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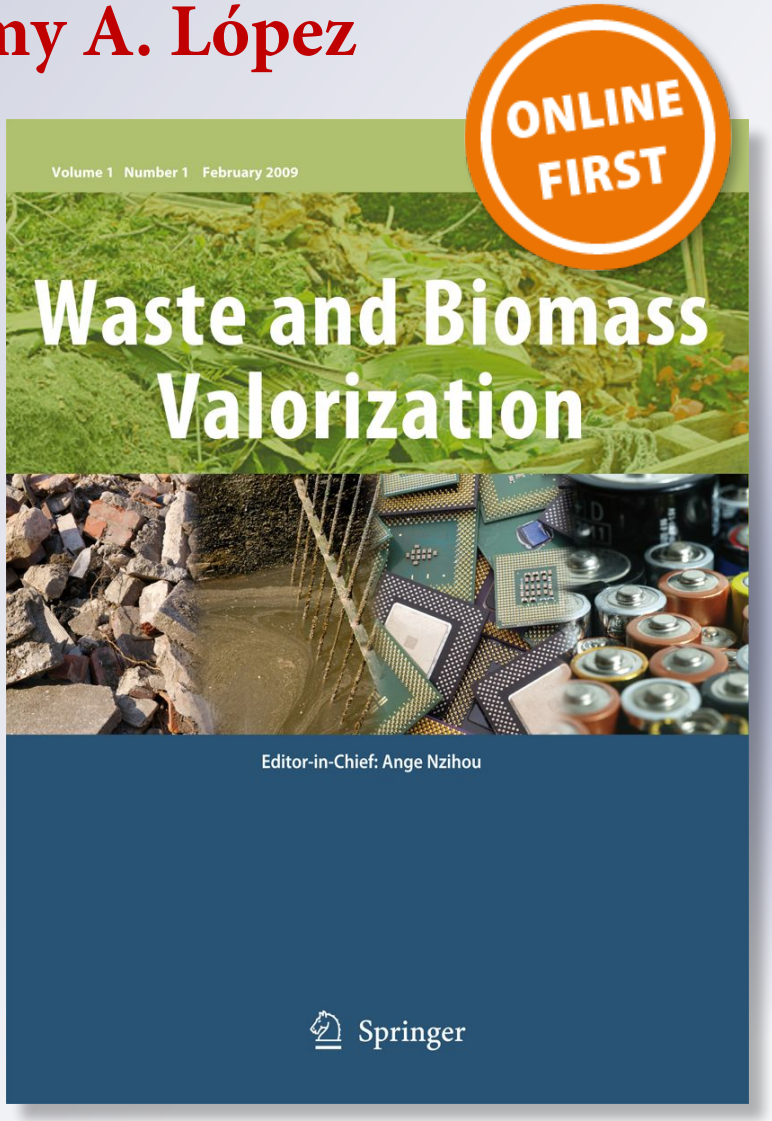

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ORIGINAL PAPER

Production of Fermentation Feedstock from Jerusalem Artichoke Tubers and its Potential for Polyhydroxybutyrate Synthesis

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Abstract A conceptual bioprocessing strategy has been developed for the production of a generic fermentation feedstock from Jerusalem artichoke (JA) tubers. Ground JA tubers were used as substrate in solid state fermentations (SSF) of *Aspergillus awamori* aiming at the simultaneous production of crude enzymes (e.g. inulinase, invertase, protease) that could be employed in the hydrolysis of JA meal macromolecules. After 48 h SSF, remaining solids that contained the crude enzymes were transferred to an aqueous solution of ground JA tubers for the hydrolysis of major macromolecules (inulin and protein) into assimilable nutrients (e.g. fructose, glucose, amino acids and peptides). Conversions of up to 70 % (w/w) inulin to fructose and glucose, among the highest reported in the literature, were achieved. This bioprocess could be employed for the production of fructose- and glucose-based fermentation media from JA tubers. Crude JA hydrolysates were tested as fermentation media for PHB production in shake flask fermentation resulting in intracellular PHB content up to 51.9 % and PHB concentrations up to 4 g L^{-1}.

Keywords Jerusalem artichoke · Bioprocess · *Aspergillus awamori* · Solid state fermentation · Generic fermentation media · Polyhydroxybutyrate

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Introduction

Jerusalem artichoke (*Helianthus tuberosus*) (JA) is a perennial herbaceous plant of the Asteraceae family. It is mainly cultivated for its tuber across the temperate zone. JA can be cultivated in environments, ranging from saline and alkaline to dry conditions, unsuitable for cultivation of food crops such as wheat, rice and potato. Furthermore, a significant advantage of JA cultivation is the high production yields achieved (40–70 tonnes/ha for JA tubers). The tubers of JA contain mainly the fructose-rich polysaccharide inulin at 70–80 % (w/w) of the total dry weight [1, 2]. The water content of JA tubers is in the range of 75–85 % [2]. Inulin contains fructose unit chains with length in the range of 3–50 linked by β-(2 → 1) D-fructosylfructose bonds that are usually terminated by a single glucose unit linked by an α-D-glucopyranosyl bond [3]. Inulin is enzymatically hydrolysed to fructose and glucose by the combined action of inulinase and invertase [4]. Apart from inulin, JA tubers also contain free reducing sugars, proteins and minerals [5]. Its suitability for cultivation, high biomass yields per hectare, favourable composition and ability to grow under extreme conditions make JA an appropriate renewable crop for producing fructose-rich hydrolysates that can be used as fermentation media. It could be considered as an alternative to glucose-rich crops and biomass that contain starch and cellulose.

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JA could be also employed in food-related applications and it has been evaluated for the production of fructose syrup, inulo-oligosaccharides and inulin that has many health benefits. Inulin and its derivatives are on a constantly increasing demand by the food industry [3]. Although chicory is the main crop utilized as raw material for industrial production of inulin, JA could be also used for inulin extraction. The utilisation of JA for food and non-food applications coupled with its cultivation under conditions unsuitable for cultivation of food crops offers a significant advantage for industrial implementation due to the flexibility provided to suit the needs of different markets. Therefore, the selection of the most suitable application or combined market alternatives for JA depends on the profitability and market/societal needs for each specific region.

