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β-GLUCANS BIOPRODUCTION TO MINIMIZE THE ECOLOGICAL IMPACT OF EFFLUENTS DERIVED FROM THE MUSSEL PROCESSING INDUSTRY.

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ABSTRACT

In the NW of Spain, the annual production of mussel is 2×10^6 tons (35% of the world). The thermal treatment in the industrial process generates between 300-400 L/t of mussel processing wastewaters (MPW) which have been continuously dumped into the sea without depuration. These effluents, relatively rich in organic matter (7 g glycogen/L and 25 g COD/L), contribute to the progressive deterioration of the marine ecosystem. Based on a laboratory optimization and industrial pre-scale trials, a biotechnological process has been developed, in which the effluents of MPW are transformed into a culture medium (acidification, clarification, ultrafiltration) and used for the production of biomass. Afterwards, the obtained biomass is isolated, fractionated and purified through different processes producing several bioproducts applicable in different sectors: 1) single cell protein (SCP); 2) an extracted cell wall material with a high content in glucans and glycoproteins and 3) purified fractions of 1,3-β-glucans and mannoproteins were obtained after several steps of alkali and acid fractionation of the yeast cell walls. The process was re-scaled and dimensioned for industrial application. In consequence, this paper provides an environmentally friendly, eco-designed and profitable solution that allows integrating the mussel industry into the ecosystem in a sustainable way.

Keywords: industrial waste; optimization; sustainable industrial process; environmental protection; bioseparation process; isolation and purification of bioproducts.

INTRODUCTION

The development of a treatment system for the effluents derived from the mussel processing industry that are being released (~100,000 m^3/year) to the estuary coastal waters at the NW of Spain has worried the Galician scientific community since many years. The focus of the solution is to transform MPW to a suitable growth medium [1-4], microbial growth with an amylolytic microorganism for the production of SCP. Additionally, the residual effluent have be used for production of several bioproducts [5-7], encouraging its industrial application. Despite the implication on the estuary marine ecosystem, in some occasions the seasonal uncertainty of raw materials, and in others, the low profitability of the products obtained, had not allowed the application of a treatment at industrial level able to reduce the environmental impact of these continuous MPW effluents. In this context, the objectives of this study were to transform the industrial MPW effluents into SCP using a the yeast *Saccharomyces fibuligera* that has been found [8] to be formed by a remarkable higher content of T_S/X and is capable of producing enzymes that hydrolyze the main source of carbon present in the mussel (glycogen) effluents, which reduces the complexity of a industrial process facilitating SCP production. Therfore, a waste material was transformed into high-value sub-products and the environmental impact from industries was minimized enhancing the socio-economic factors and foster a sustainable behavior to integrate the ecosystem with the production patterns in a long run.

MATERIAL AND METHODS

Fundamental analytical methods

Dry solids (D_W) and ashes (CZ) were conducted following indications of [9]. *Total sugar (T_S)* using phenol-sulfuric method [10, 11] and r*educing sugars (R_S)* by the dinitrosalicylic acid method (DNS) according to [12], in both with glucose:mannose (0.6:0.4). *Proteins (Pr)* with the Lowry [13] method. *Total nitrogen (Nt)* by the Kjeldahl method and quantify by the spectrophotometer method developed by [14]. *Total hexosamines (Ht)* following the method described by [15] with N-acetylglucosamine as standard. *Fatty acids (FA)* by gravimetric difference between the extracted and non extracted residue in Soxhlet with ether [16]. Enzymatic determination of glycoside linkages β-D-1,3. The analysis was conducted with the commercial product β-D-1,3 glucanase Glucanex® 200G (Novozymes) as described by [17]. *Total amylolytic activity (AAT)* was determined using the R_S production from starch following the description of [18]. The GC monosaccharide determination used was conducted following the work of [8].

