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Bioprocessing of seaweed into protein enriched feedstock: process optimization and validation in reactor

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Abstract

Solid state bioconversion (SSB) of *Ulva* seaweed for protein enrichment was conducted over 7-days using *Phanerochaete chrysosporium (P. chrysosporium)*. One-factor-at-a-time (OFAT) optimization of process parameters revealed 70% for moisture content, 9% for inoculum size and 7% for minerals. Optimum substrate depth in 1 kg reactor was 12 mm and the protein increased from 88.51 mg/g to 107.89 mg/g. Fourier transformation infrared spectroscopy (FTIR) analysis showed modifications in the polysaccharide resulting from SSB. Soluble and reducing sugars concentrations improved by 168.88% (from 33.74±0.51 mg/g to 90.72±1.38 mg/g) and 198.95% (from 0.13±0.05 mg/g to 0.40±0.01 mg/g), while total carbohydrate reduced to 107.15±1.21 mg/g from initial concentration of 109.39±1.33 mg/g.

Introduction

Seaweeds by their natural composition are important algae colonizing several marine coastal areas. Many species of seaweeds are normally used in their unprocessed forms in folk medicine, human diets, animal feed and manure for agricultural land improvements (Felix, 2011). The rheological properties of such seaweed species such as gelling properties of their sulphated polysaccharides enables them to carry out such biological activities for improved utilization beyond folk medicine. *Ulva* is a seaweed species with bright green sheets and domiciled in marine environments and in brackish water; most especially in estuaries. This species is one of the important types of seaweed found abundantly in many coastal areas of many countries and contains abundant minerals, protein and vitamins (Burtin, 2003).

Utilization of *Ulva* seaweed as animal feed or supplement is a daunting task since bioavailability of the nutrients embedded in the polysaccharide remained elusive due to inefficient metabolism by animals. This chemical limitation generally impedes efficient use of *Ulva* seaweed as sole animal feed. Although several seaweed species contain valuable amino acids of immense nutritional efficacy; their release can be poor due to crosslinking within the polysaccharide matrix of the algae mass.

Bioconversion process involving fungi, bacteria and yeast remained a viable option in metabolizing polysaccharide molecules present within the matrix of seaweed. Bacteria strains are less utilized for bioconversion since they lack specialized enzymes that can degrade algae polysaccharide membranes. Similarly, high concentration of nucleic acid within bacterial cells limits their use as feed sources for animals. Therefore, fungi and yeast are preferred microbial classes for bioconversion of a complex solid matrix like seaweed. Similarly, fungi cells harbor high protein, vitamins and low nucleic acid after successful bioconversion process (Dhanasekaran *et al.*, 2011). *P. chrysosporium* is a filamentous fungus that can break down seaweed polysaccharides through its multi-enzyme secretion during bioconversion process (Gad *et al*., 2010).

Solid-state bioconversion (SSB) entails the growth of microorganisms on a solid matrix devoid of absolute aqueous medium. It has been widely studied over years because of its economical technology, high through put, low contamination issues and high potential of successfully converting inexpensive agro-industrial wastes, as well as plant substrates, in a large variety to value added industrial compounds

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(Rodríguez-Jasso *et al*., 2013). When compared to the liquid media used in submerged fermentation systems, the solid media used in SSB contains less water, but they present an important gas phase between the particles. This condition favours growth and development of filamentous fungi, which have great capacity to colonize the inter-particle spaces of solid matrices (Raghavarao *et al*., 2003; Rodríguez-Jasso *et al*., 2013). Therefore, the objective of this paper is to examine the conditions necessary for optimization of protein synthesis of *P. chrysosporium* using *Ulva* seaweed as carbon source.

Materials and methods

Material collection and cleaning

Seaweeds (*Ulva* sp.) were collected from the laboratory stock of Universiti Kebangsaan Malaysia. The samples were thoroughly washed with clean tap water to separate stones and dirt before oven drying at 60°C. Dried seaweed was then grinded through 2 mm sieve size and stored in air tight container for future use.