JA can be characterised as an energy crop, but its cultivation imposes important disadvantages that could outweigh its potential as fermentation feedstock [6]. Although JA cultivation is easy, its harvest and storage imposes certain difficulties. JA harvesting is a labour intensive process because after mechanical collection the tubers cannot be easily separated from crown tissue and soil. In addition, the tubers are easily spoiled during storage. These are also important disadvantages that have prevented the widespread utilisation of JA as a source of inulin for food related applications [7]. A solution to this problem could be given by storing JA tubers in the field as they are preserved in a good condition as long as the soil is not too wet [6]. The ability to cultivate JA under desert conditions and frost could outweigh the problems with collection and storage.

There is an imminent need to evaluate various renewable resources (e.g. agricultural crops, non-food crops, residues and waste) as feedstock for the production of chemicals, biopolymers and biofuels via fermentation or green chemical conversion routes. Each region or country should evaluate different renewable resources depending on sustainability issues. JA has been used in previous studies for fermentative production of 2,3-butanediol [8], ethanol [9], single cell protein [1], microbial lipids [10, 11], butanol [12] and lactic acid [13]. In some studies, mixed cultures in simultaneous saccharification and fermentation experiments have been employed for the production of extracellular products (e.g. 2,3-butanediol, lactic acid) from JA tubers [8, 13]. JA extracts have been employed for the production of intracellular products, such as microbial lipids [10, 11]. JA hydrolysis and fermentation could be achieved either simultaneously of sequentially. The production of intracellular metabolic products prevents the utilisation of the simultaneous process because remaining solids that are not hydrolysed prevent the separation of intracellular products from microbial mass. The simultaneous process is industrially feasible in the case of extracellular metabolic products.

Previous studies utilised JA only as a source of carbon neglecting the presence of other nutrients (e.g. protein, minerals) that are essential in the formulation of nutrient-complete fermentation media. In this study, JA was evaluated not only as a renewable resource for the production of carbon sources (i.e. fructose and glucose) but also for the generation of sources of nitrogen and phosphorus.

The fungal strain *Aspergillus awamori* 2B. 361 U2/1 has been employed extensively at the SATAKE Centre for Grain Process Engineering (SCGPE) in the development of wheat-based biorefineries for the production of succinic acid, bioethanol and polyhydroxybutyrate (PHB) [14–16]. Previous studies have shown that it is a prolific producer of glucoamylase and to a lesser extent protease and phosphatase [17, 18]. This study demonstrates that this strain of *A. awamori* can also produce significant quantities of inulinase and invertase that could be employed in the hydrolysis of inulin. The extent of inulin hydrolysis achieved in this study is among the highest reported in the literature using a single fungal strain.

To evaluate the efficiency of the medium derived from JA, preliminary shake flask fermentations were carried out with *Cupriavidus necator* for the production of polyhydroxybutyrate (PHB). PHB belongs to the family of polyhydroxyalkanoates (PHAs) that are considered as candidates to replace petroleum-derived synthetic plastics due to their structural diversity, biodegradability, biocompatibility and the fact that they can be produced from renewable carbon sources via fermentation [19]. Biosynthesis of PHAs by *C. necator* is carried out as secondary anabolic activity products where cellular growth occurs in the first growth stage that takes place without nutrient limitation (balanced growth phase) and PHAs are accumulated as intracellular storage compounds in the second stage under limiting conditions of a specific nutrient (e.g. N, P) and abundant availability of a carbon source. Recent research on PHB production focus on the substitution of purified carbon sources (e.g. purified glucose, sucrose and vegetable oils) and commercial nutrient supplements by renewable resources such as wheat, grass and silage residues, whey, molasses, meat and bone meal, various sources of triacylglycerols, lignocellulosic wastes and crude glycerol [20–22]. Some research efforts have concentrated on the utilisation of pure fructose for the production of PHB [23, 24]. However, there is no published record of the utilisation of crude agricultural resources that contain fructose for the production of PHB.

It should be stressed that this study focuses on the presentation of a conceptual bioprocess investigating the potential to produce nutrient-rich fermentation media from JA. The potential of *A. awamori* as microbial factory for JA meal hydrolysis was considered. Preliminary shake flask fermentations were carried out to evaluate JA hydrolysates

as fermentation media for PHB production by *C. necator*. Future studies will focus on bioprocess design to assess the commercial potential of this bioprocess.

Materials and Methods

Microorganisms

Fungal fermentations were carried out using the strain *A. awamori* 2B. 361 U2/1. *A. awamori* spores were stored dry in sand at 4 °C. Prior to experimental work, spores were purified, sporulated and stored in slopes containing a solid medium consisted of 50 g L^{-1} lyophilized JA meal and 20 g L^{-1} agar. The inoculum employed in SSF contained approximately 10^6 spores per g of JA on a wet basis (wb).

Submerged bacterial fermentations for PHB production were conducted with *C. necator* DSM 4058. Lyophilized bacterial cells were reactivated at 30 °C for 24 h in a growth medium containing 10 g L^{-1} fructose, 10 g L^{-1} yeast extract, and 5 g L^{-1} peptone. Bacterial cultures were stored at 4 °C in slopes containing the previous growth medium and 20 g L^{-1} agar. Fermentation inocula were prepared by transferring bacteria cells using a wire loop into a shake flask containing 50 mL of the growth medium that were cultivated at 30 °C for 20 h.

Jerusalem Artichoke Tubers

JA tubers were directly purchased from Grenoble (Isère Province, France). The tubers were washed, cut and lyophilized for 24 h. JA meal was produced by grinding dried JA pieces with a Universal Grinding Mill. JA meal was used as the sole nutrient source for enzyme production by SSF and as the sole substrate for the production of hydrolysates that were subsequently employed for PHB production. The composition of JA tubers is presented in Table 1.