Transforming MPW to a suitable growth medium

The mussel process wastewaters (MPW) were kindly supplied by Marcelino S.A. (Galicia, Spain), and their chemical composition was as follows: 7 g/L glycogen, 0.10 g/L reducing sugars, 3.5 g/L proteins and 1.6 g/L total nitrogen. Sediments were not observed in these effluents, and the initial pH was 7.2. The effluents were clarified by centrifugation after acidification (5N HCl to pH 4.5) to precipitate the greater part of their protein content, and concentrated to 20 g/L of glycogen by ultrafiltration with a 100 kDa cut-off membrane [4, 19]. Afterwards, the media was supplemented with 400 mg/L of P (as KH_2PO_4) and 1.200 mg/L of N (as $NaNO_3$ and NH_4Cl), the pH was brought to an initial value of 6.0 and the resulting culture media was freeze until its use. At the time of use, the culture media was sterilized in an autoclave with steam vapor during 60 min.

Microbial methods

The microbial amylolitic specie used (all of them GRAS: Generally recognized as safe) was the yeast *Saccharomyces fibuligera* (other non-used synonyms: *Endomyces fibuliger*, *Saccharomycopsis fibuligera* CBS 2521). The culture growth was conducted in 250 mL flasks with orbital shaking at 200 rpm. At appropriate times, medium and biomass were separated by centrifugation. The supernatant was used for the basic composition analysis, and the biomass pellet was washed twice with distilled water, lyophilized and stored at -18 °C until processing.

The growth analysis was carried out by adjusting the values of the produced biomass (as g/L of dry weight) to the logistic-growth equation:

$$X(t) = K\left[1+\exp\left(c-\mu_m \cdot t\right)\right]^{-1} \;;\; c = \ln\left[\left(K/X_0\right)-1\right] \;;\; v_m = \left(K\cdot\mu_m\right)/4 \;;\; \lambda = \left(c-2\right)/\mu_m \;;\; \tau = c/\mu_m \qquad (1)$$

where X is the biomass (with X_0 and K as initial and maximum values, respectively), t the time and μ_m the maximum specific rate. Other values of interest defined from (1) were the maximum growth rate (v_m), the lag-phase (λ) and the time (τ) corresponding to the production of the half maximal biomass.

Cell walls extraction and treatment

All fractions (except for the CIT one, in which dialysis and lyophilization steps were omitted) were treated as follows: 1) dialysis with a cut-off membrane of 12 kDa using ~50 L of distilled water; 2) lyophilization; 3) drying over KOH at 50 °C/24 hr, gravimetric analysis and grinding; 4) determination of main compositional analysis (T_S, R_S, *Pr*, *Nt*, *Ht* and *FA*); 5) GC identification and quantification of the main monosaccharide components; and 6) quantification of the proportion of β-D-1,3 links by enzymatic analysis.

The method for CW extraction was described previously [8,20], which is based on the cell autolysis in autoclave and a CW separation by centrifugation as follows: a) a suspension of 30 g/L biomass in distilled water at pH 6.0 (200 mM TRIS) was treated in a previously heated (at 80 ºC to avoid the initial states) autoclave during 20 min at 120 °C; b) the suspension was centrifuged, the sediment was resuspended in distilled water at pH 6.0 (200 mM TRIS); c) ten treatments (1 min, 3 min intervals) with a double volume of glass beads were performed to remove CW impurities as much

as possible; and d) the solution was centrifuged, all the supernatants were collected conforming the cytoplasm fraction (CIT), and the pellet fraction was taken as the CW fraction.
The alkali treatment was carried out at a concentration of 10 g/L, with 1 M KOH for 24 h with gentle magnetic stirring. The suspension was centrifuged and the pellet was re-extracted two consecutive times during 2 h under the same conditions. The supernatants were taken as the alkali-soluble fraction (AS), and the sediments as the alkali-insoluble fraction (AI). The temperature effect of process was analyzed in detail at 10, 30, 50, 70 and 90 °C.
The acid treatment was carried out at a concentration of 20 g/L, with 1M acetic acid at 90 °C for 90 min with magnetic stirring. The sediment and supernatant were separated by centrifugation and collected. The process was repeated three times for the sediment. The supernatants collected are considered as the alkali-insoluble acid soluble fraction (AIAS) and the sediment as the alkali-insoluble acid-insoluble (AIAI).
The pH precipitation treatment was carried out adding acetic acid (50 % w/v) to the AS fraction until the pH was 4.5, at which most of the protein content can be removed easily by centrifugation. The sediment fraction is named as ASpH and the supernatant is used in the next purifying step.
The alcohol precipitation of the above supernatant was treated with 2 volumes of ethanol and was centrifuged. The discarded supernatant and sediment fraction is named as ASET.

