained with the system developed have been tested for PHB and ethanol production, confirming the suitability of particulate bioprocessing as a useful tool in future biorefinery systems for the production of biofuels and value-added chemicals.

Acknowledgment

The authors acknowledge the financial support provided to Carolina Botella by the European Commission under the Marie Curie Host Fellowship for Early Stage Research Training Programme.

References

[1] Kirby A. Energy: meeting soaring demand. BBC BBC News, Special Reports: Planet Under Pressure; 2004.
[2] Energy_Information_Administration. International energy outlook. Energy Information Administration; 2003.
[3] UN. Kyoto protocol to the United Nations framework convection on climate change. United Nations; 1998.
[4] EurActiv. Alternative fuels for transport Environmetal Policy Section; 2005.
[5] Klass DL. Biomass for renewable energy, fuels and chemicals. Academic Press; 1998.
[6] FAO. Food outlook: global market analysis. Food and Agriculture Organisation of the United Nations (FAO); 2006.
[7] Kamm B, Kamm M, Gruber PR, Kromus S. Biorefinery systems an overview. In: Kamm B, Gruber PR, Kamm M, editors. Biorefineries—industrial processes and products. Wiley-VCH; 2005. p. 3–41.
[8] Kamm B, Kamm M. Principles of biorefineries. Appl Microbiol Biotechnol 2004;64:137–45.
[9] Clark J, Macquarrie D. Handbook on green chemistry & technology. Blackwell Publishing; 2002.
[10] Mulvihill P. Crystalline and liquid dextrose products: production, properties and applications. In: Schenck F, Hebera R, editors. Starch hydrolysis products—worldwide technology production and applications. New York: VCH; 1992.
[11] Crabb W, Shetty J. Commodity scale production of sugars from starch. Curr Opin Microbiol 1999;2:252–6.
[12] Webb C, Wang R. Development of a generic fermentation feedstock from whole wheat flour. In: Campbell GM, Webb C, McKee SL, editors. Cereals: novel uses and processes. New York and London: Plenum Press; 1997.
[13] Koutinas AA, Wang R, Campbell GM, Webb C. A whole crop biorefinery system: a close system for the manufacture of non-food products from cereals. In: Kamm B, Gruber PR, Kamm M, editors. Biorefineries—industrial processes and products. Wiley-VCH; 2005. p. 165–93.
[14] Koutinas AA, Xu Y, Wang R, Webb C. Polyhydroxybutyrate production from a novel feedstock derived from a wheat-based biorefinery. Enzyme Microb Technol 2006;40:1035–44.
[15] Wang R, Dominguez-Espinosa RM, Leonard K, Koutinas A, Webb C. The application of a generic feedstock from wheat for microbial fermentations. Biotechnol Prog 2002;18(5):1033–8.
[16] Koutinas AA, Wang R, Webb C. Evaluation of wheat as generic feedstock for chemical production. Ind Crops Prod 2004;20(1):75–88.
[17] Koutinas AA, Wang R, Webb C. Restructuring upstream bioprocessing: technological and economical aspects for production of a generic microbial feedstock from wheat. Biotechnol Bioeng 2004;85(5):524–38.
[18] Mousia Z, Edherly S, Pandiella SS, Webb C. Effect of wheat pearling on flour quality. Food Res Int 2004;37(5):449–59.
[19] Mitchell DA, Kreiger N, Berovic M. Solid-state fermentation bioreactors. In: Fundamentals of design and operation. Springer; 2006.
[20] Moo-Young M, Moreira AR, Tengerdy RP. Principles of solid-substrate fermentation. In: Smith JE, Berry DR, Kristiansen B, editors. The filamentous fungi. London: Edward Arnold; 1983. p. 117–44.
[21] Mitchell DA, Berovic M, Krieger N. Biochemical engineering aspects of solid state bioprocessing. In: Scheper T, editor. Advances in biochemical engineering biotechnology. Springer; 1999. p. 61–138.
[22] Ustok FI, Tarin C, Gogus N. Solid-state production of polygalacturonasa by *Aspergillus sojae* ATCC 20235. J Biotechnol 2007;127(2):322–34.
[23] Botella C, de Ory I, Cantero D, Blandino A. Hydrolytic enzyme production by *Aspergillus awamori* on grape pomace. Biochem Eng J 2005;26(2–3):100–6.
[24] Pandey A, Selvakumar P, Soccol CR, Nigam P. Solid state fermentation for the production of industrial enzymes. Curr Sci 1999;77(1):149–62.
[25] Botella C, Diaz A, De Ory I, Webb C, Blandino A. Xylanase and pectinase production by *Aspergillus awamori* on grape pomace in solid state fermentation. Process Biochem 2007;42:98–101.
[26] Viniegra-González G, Favela-Torres E, Aguilar CN, Rómero-Gomez SJ, Díaz-Godínez G, Augur C. Advantages of fungal enzymes production in solid state over liquid fermentation systems. Biochem Eng J 2003;13(2–3):157–67.
[27] Pandey A, Soccol CR, Rodriguez-Leon JA, Nigam P. Solid-state fermentation in biotechnology: fundamentals and applications. Asiatech Publisher Inc.; 2001.
[28] Holker U, Lenz J. Solid-state fermentation – are there any biotechnological advantages? Curr Opin Microbiol 2005;8:301–6.
[29] Hardin MT, Howes T, Mitchell DA. Mass transfer correlations for rotating drum bioreactors. J Biotechnol 2002;97(1):89–101.
[30] Harding KG, Dennis JS, von Blottnitz H, Harrison STL. Environmental analysis of plastic production processes: comparing petroleum based polypropylene and polyethylene with biologically-based poly-β-hydroxybutyric acid using life cycle analysis. J Biotechnol 2007;130(1):57–66.
[31] Lee J. Biological conversion of lignocellulosic biomass to ethanol. J Biotechnol 1997;56(1):1–24.
[32] Lie S. The EBC-ninhydrin method for determination of free alpha amino nitrogen. J Inst Brew 1973;79:37–41.
[33] Riis V, Mai W. Gas chromatographic determination of poly-β-hydroxybutyric acid in microbial biomass after hydrochloric acid propanolysis. J Chromatogr 1988;445:285–9.
[34] Lonsane BK, Sucedo-Castaneda G, Raimbault M, Roussos S, Viniegra-González G, Ghildyal NP, et al. Scale-up strategies for solid-state fermentation. Process Biochem 1992;27:259–73.
[35] Ryoo D, Murphy VG, Karim MN, Tengerdy RP. Evaporative temperature and moisture control in a rocking reactor for solid substrate fermentation. Biotechnol Tech 1991;5:19–24.
[36] Nagel FJI, Tramper J, Bakker MSN, Rinzema A. Temperature control in a continuously mixed bioreactor for solid-state fermentation. Biotechnol Bioeng 2000;72(2):219–30.