Solid State Fermentations

Prior to each SSF, cultures of *A. awamori* were sporulated on solid medium, consisting of 5 % (w/v) JA meal and 2 % (w/v) agar, in 20 mL test tubes for 5 days. Subsequently, an aqueous spore suspension was formed by adding 10 mL of sterile tap water with two drops of Tween 80 (0.01 % v/v) to each test tube containing *A. awamori* spores. Spore suspensions were used as inocula for SSF, which were carried out in 250 mL shake flasks containing 5 g of dried JA meal as the sole nutrient source. Shake flasks were incubated at 30 °C and natural pH. The main objective of the SSF was the production of crude enzyme-rich extracts for subsequent enzymatic hydrolysis of JA meal.

Table 1 Composition of Jerusalem artichoke tubers

Component	Content (%, w/w)[a]
Total dry matter (wet basis)	22.8 ± 0.81
Total carbohydrate (dry basis)	77.5 ± 2.32
Reducing sugars (dry basis)	3.4 ± 0.13
Inulin (dry basis)	74.1 ± 1.76
Kjeldahl nitrogen (dry basis)	1.4 ± 0.35
Protein (6.25 × Kjeldahl nitrogen)	8.75 ± 0.49
Phosphorus (dry basis)	0.31 ± 0.08

[a] Data is expressed as mean ± 1 standard error

Production of Medium for Bacterial Fermentation

Prior to the production of medium for bacterial fermentation by enzymatic hydrolysis, inulin extraction was carried out in 1 L Duran bottles containing 40 g L^{-1} of JA dried meal. The conditions used for inulin extraction were taken from Lingyun et al. [7]. A JA aqueous suspension consisting of 11 mL of water per g of lyophilised JA meal was heated to 77 °C for 25 min and agitated using a magnetic stirrer.

Enzymatic hydrolysis was carried out by mixing fermented solids from SSF with an aqueous suspension of JA meal that was previously processed for inulin extraction. The total volume of the reaction mixture was 200 mL. The fermented solids mixed with JA meal aqueous suspensions were produced at different SSF times. This means that varying quantities of JA macromolecules were still unconsumed when fermentations were stopped at different times. Especially at early fermentation times, significant quantities of JA macromolecules should be still intact during enzymatic hydrolysis. For this reason the calculation of fructose production yield and inulin hydrolysis yield was based on the total amount of inulin that was present in the total JA meal that was used in both SSF (5 g) and aqueous JA meal suspensions (40 g L^{-1} in 200 mL reaction mixture). Thus, a theoretical maximum reducing sugar equivalent of 53 g L^{-1} can be produced from a total JA meal concentration of 65 g L^{-1} (an inulin to reducing sugar conversion factor of 1.1 and an inulin content of 74.1 %, see Table 1, have been assumed).

The pH of the solution during hydrolysis was kept natural. Hydrolytic reactions were carried out in a water bath at 55 °C, with constant agitation by a magnetic stirrer. The temperature employed during hydrolytic reactions was based on optimum values reported in the literature for fungal strains belonging to the genus of *Aspergillus* [25–27]. Hydrolytic reactions were carried out for 48 h.

At the end of the hydrolysis period, remaining solids were separated by vacuum filtration (Whatman No. 1). The fructose- and nutrient-rich solution was filter sterilized

using a 0.2 μm filter unit (POLYCAP 36 AS, Fisher). The pH of the medium was adjusted to the optimal value (6.8) for *C. necator* growth with 10 M KOH.

Bacterial Fermentations

Three sets of bacterial fermentations were carried out in 250 mL Erlenmeyer flasks on a 200 rpm rotary shaker (ZHWY-211C Series Floor Model Incubator, PR China) at 30 °C and an initial pH of 6.8. Fermentation medium of 40 mL and inoculum of 1 mL were used in each flask. The first set of shake flask fermentations was carried out with commercial sources of nutrient supplements (*i.e.* 10 g L^{-1} yeast extract and 5 g L^{-1} peptone) employing a constant free amino nitrogen (FAN) concentration (around 600 mg L^{-1}) and different fructose concentrations (10, 20 and 30 g L^{-1}). The other two sets of shake flask fermentations were carried out with JA hydrolysates as fermentation medium. This experiment was carried out in order to compare the efficiency of commercial sources of nutrients with JA hydrolysates on bacterial growth and PHB accumulation.

It was observed that the nutrient concentration in JA hydrolysates was not adequate to promote sufficient microbial growth and PHB formation. For this reason, different amounts of yeast extract were added to the medium in order to enhance the nutrient content of the JA hydrolysates. As a result, the second and third sets of shake flask fermentations were carried out using two initial fructose concentrations (14 and 28 g L^{-1}) and five individual FAN concentrations in the range of 100–900 mg L^{-1}.

Analytical Methods

Fructose, glucose and sucrose concentrations were analyzed by HPLC (Waters 600E) with an Aminex HPX–87H (300 mm × 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 deionized water and filtered through a 0.45 μm membrane filter.

Total carbohydrates were analysed with the modified colorimetric method reported by Mullin et al. [28] using pure inulin (Sigma Aldrich Co.) as standard. Reducing sugars concentration was measured by the dinitrosalicylic acid (DNS) method [29] using fructose as standard. The inulin content was calculated as the difference between total carbohydrate and reducing sugars [3, 7]. FAN concentration was determined by the ninhydrin colorimetric method promulgated in the European Brewery Convention [30]. Phosphorus and Kjeldahl nitrogen (KN) in lyophilised JA meal were determined via digestion of solid samples with a DK6 Digester (Velp Scientifica, Italy) using concentrated H_2SO_4 and 35 % H_2O_2. Phosphorus content in the digest and in any liquid sample was measured using the method described by Herbert et al. [31]. KN content was measured using a KjeltekTM 8100 distillation Unit (Foss, Denmark). The moisture content in JA meal was analyzed by drying three samples of approximately 1 g pre-weighed JA meal at 105 °C until constant weight.

During enzymatic hydrolysis, samples (5 mL) were taken at random intervals. They were centrifuged at 3,000×*g* for 10 min to separate the supernatant from unhydrolysed solids. The supernatant from hydrolytic reactions was employed for the analysis of fructose, glucose, sucrose, reducing sugars, total carbohydrates, FAN and phosphorous.