RESULTS AND DISCUSSION

Microbial growth on residual media

Figure 1 shows the experimental results of *E. fibuliger* fermentation on the MPW based medium. Maximum growth was obtained at 55 h with 12 g/L of biomass (as dry weight). At that time, the glycogen from the residual culture media was completely consumed and the pH increased from 5.3 to 6.8. From 30 h the yeast amylases were deactivated due to the drop in the concentration of the reducing sugars. In addition, 1.3 g/L of protein-Lowry was consumed by the yeast. Therefore, 55 h of culture were selected in order to obtain the maximum biomass.

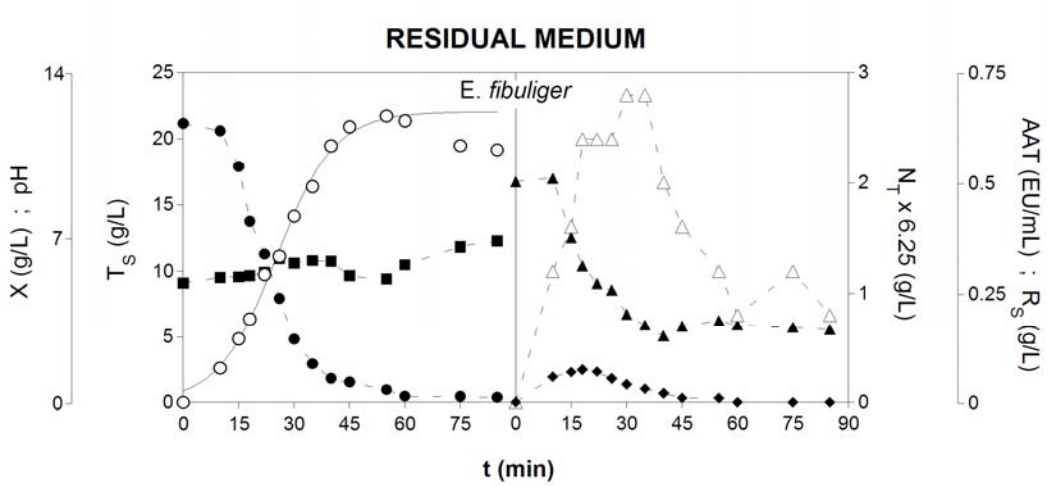

Figure 1: Kinetic growth of biomass (○) and behavior of the main compositional products (▲ T_N x 6.25, ● T_S, △ R_S y ■ pH) of S. *fibuligera* fermented in residual medium at 30 °C.

The parameter estimations from equations (1) describe a maximum asymptotic value (K) with 12.37 g L^{-1}, a specific velocity (μ_m) of 0.126 hr^{-1}, an adjusting c coefficient of 3.19, a maximum velocity (V_m) of 0.391 g X L^{-1}, a lag phase (λ_m) at 9.38 hr^{-1}, the time to achieve the half maximum growth (τ) of 25.22 hr^{-1} and an overall r^2 coefficient of 0.9956. All parameters were statistically significant.