Inoculums preparation and Solid state bioconversion (SSB)

Laboratory stock strain of *P. chrysosporium* (ATCC 20696) was cultured on Potato dextrose agar (PDA) plates at 32°C for 5-7 days until the fungi covered the plates. The plates were washed with 25 ml sterilized distilled water using L-shaped rod then poured into sterilized flask. Excess inoculum was stored at 4°C for further use. The fungi strain was subcultured every fortnight to keep it in good shape. SSB was carried out in 250 ml Erlenmeyer flasks with 30% solid substrate and 70% liquid containing mineral supplements (KH_2PO_4 0.5 mg/L, $NH_4H_2PO_4$ 0.4 mg/L, $FeSO_4$ 0.3 mg/L, KCl 0.5 mg/L, $MgSO_4$ 0.6 mg/L and Sucrose 5 mg/L). Inoculums were added after sterilization of media at 121°C for 15 min in an autoclave.

Protein assay

Protein content of the sample was determined using folin phenol reagent method by (Lowry *et al*., 1951). 100 mg of sample was dissolved in 1 M NaOH and allowed to stand for 24 h. samples were centrifuged at 6000 rpm for 15 min and supernatant was assayed for protein using bovine serum albumin (BSA) for standard curve and absorbance was read at 660 nm.

Determination of total soluble sugar and total carbohydrate

Total soluble sugar and total carbohydrate of samples were determined according to phenol sulphuric acid standard methods previously described (Dubois *et al*., 1956). For total carbohydrate, samples were hydrolysed with 2.5 N HCl for 3 hours before carrying out the experiments for phenol sulphuric acid standard method for sugars. All absorbance were measured at 490 nm, triplicate samples were analyzed and glucose solution was used for standard curve.

Determination of total reducing sugar

Aqueous extraction of reducing sugar from grinded seaweed was performed in 50 ml stoppered conical flask containing 5 g seaweed powder. 10 ml of 0.2 (mol/L) of disodium hydrogen phosphate, 0.1 (mol/L) of citrate buffer (pH 4.8) was added. The sample was allowed to equilibrate at room temperature for 24 hrs followed by centrifugation at 6000 rpm for 15 min. Total reducing sugar of the supernatant was determined by dinotrosylsalicylic acid (DNS) method (Miller, 1959).

Fourier Transformation Infra-red (FT-IR) analysis

Samples for the FT-IR analysis were grinded finely into powder and mixed with potassium bromide (KBr) before pressing into pellet discs of 10 mm diameter. FT-IR spectra were obtained from computer connected with the spectrophotometer (Bruker Tensor 27). Spectra were collected within pre-set wave number region of 400-4000 cm^{-1}, with a spectral resolution of 4 cm^{-1}. Functional groups characteristics of samples were investigated using infrared spectroscopy.

Protein enrichment in tray reactor and scale up process

Validated process conditions in shake flask was initially transferred to a plastic reactor with 160 mm x 110 mm x 58 mm dimensionand 3 kg capacity. Five equidistant circular holes of about 5 cm diameter were drilled on the reactor cover and plugged with cotton to facilitate sterilized air transfer. Substrate depth was varied between 8 mm and 24 mm to leave one third space for air circulation. The reactor and content was sterilized and inoculum was added from each hole to facilitate even distribution. The reactor was incubated in under constant humidity (~95%) and 32°C. The whole experiment was performed in triplicate.

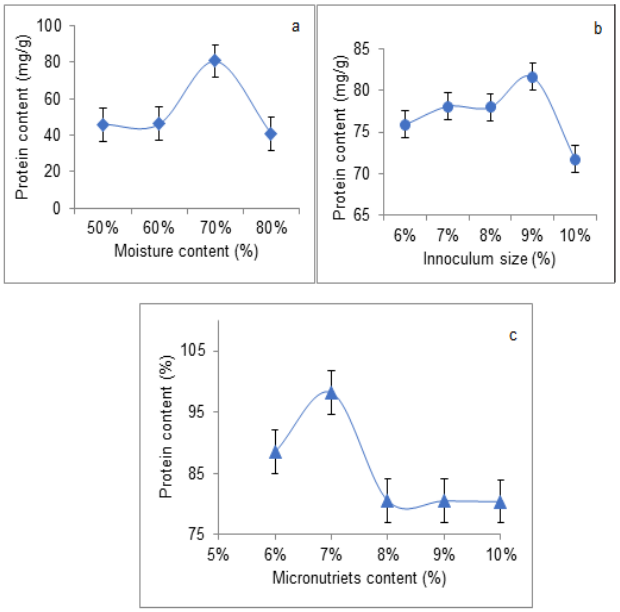

Figure 1. Protein profile of *P. chrysosporium* (a) on moisture content (b) on inoculum size (c) on micronutrients