Samples (2 mL) from bacterial fermentations were also taken at random intervals. Each sample was centrifuged at 3,000×*g* for 10 min and the sediment was washed with distilled water and centrifuged twice. The solids were re-suspended in acetone and transferred into 14 mL universal bottles. The total dry weight was measured by drying the solids at 50 °C and cooling in a desiccator until constant weight. Residual bacterial cell concentration (non-PHB bacterial mass) in each sample was calculated by substracting the PHB concentration measured by GC analysis from the total dry weight. The supernatant from bacterial fermentations was used for the analysis of fructose, glucose, FAN and phosphorus.

PHB was measured by the chromatographic method proposed by Riis and Mai [32] using benzoic acid as internal standard. A gas chromatographic analyzer Fisons 8060 equipped with a Flame Ionization Detector (FID) and a Chrompack column (60 m × 0.25 mm, film thickness 0.25 μm, J&W Scientific) was used for PHB analysis. Helium was used as carrier gas at a flow rate of 2 mL min^{-1}. The injector and detector temperatures were 230 °C and the initial temperature was 120 °C.

Enzyme Activities

The quantification of inulinase activity was based on the hydrolysis of 25 g L^{-1} pure inulin for the production of fructose in 200 mM phosphate buffer at pH 6 and 55 °C within 15 min. After each sample was taken, enzymatic hydrolysis was terminated via boiling at 100 °C for 10 min. One unit (U) of inulinase activity was based on the amount of enzyme required for the release of 1 mg fructose in 1 min under the reaction conditions mentioned above. Invertase activity was assayed as the production of glucose (or fructose) during hydrolysis of 25 g L^{-1} sucrose in 200 mM phosphate buffer at pH 6 and 55 °C within

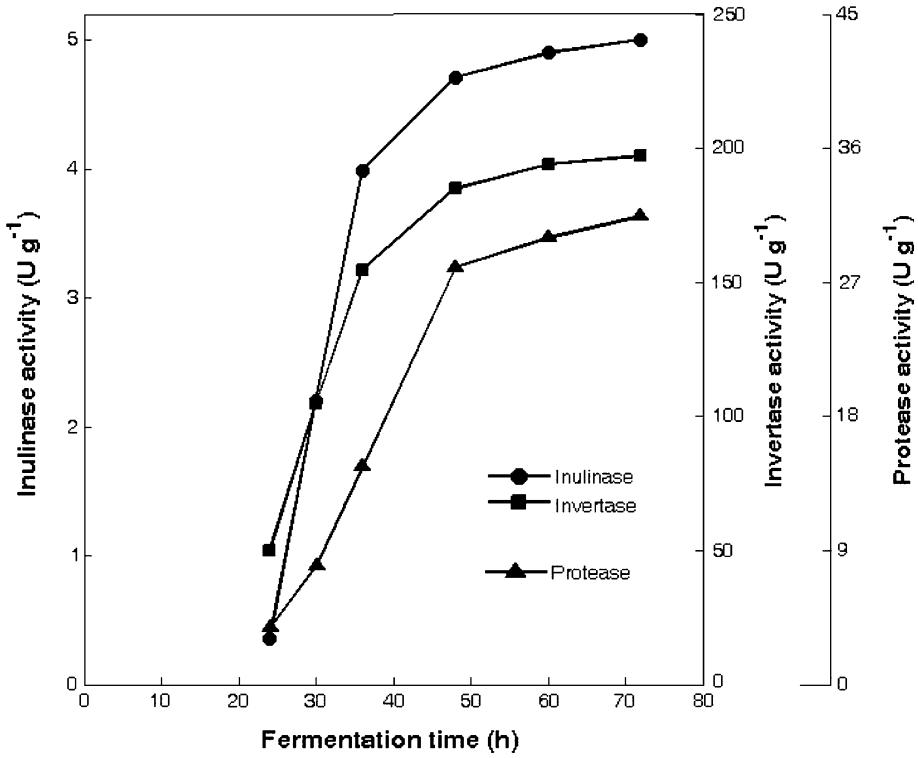

Fig. 1 Inulinase, invertase and protease production during SSF of *A. awamori* on JA meal

10 min. One unit (U) of invertase activity was defined as the amount of enzyme required for the release of 1 μg glucose in 1 min under the reaction conditions mentioned above. Protease activity was assayed as the production of FAN at 55 °C in 30 min using 15 g L^{-1} casein as substrate in 200 mM phosphate buffer. One unit (U) of proteolytic activity was defined as the amount of enzyme required to release 1 μg FAN within 1 min under the reaction conditions mentioned above.

Results and Discussion

Enzymatic Hydrolysis of Jerusalem Artichoke Meal

The development of a JA-based bioprocess is dependent on the efficient enzymatic hydrolysis of macromolecules contained in JA. The production of a nutrient-rich feedstock from JA would require the utilisation of inulinase to hydrolyse inulin into fructose and sucrose, invertase to hydrolyse sucrose into glucose and fructose and protease to hydrolyse protein into directly assimilable amino acids and peptides. The production of these enzymes could be efficiently achieved via SSF eliminating the need to purchase commercial enzymes that increase significantly processing costs. JA meal was employed as substrate in SSF with *A. awamori* because inulin is the most suitable substrate for inulinase production [33].

The fungal strain of *A. awamori* employed in this study has never been evaluated for its ability to hydrolyse JA meal. It has been extensively studied for the production of wheat hydrolysates due to its ability to produce amylolytic (mainly glucoamylase) and proteolytic enzymes [17, 18]. It should be stressed that this study was focused on illustrating the efficiency of this *A. awamori* strain in the production of JA meal hydrolysates that could be subsequently employed as fermentation media. Figure 1 presents kinetics of inulinase, invertase and protease activities produced during SSF of *A. awamori*. The activities of all enzymes increased significantly up to 48 h SSF. After this point, enzyme activities either reached a plateau (invertase) or increased slightly (inulinase and protease).

In the first set of SSF experiments, the main target was the identification of the optimum duration of SSF that resulted in the production of JA hydrolysates containing the highest amount of nutrients. The efficiency of hydrolysis was evaluated through the determination of inulin and protein hydrolysis products. Fermented solids taken at five different SSF times (24, 36, 48, 60 and 72 h) were employed in hydrolytic reactions of JA meal. It is important to mention that both SSF experiments and enzymatic hydrolysis experiments were carried out two to three times in order to verify the validity of the results.