Biomass and CW yields and composition

The composition of the dry solids (D_W) of the biomass at 55 h of cultivation was (in %): 59.2 ± 2.5 total sugars (T_S), 7.63 ± 0.77 reducing sugars (R_S), 14.9 ± 0.7 of fatty acids (*FA*), 1.6 ± 0.2 ash (*CZ*) and 6.4 ± 0.4 proteins (*Pr*) as N_T × 6.25. The chemical composition is presented in Table 1.

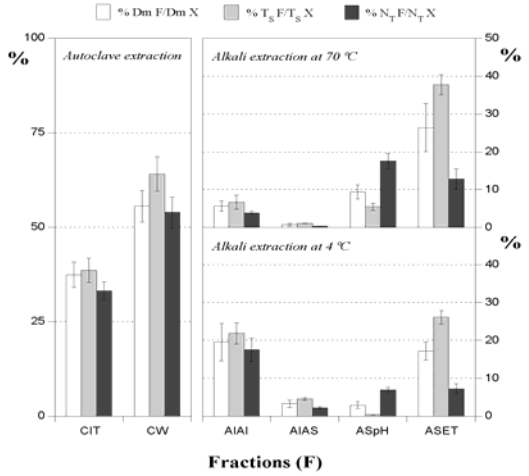

Figure 2: Polysaccharides structures and compositional analysis of the of the fractions obtained: (A) Extraction of the CW; (B) Alkaline extraction at different temperatures, and (C) Purification of AS and AI fractions obtained at selected extraction temperatures. Note that the data for the fractions compositional analysis are referred to the % X and the structure in % T^S. Note that the confidence intervals (±) are in percentages.

The extraction of the CW by the combination of both treatments (autoclave and glass beads) may not be as efficient as other more laborious methods [15], but produces stable and comparable fractions, and it is a rapid analytical tool and easily incorporated into industrial processes. Dry biomass (500 g) was obtained in a residual media for the compositional analysis of *S. fibuliger* CW. The results are shown in the first part of Table 1 and Figure 2. The values for the CW analysis were (in % of CW dry solids): 69.3 ± 1.2 of T_S, 25.9 ± 1.4 of *Pr*, 0.7 ± 0.1 of *FA* and 3.3 ± 0.2 of *CZ*. No R_S were detected in CW. A 54.7% yield of CW was obtained from the *S. fibuliger* biomass. This value was higher than those reported in the bibliography [21,8]. We want to highlight the 51 % of total sugars and the 29 % of proteins. The CW obtained exhibits a whitish color with slight brownish and has the typical rigidity of yeast cell walls. The brown tone is attributed to the protein content in the CW, which is mostly accepted to be covalently bonded to Man molecules [21]. The CW rigidity is attributed to the presence of 1.3-β-D-glucan links [22] that would form a three-dimensional platform that provides the structural basis of CW. Emphasize that the total sugars composition of CW was 55 %. This value was higher than those reported in the bibliography [21]. This biomass was then used for the compositional analyses and production of all different fractions described in the material and methods section.

Alkaline treatment. Optimization and performance.

In general, most of the studies focus on the insoluble glucan fraction [23] due to their properties as stimulators of the immune system, reducing agents of cholesterol level in blood stream, antitumor activity and application in cosmetic products [21]. In addition, recently the food industry has shown interest in the soluble glucan fractions as fattening agents or as sources of dietary fiber. Apparently the solubility or insolubility of the glucan fraction is closely related to the presence of the β(1→6) links [24]. In addition, the cell wall is divided chemically in different fractions and the resulting composition is described in detail (Table 1 and Figure 2).

The alkaline treatment is commonly the first step applied in the CW chemical separation, in which the following fractions are obtained: AS composed mainly by soluble glucans, mannans, proteins and lipids; and AI composed by insoluble glucans and chitin .When insufficient alkali treatments are applied, proteins and polysaccharides of Man may also be found. A 1 M of KOH was used and the effect of T (4 and 70 °C) is assessed. The results are displayed in the second part of Table 1 and summarized bellow:

- The increase of T causes a decrease in the total DW of the AI fraction and in the proportion of T_S/AI, increasing almost proportionally in the AS fraction. Overall, the recovery rate does not undergo significant changes between the two fractions.
- The T_H (chitin) shows a decrease as the T increases, which is attributable to the thermo-alkaline deacetylation of the N-acetylglucosamine links (chitosan).
- As T increases the remaining proteins (T_N) move from the AS to the AI fraction.
- Theβ-D-1,3 links and the presence of Glu increases in the AI fraction at higher T. The proportion of Man increases in the AS fraction as the T of the extraction increases.