Results and discussion

Process optimization

In order to ascertain the optimum level of each process condition on protein enrichment of the seaweed, each process parameter was pre-optimized using OFAT method.

Effect of moisture content

The most contributing point for moisture content was elucidated by varying the percentage moisture content of the substrate bed between 50% and 80%. Result obtained showed a gradual change in protein content from 50% (46.0 mg/g) up to 70% (80.65 mg/g) moisture content where synthesis peaked; protein enrichment plummeted with further increase in moisture content to 80% (40.99 mg/g) (Figure 1a). Increase in moisture content of substrate bed above 70% encourages anaerobiosis through oversaturation of the void spaces within the substrate particles (Pandey, 2003). Similarly, current result agreed with other reports which opined that at 70% moisture content enhanced aeration, optimum nutrient release and efficient mass transfer are peaked for synthesis of crude protein by white rot fungi (Ruqayyah *et al.*, 2014).

Effects of Inoculum size on protein profile of P. chrysosporium

The volume of inoculum (live cells) used in any bioconversion process is of paramount importance to successful cell growth initiation, propagation and enrichment of the substrate bed. Therefore, the percentage inclusion in the fermentation bed was varied between 6% and 10%. A steady increase in protein content of the seaweed was recorded with maximum protein secretion at 9% inoculum size (Figure 1b). Although elevated amount of inoculum has been reported to trigger accelerated substrate consumption without commensurate product synthesis (Mahanama *et al*., 2011); however in the present study, positive influence of higher inoculum concentration could be attributed to high content of metabolizable polysaccharides (cellulose, lignin, hemicellulose) within the matrix of the substrate. Similar trend has been reported concerning highly lignocellulosic substrates where presence of higher microbial population was necessary to percolate the inner voids on the substrate for improved cellular secretions (Raghavarao *et al*., 2003; Mahanama *et al*., 2011).

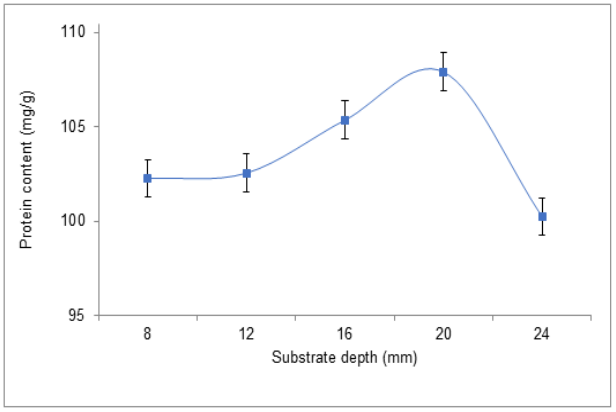

Figure 2. Effect of substrate depth on Protein synthesis

Effects of micronutrients on protein profile of P. chrysosporium

To investigate the required amount of micronutrients necessary for increased protein enrichment of seaweed, the micronutrients concentration was varied between 6% and 10%. Results obtained showed that fungal protein synthesis increased sharply between 6% and 7% but dropped at concentrations above 7% (Figure 1c). Fungi cells require sufficient amount of minerals to accelerate cell walls formation through crosswall movement of essential minerals that facilitate ion exchange. This process usually caused synthesis, progression and merging of fungi hypae that penetrate substrate bed during SSB. Increased concentration of minerals above acceptable threshold could therefore impede fungal performance for extracellular product synthesis due to slow cellular growth and development (Saheed *et al*., 2012). Current result supported this assertion as evident in the sharp drop in protein production as the mineral content increased above 7%.