The lyophilised JA powder used in these experiments will not be employed in industrial applications as drying the wet tuber will contribute a significant expenditure.

Table 2 Evaluation of hydrolysis performance using crude enzymes produced at different SSF times

SSF time (h)	Maximum concentration					Maximum yield	
	Sugars (g L^{-1})[a]			Nutrients (mg L^{-1})[a]		Fructose production	Inulin hydrolysis
	Fructose	Glucose	Sucrose	FAN	Phosphorus	$Y_{F/S}$ (g g^{-1})[b]	$Y_{FG/S}$ (g g^{-1})[c]
24	21.4 ± 1.3	2.1 ± 0.1	0.6 ± 0.1	111 ± 8.5	66.8 ± 4.5	0.4	0.44
36	25.7 ± 0.9	4 ± 0.2	0.75 ± 0.1	117.3 ± 6.4	84.8 ± 5.6	0.49	0.56
48	32.6 ± 2.3	4.7 ± 0.1	1 ± 0.1	129.8 ± 7.9	101.6 ± 9.1	0.62	0.7
60	27.5 ± 1.7	3.7 ± 0.3	0.6 ± 0.1	100.15 ± 9.2	92 ± 7.2	0.52	0.59
72	26.9 ± 0.4	3.6 ± 0.5	1.6 ± 0.2	100.6 ± 4.3	90.85 ± 6.9	0.51	0.58

[a] Data is expressed as mean ± 1 standard error

[b] $Y_{F/S} = F_t/TRS$, where F_t is the final fructose concentration achieved (the initial fructose concentration has already been subtracted from these values) and TRS is the total reducing sugar equivalent (53 g L^{-1}) from the JA meal (65 g L^{-1}) used in both SSF and hydrolytic reactions

[c] $Y_{FG/S} = FG_t/TRS$, where FG_t is the final fructose and glucose concentrations achieved (the initial fructose and glucose concentrations have already been subtracted from these values) and TRS is the total reducing sugar equivalent (53 g L^{-1}) from the JA meal (65 g L^{-1}) used in both SSF and hydrolytic reactions

Processing of tubers or roots with similar moisture contents is not an uncommon practise in either food or fermentation industry. For instance, sugar beet has a moisture and sugar content of 75 and 19 %, respectively. Prior to the extraction of sugar from wet sugar beet roots, they are mechanically sliced into thin strips. A similar process could be followed as an initial step in the case of JA tubers. Thus, production of thin strips could be followed by boiling of JA strips to release inulin. The suspension will be finally enzymatically hydrolysed by adding the solids from solid state fermentation that will contain all necessary enzymes.

Table 2 presents the results considering product formation (*i.e.* fructose, glucose, sucrose, FAN and phosphorus) and overall conversion yields (*i.e.* fructose and inulin) at the end of each hydrolytic reaction using aqueous suspensions of SSF solids taken at five individual SSF times. The concentrations and yields presented in Table 2 were taken at the end of each hydrolytic reaction that lasted up to 48 h. Fructose production yield and inulin hydrolysis yield were calculated by taking into consideration the total JA meal used during both SSF and hydrolysis (initial JA meal concentration of 65 g/L on a dry basis). It should be stressed that each hydrolytic reaction presented in Table 2 was carried out in parallel with a hydrolytic reaction using only SSF solids so as to evaluate the nutrients released from JA macromolecules that were not consumed during SSF by fungal cells. It was observed that hydrolytic reactions with SSF solids taken at fermentation times higher than 48 h resulted in significantly lower fructose and FAN production.

The highest concentration of fructose, glucose, FAN and phosphorus as well as the highest degree of inulin hydrolysis was observed at 48 h of SSF duration. Figure 1 showed that all enzyme activities increased significantly during SSF up to 48 h. Although enzyme activities were still high after 48 h SSF, hydrolytic reactions using SSF solids after 60 and 72 h resulted in lower nutrient concentrations (Table 2). As presented in Table 2, using crude enzymes produced at 48 h SSF resulted in a fructose production yield of 0.62 g g^{-1} and an inulin hydrolysis yield of 0.7 g g^{-1}. The highest conversion of total Kjeldahl nitrogen to FAN concentration was 11.5 %. The maximum phosphorus released as related to the initial phosphorus in JA meal was 50.9 %. The nitrogen (FAN) and phosphorus conversion yields were calculated as related to Kjeldahl nitrogen and phosphorus present in the total JA meal used in both SSF and hydrolytic reactions.

In the case that crude enzymes produced at 48 h SSF were used, approximately 14 g L^{-1} of solid concentration remained unhydrolysed. As compared to the total weight of JA used in SSF and hydrolysis, the total dry weight reduction achieved was approximately 78.5 %. The solid residue contained 35.7 % inulin and 29 % protein. This means that 10.4 and 71.2 % of inulin and protein remained unhydrolysed.

Figure 2 presents the concentration profile of fructose, glucose, sucrose, FAN and phosphorus during JA hydrolysis using SSF solids after 48 h fermentation. The production of fructose during hydrolysis indicates the existence of inulinase that hydrolyse inulin into fructose and sucrose. The reduction of sucrose concentration and the increase of glucose concentration during hydrolysis are two factors directly associated to the presence of invertase activity that hydrolyse sucrose into glucose and fructose. This means that *A. awamori* produces both inulinase and invertase. Figure 2 shows that inulin hydrolysis and phosphorus release were terminated at approximately 24 h, while protein hydrolysis continued at a very low rate. The increase of phosphorus content should be attributed to fungal autolysis that occurs simultaneously with JA