[37] Von Mein OF, Mitchell D. A two-phase model for water and heat transfer within an intermittently-mixed solid-state fermentation bioreactor with forced aeration. Biotechnol Bioeng 2002;79(4):416–28.
[38] Raimbault M. General and microbiological aspects of solid substrate fermentation. Electron J Biotechnol 1998;1(3):174–88.
[39] Rahardjo YSP, Weber FJ, Haemers S, Tramper J, Rinzema A. Aerial mycelia of *Aspergillus oryzae* accelerate α-amylase production in a model solid-state fermentation system. Enzyme Microb Technol 2005;36:900–2.
[40] Oostra J, le Comte EP, van den Heuvel JC, Tramper J, Rinzema A. Intra-particle oxygen diffusion limitation in solid-state fermentation. Biotechnol Bioeng 2001;75(1):13–24.
[41] Ramadas M, Holst O, Mattiasson B. Production of amyloglucosidase by *Aspergillus niger* under different cultivation regimens. World J Microbiol Biotechnol 1996;12(3):267–71.
[42] Te Biesebeke R, Ruijter G, Rahardjo YSP, Hoogschagen MJ, Heerikhuisen M, Levin A, et al. *Aspergillus oryzae* in solid-state and submerged fermentations. Progress report on a multi-disciplinary project. FEMS Yeast Res 2002;2:245–8.
[43] Webb C, Koutinas WR, Wang R. Developing a sustainable bioprocessing strategy based on a generic feedstock. Adv Biochem Eng/Biotechnol 2004;87:195–268.
[44] Abd-Aziz S. Sago starch and its utilisation. J Biosci Bioeng 2002;94(6):526–9.
[45] Viesturs UE, Strikauska S, Leite M, Berzins A, Tengerdy RP. Combined submerged and solid substrate fermentation for the bioconversion of lignocellulose. Biotechnol Bioeng 1987;30(2):282–8.
[46] Laukevics JJ, Apsite AF, Viesturs UE, Tengerdy RP. Solid substrate fermentation of wheat straw to fungal protein. Biotechnol Bioeng 1984;26(12):1465–74.
[47] Wang R, Webb C. Autolysis of *Aspergillus awamori*: an approach to nutrient regeneration. IchemE Research Event, Newcastle, 7–8 April; 1998.
[48] Aikat K, Bhattacharyya BC. Protease production in solid state fermentation with liquid medium recycling in a stacked plate reactor and in a packed bed reactor by a local strain of *Rhizopus oryzae*. Process Biochem 2001;36:1059–68.
[49] Diaz AB, Caro I, De Ory I, Blandino A. Evaluation of the conditions for the extraction of hydrolytic enzymes obtained by solid state fermentation from grape pomace. Enzyme Microb Technol 2007;41(3):302–6.