Table 1. Effect of bio-processing on sugar composition of seaweed

Sugar component	Control sample (mg/g)	Processed sample (mg/g)	Difference (%)
Soluble sugar	33.74±0.51	90.72±1.38	168.88
Reducing sugar	0.13±0.05	0.40±0.01	198.95
Acid soluble carbohydrate	109.39±1.33	107.15±1.21	2.05

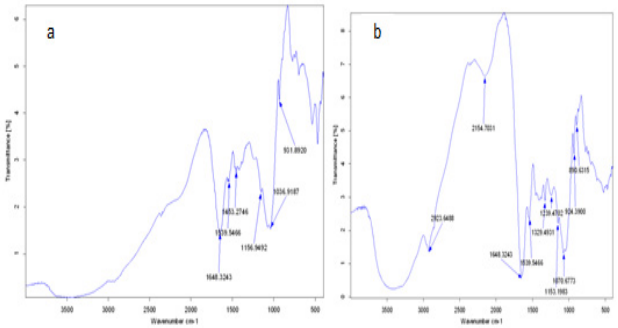

Figure 3. FT-IR spectral of samples (a) raw seaweed (b) protein enriched seaweed

Evaluation of protein enrichment of seaweed in try reactor

At this level, all the values of OFAT optimized parameters were adopted and the substrate bed was varied. Result showed a gradual increase in protein synthesis by the fungi as the depth increased from 8 mm to 20 mm, while sharp decline was recorded afterwards (Figure 2). The sharp decrease in production at elevated depth could be attributed to reduced mass and energy transfer caused by loss in void spaces within substrate particles. Increase in substrate thickness above 18 mm have been reported to reduce enzyme production by Oyster Mushroom with corresponding gaseous space reduction withing the bed (Raghavarao *et al*., 2003; Nitin Prakash and Sanjiv Kumar, 2012).

The effect of bioprocessing on sugar components of the samples showed that soluble sugar and reducing sugar content of the converted substrate increased while acid soluble total carbohydrate reduced (Table 1). The increase or decrease in soluble and reducing sugar content of plant materials during fermentation could be a signal for fungal metabolism of the substrate as previously reported for lignocellulosic materials (Saheed *et al*., 2013), the quantitative measurement of the two parameters remained elusive during protein enrichment of seaweed.

In order to elucidate the effect of SSB on the protein enrichment of seaweed, FT-IR analysis was conducted to visualize spectra characteristics of the original and fermented samples. The spectra peaks were assigned according to fingerprints of lignocellulosic materials as previously described (Kannan, 2014; Jamal *et al.*, 2015). Results obtained revealed a profound fungal attack on the seaweed chemistry causing modifications and bond re-organization (Figures 3a and b).

Glucose units of the substrate was suspected to be consumed during the fermentation as 1036 cm^{-1} (S=O stretching) peak quoting for it disappeared from the spectra of the fermented sample. Similarly, polysaccharide components of the substrate appeared to be converted to simple sugars due to presence of a peak at 1070 cm-1 (-O- ether group of glucose formation) on the fermented sample (Figure 3b). The appearance and absence of peaks quoting for certain molecule on FTIR spectra signified its absence. The stretching of ether group recorded in the fermented sample could be due to white rot fungi metabolism of the substrate constituents (Tomak *et al*., 2013). The presence of peak at 1153 cm^{-1} (C-O stretching) on the spectra showed cellulose modification and possible consumption by the microorganism while pronounced peak at 1539 cm^{-1} (C=O stretching) signal attack on lignin by the fungi. Results recorded are consistent with previous opinion that *P. chrysosporium* posses the ability to modify complex polysaccharides during bioconversion processes (Pandey and Pitman, 2003).

Conclusion

OFAT methodology revealed optimum values of process parameters investigated for protein enrichment of seaweeds through cultivation of *P. chrysosporium* under SSB. Moderate substrate depth in reactor was necessary to maintain protein enrichment with impact on concentration of soluble and reducing sugars. Bond streaching was predominant process utilized by P. chrysosporium during conversion of the substrate for protein synthesis.

Conflict of Interest

There is no potential conflict of interest.

Acknowledgments

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