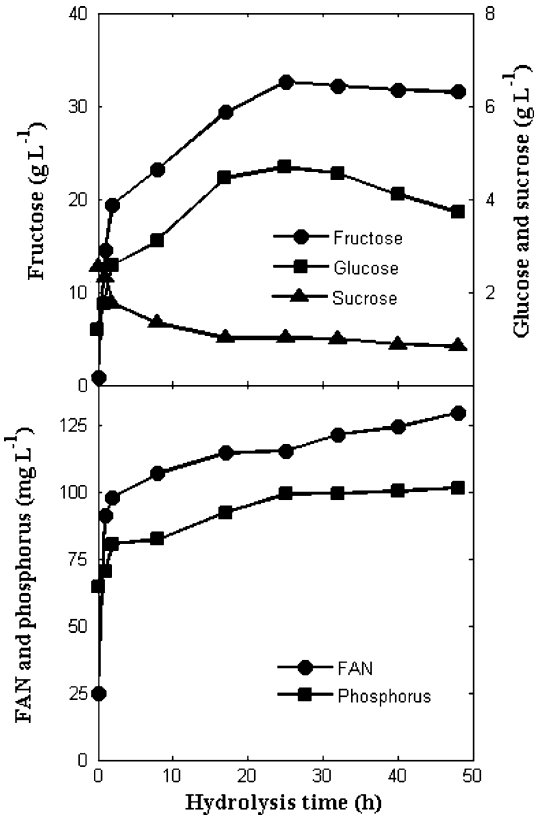

Fig. 2 Concentration profiles of sugars and nutrients during hydrolysis of JA meal carried out with solids taken from 48 h solid state fermentation

hydrolysis. This has been identified by Koutinas et al. [34] that presented optimisation of *A. awamori* autolysis where a nutrient-rich solution containing 1.6 g L^{-1} free amino nitrogen, 5.3 g L^{-1} total nitrogen and 0.5 g L^{-1} phosphorus was produced. A similar autolytic process of *A. awamori* takes place during JA hydrolysis presented in this work. The different prosphorus concentration achieved during hydrolytic experiments carried out using enzymes produced at different SSF times should be attributed to the varying ability of fungal biomass to autolyse at different SSF times or the different growth stages of the fungus during SSF.

Various fungal (e.g. *A. niger*) and yeast strains (*e.g. Kluyveromyces marxianus*) have the ability to produce high quantities of inulinase [13, 25, 26, 35, 36]. Mixed cultures of fungal and yeast strains have been employed for the production of crude inulinase and invertase that could be used for efficient JA hydrolysis [35, 37]. Öngen-Baysal and Sukan [37] reported that enzymatic hydrolysis experiments of JA meal carried out at 50 °C resulted in the hydrolysis of inulin at 34.7, 62.6 and 87.9 % in the case that crude enzymes from *A. niger* A42, *K. marxianus* NCYC 587 and a mixed culture of the two strains were used, respectively. Sirisansaneeyakul et al. [35] also reported that mixed crude enzymes produced by *A. niger* TISTR 3570 and *C. guilliermondii* TISTR 5844 proved superior to individual crude inulinases in hydrolysing inulin to fructose. There are limited studies on the utilisation of single cultures of *A. niger* for the production of enzymes that resulted in satisfactory inulin hydrolysis that could be subsequently employed as fermentation medium, as in the case of lactic acid production reported by Ge et al. [13]. The highest inulin conversion yield (0.7 g g^{-1}) achieved in this study is significantly higher than the respective yields achieved by single cultures of *A. niger* employed in the studies mentioned above [35, 37]. Such a high inulin conversion yield is usually achieved when mixed cultures are used that contain sufficient quantities of both inulinase and invertase. Further optimisation of enzyme production by this strain of *A. awamori* is likely to increase production yields. Future studies should also focus on the production and characterisation of inulinase and invertase produced by this strain of *A. awamori*.

Another important advantage of JA hydrolysates is the fact that they contain complex sources of nitrogen such as amino acids and peptides, which provide not only nitrogen but also an additional carbon source. Previous studies have demonstrated that amino acids have the ability to increase fermentation yields, enhance productivities and reduce reaction times [16, 38].

A statistical analysis on the experimental results of enzymes production via SSF and JA hydrolysis showed that all experimental data have a good reproducibility. For both cases, standard error values between 1 and 4 % of the reported value were obtained. These results coincide with values reported in Table 2, where standard errors show a similar trend. This indicates that SSF and hydrolysis experiments are statistically valid.

Bacterial Fermentations for PHB Production

Table 3 presents the initial fructose and FAN concentrations as well as the final fermentation time, total dry weight, PHB concentration, residual biomass concentration and PHB content during three sets of shake flask fermentations carried out with commercial sources of nutrients (i.e. fructose, yeast extract and peptone) and JA hydrolysates supplemented with yeast extract. In the case that a commercial fermentation medium was used, 3 shake flask fermentations were carried out with a constant FAN concentration of 600 mg L^{-1} and three different fructose concentrations (10, 20 and 30 g L^{-1}). The total dry weight, PHB concentration and residual microbial biomass concentration were increased gradually with fructose concentration. A maximum PHB concentration of 4.4 g L^{-1} was achieved when 30 g L^{-1} fructose concentration was used. However, the highest PHB content (42.9 %) was reached

Table 3 Effect of different fructose and FAN concentration on *C. necator* growth and PHB production during shake flask fermentations using commercial sources of nutrients and JA hydrolysates

Initial fructose (g L^{-1})	Initial FAN (mg L^{-1})	Fermentation duration (h)	Total dry weight (g L^{-1})	PHB concentration (g L^{-1})	Residual microbial biomass (g L^{-1})	PHB content (%)
Commercial fermentation medium (fructose, 10 g L^{-1} yeast extract, 5 g L^{-1} peptone)						
10	600	19	5.2	1.7	3.5	32.7
20	600	40	9.8	4.2	5.6	42.9
30	600	48	11.5	4.4	7.1	38.3
JA hydrolysate supplemented with yeast extract						
14	100	32	5.3	1.5	3.8	28.3
14	290	34	7.1	3	4.1	42.2
14	490	50	8.3	3.8	4.5	45.8
14	560	50	8.2	3.1	5.1	37.8
14	900	50	8.5	1.5	7	17.6
28	120[a]	56	5.4	1.4	4	25.9
28	340	56	7.5	3.2	4.3	42.7
28	430	56	7.7	4	3.7	51.9
28	540	54	8.5	1.9	6.6	22.4
28	800	49	9.2	1.4	7.8	15.2

[a] No yeast extract supplementation

when an initial fructose concentration of 20 g L^{-1} was used.

The other two sets of shake flask fermentations were carried out with mixed JA hydrolysates (Table 2). Fructose and FAN concentrations in the mixture of JA hydrolysates were 28 g L^{-1} and 120 mg L^{-1}, respectively. In the second set of shake flask fermentations, the original JA hydrolysate was diluted twice to reach a fructose concentration of 14 g L^{-1}. Due to the low FAN concentration, shake flask fermentations were supplemented with yeast extract to reach the initial FAN concentration presented in Table 3. Total dry weight and residual biomass concentration were gradually increased with increasing FAN concentration indicating that higher nutrient concentrations promote microbial growth. The PHB concentration was increased up to a FAN concentration of 490 mg L^{-1} and was gradually decreased at higher FAN concentrations. Higher FAN concentrations may facilitate microbial growth because PHB accumulation in *C. necator* is usually triggered under nutrient limiting conditions. It was also observed that PHB accumulation occurred during bacterial growth. This most probably could be attributed to oxygen limitation that cannot be avoided during shake flask fermentations.

In the third set of shake flask fermentations, the original mixture of JA hydrolysates was used only in the first shake flask experiment where the FAN concentration was 120 mg L^{-1}. In the other four shake flask fermentations, JA hydrolysates were supplemented with yeast extract in order to increase the initial FAN concentration. PHB concentration and content were increased up to 430 mg L^{-1} and then decreased at higher FAN concentrations. The highest PHB concentration (4 g L^{-1}) was achieved when fructose and FAN concentrations of 28 g L^{-1} and 430 mg L^{-1} were used.

Koutinas et al. [39] reported PHB production in shake flask fermentations using *C. necator* NCIMB 11599 and mixtures of wheat hydrolysates and fungal extracts as fermentation media. The PHB produced with 16.5 g L^{-1} glucose concentrations and 450 mg L^{-1} FAN concentration was 4.9 g L^{-1}. In addition, current studies by our research group is focused on PHB production using *C. necator* DSM 545 and crude glycerol mixed with rapeseed cake hydrolysates. Shake flask fermentations resulted in lower than 4.5 g L^{-1} PHB concentration when glycerol and FAN concentrations of approximately 16 g L^{-1} and 450 mg L^{-1} were used (results not published yet). This means that the results reported in this study when a JA hydrolysate of 14 g L^{-1} fructose concentration was employed are comparable to both wheat-based fermentation media and media derived from by-products of biodiesel production plants. However, at glucose and glycerol concentrations higher than 20 g L^{-1}, the PHB concentrations produced in previous studies were higher than the PHB concentration achieved in this study using JA hydrolysates with 28 g L^{-1} initial fructose concentration.

Figure 3 presents kinetics of total dry weight, PHB concentration and fructose concentration during shake flask fermentations carried out with a medium containing commercial sources of nutrients (20 g L^{-1} fructose and

600 mg L^{-1} FAN) and two JA hydrolysates (14 and 28 g L^{-1} fructose concentration and 490 and 430 mg L^{-1} FAN concentrations, respectively). Fructose was consumed at a higher rate when commercial medium was used. When a JA hydrolysate of 28 g L^{-1} fructose concentration was used, fructose was not entirely consumed when microbial growth and PHB production stopped after 56 h fermentation. In the experiment with JA hydrolysate of 14 g L^{-1} fructose concentration, fructose was completely consumed at approximately 50 h fermentation. This indicates that JA hydrolysate does not provide a sufficient source of nutrients to support microbial growth and PHB production. This could be also indicated by the trend of total dry weight and PHB concentration. In the case of the commercial medium both profiles are increasing linearly until complete fructose consumption, whereas in the case of JA hydrolysates the linear trend changes at approximately 30 h fermentation. The linear trend also indicates oxygen limitation during shake flask fermentations.

In Fig. 3, total dry weight represents the summation of microbial mass and intracellular PHB. Total dry weight increases constantly during fermentation because of either microbial growth or PHB accumulation. It should be stressed that the highest PHB concentration produced by using either commercial media or JA hydrolysates were similar. This indicates that optimisation of JA-based fermentation media should be employed so as to improve PHB fermentations. Future experiments should also focus on fermentations using JA hydrolysates in bioreactor experiments in order to control aeration and pH that will lead to concrete conclusion on the effect of JA hydrolysates on PHB accumulation by *C. necator*.

Future studies focused on further optimisation of JA meal hydrolysis to increase the concentration of fructose (and glucose) in the final hydrolysate will be necessary in order to develop fed-batch fermentations in a bioreactor. In addition, the results presented in Table 2 indicate that the FAN and other nutrient content of JA hydrolysates are too low to support sufficient bacterial growth for high levels of PHB accumulation. This problem could be solved either by enhancing protein hydrolysis in JA meal or by using a crude protein-rich industrial supplement. The first option has been explored by exploiting in the hydrolysis of JA a fungal strain (namely *Aspergillus oryzae*) that has been characterised as a prominent protease producer [18, 40]. Protein hydrolysis was not, however, improved significantly (results not presented). It should be stressed that the low protein content in JA meal will not provide a sufficient amount of nitrogen for subsequent PHB fermentations. For this reason, future studies should focus on the utilisation of crude protein-rich industrial by-products as raw material for the production of nitrogen-rich as well as other nutrient-rich fermentation supplements. Oilseed residues from

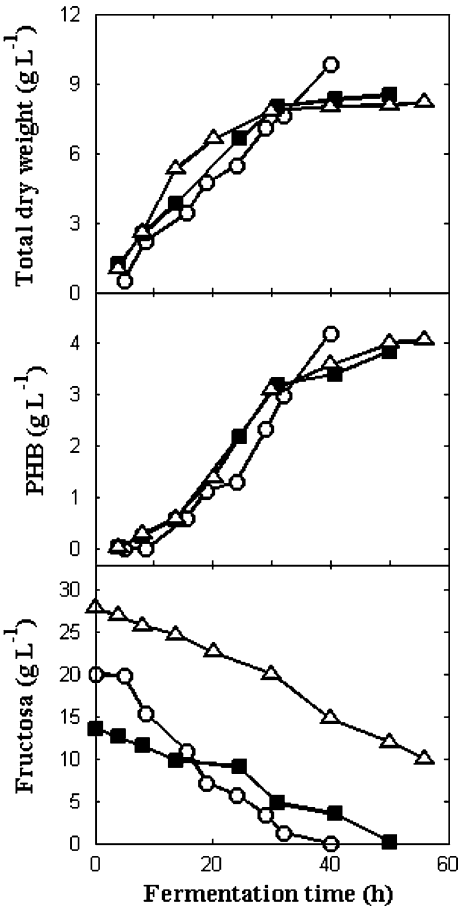

Fig. 3 Concentration profiles of total dry weight, PHB and fructose during shake flask fermentation of *C. necator* using a medium with commercial sources of nutrients and two JA hydrolysates. Symbols: (*open circle*), Commercial medium with 20 g L^{-1} fructose and 600 mg L^{-1} FAN; (*filled square*), JA hydrolysate with 14 g L^{-1} fructose and 490 mg L^{-1} FAN; (*open triangle*), JA hydrolysates with 28 g L^{-1} fructose and 430 mg L^{-1} FAN

biodiesel production plants (e.g. sunflower, soybean and rapeseed) could be regarded as ideal renewable resources for the production of such crude nutrient supplements [41, 42]. Oilseed residues are particularly rich in protein and minerals including phosphorus, thus offering high biotechnological potential. Rapeseed hydrolysates with up to 2,061.2 mg L^{-1} FAN and 304 mg L^{-1} inorganic phosphorus could be produced via a novel SSF-based bioprocess [42].

Potential of JA for PHB Production

The economic perspective of PHB production from JA cannot be estimated with accuracy because the whole bioprocess has not been optimized yet. At this stage, it is more meaningful to present the potential of PHB production from JA as compared to other agricultural crops.

Table 4 PHB production yield and market price for wheat, corn, sugarbeet and JA

Crop	Country	Crop production yield (tn/ha)[a]	Carbohydrate content (g/g)[b]	Carbohydrate yield (tn/ha)	Potential PHB production (tn/ha)[c]	Market Price ($/tn)[d]
Wheat	World	3	0.65	2	1	–
	USA	3.12		2	1.1	215
	China	4.75		3.1	1.6	–
	Argentina	3.41		2.2	1.2	144
Corn	World	5.21	0.6	3.1	1.7	–
	USA	9.60		5.8	3	148
	China	5.46		3.3	1.7	–
	Argentina	7.81		4.7	2.5	117
Sugarbeet	World	48.86	0.19	9.3	4.8	–
	USA	61.88	0.145	9	4.7	73.5
	China	42.45	0.19	8.1	4.2	–
JA	[43]	40–70	0.15–0.18	6–12.6	3.2–6.7	–
	[44]	90	0.04–0.15	3.6–13.5	1.9–7.2	180

[a] The crop production yield for wheat, corn and sugarbeet were taken from FAO for 2010 (http://faostat.fao.org/site/567/DesktopDefault.aspx?PageID=567#ancor)

[b] Based on average carbohydrate content (wheat, corn, JA) or recovery rate achieved (only for sugarbeet in USA)

[c] Based on a glucose to PHB conversion yield of 0.48 g g^{-1}. An inulin, starch and sucrose to sugar conversion yield of 1.1 has been used

[d] The market price for wheat and corn were taken from FAO Statistical Yearbook 2010. The market price for sugarbeet were taken from USDA (*source*: "Agricultural Prices," Agricultural Statistics Board, NASS, USDA) for 2010/11. The market price for JA refers to JA powder and was given by Cheng et al. [45]

Table 4 presents the prospects of JA as a renewable feedstock for PHB production in comparison to corn, wheat and sugarbeet. The crop production yield per hectare, carbohydrate content and market price for JA were taken from literature-cited publications [43, 44]. Table 4 shows that potential PHB production per hectare of cultivated land is higher in the case of JA provided that optimum yields are achieved. Furthermore, as calculated in several publications investigating the potential of JA utilisation for bioethanol production as compared to other roots and tubers, the bioethanol yield was much higher. For instance, Mays et al. [6] identified JA as the second best crop among various carbohydrate producing crops (sweet potato, sweet sorghum, potato, sugar beet and fobber beet) regarding bioethanol production yield per hectare of cultivated land. This means that the potential of JA utilisation for PHB production should be investigated further.

The price of crude JA powder was found only in the case of China as $180 per tn, which is lower than the respective price of glucose [45]. This price will be lower in the case that the crude JA tuber is used. Table 4 shows that this market price is comparable to wheat and corn and higher than sugar beet. Cheng et al. [45] claimed that JA is a drought-resisting crop that can be cultivated in desertified land for soil improvement and water conservation. In addition, JA has low requirements for fertilizer, pesticides and water supply. For this purpose, JA is more resistant crop than wheat, corn and sugar beet in desert conditions and frost. This is an important advantage of JA utilization for chemical and biomaterial production.

Conclusions

JA has been evaluated as a potential renewable feedstock for PHB production. A fungal strain of *A. awamori* was employed to produce enzyme consortia via SSF that could efficiently produce a JA hydrolysate rich mainly in fructose with lower concentrations of glucose, FAN and phosphorus. Shake flask fermentations using JA hydrolysate as fermentation medium showed that PHB production is feasible. However, enrichment of JA hydrolysates with nutrient-rich supplements (e.g. oilseed meal hydrolysates) should be carried out in bioreactor applications.

It should be stressed that industrial implementation of PHB production has not been established yet due to the high cost of conventional raw materials and processing stages. The utilisation of non-food crops or industrial waste streams could assist in the commercialisation of PHB production. JA cultivation for chemical and biopolymer production could be achieved in desertified land where water shortage is unavoidable and irrigation is not effectively achieved. Lal [46] reported that the worldwide land area prone to desertification is approximately 3.5–4 billion ha, while the current annual rate of desertification is approximately 5.8 million hectares.

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