In general, the increase of T affects the AI fraction by increasing the proportion of polysaccharides of Glu with β-D-1,3 links, solubilizing the protein and polysaccharides of Man -probably as part of

larger structures (mannoproteins)- to the AS fraction. Thus, both temperatures for the alkali treatment were found suitable for further analysis. At low T the process is less costly on an industrial scale, but also less effective. At high T the a more optimized extraction process is obtained, but more expensive than at lower T.

Purification steps

The obtained insoluble and soluble fractions could be used directly for some industrial sectors, but with some chemical, mechanical and enzymatic treatments could be purify and used in more refined and expensive products. In this work, the basic purifications processes were explored. On the bases of this analysis, the simple purifications steps were used: an acid treatment for the AI fraction and a pH and ethanol precipitation for the AS fraction, allowing to establish the bases for more complex treatments. The compositional transformations of the products were described and summarized in Table 1.

Purification of the AI fraction. Isolation of 1,3-β-glucans fraction

The most interesting compounds in this fraction are the β-glucans. There are polysaccharides formed mainly by β(1→3) and those containing β(1→6) and β(1→3). The Common chemical process to separate both is by treating the AI fraction with a weak acid (more details see material and methods section). The acid extraction of the AI fraction (10 to 70 °C) produces the results are summarized below (Table 1):

- The results shows that after the acid treatment, independently of the temperature of the alkali treatment, the produced AIAI fraction mainly contains Glu polysaccharides linked β(1→3) In the other hand the AIAS is formed mainly by polysaccharides of Glu with β(1→6) and β(1→3) links.
- When alkali treatment was conducted at 10 °C the acid extraction is much less effective than the results obtained when is performed at 70 ºC
- At 70 ºC a purified product is product is produced formed by an 80 % of 1,3-β-glucans. Higher results are obtained if a series of enzymatic treatments are added.

These results suggest that to obtain higher purifications the alkali treatment should be run at higher temperatures.

Purification of the AS fraction. Isolation of a glycoprotein fraction

Some the easy and typical purification steps involve the precipitation of the protein content at pH close to 4.5 to remove the higher molecules of protein, and afterwards, to the remaining fraction material a precipitation with ethanol will isolate the glycoprotein structures. These purification steps were able to isolate a fraction ASET that presents a higher content on glycoprotein.

Table 1: Polysaccharides structures and compositional analysis of the of the fractions obtained: (A) Extraction of the CW; (B) Alkaline extraction at different temperatures, and (C) Purification of AS and AI fractions obtained at selected extraction temperatures. Note that the data for the fractions compositional analysis are referred to the % X and the structure in % T^S. Note that the confidence intervals (±) are in percentages.

fractions		***compositional analysis (%)***				***sugar composition***		
treated	***obtained***	**D_W** *% g F/g X*	**T_S** *% T_S F/T_S X*	**T_N x 6.25** *% T_N F/N_T X*	**T_H** *% T_H F/H_T X*	**Glu** *% Glu/T_S F*	**Man** *% Man/T_S F*	**βD-1,3** *% βD-1,3/T_S F*
(A) Cell wall treatment								
BIO	**CIT**	37.36±5,0	38.49±4,8	33.07±3,5	–	95.35±4.2	4.65±3.6	–
	CW	55.56±6,3	64.06±6,8	53.92±6,0	96.23±5,4	62.12±3.7	37.88±5.5	44.91±3.1
(B) Alkali treatment and temperature effect								
CW (4 ºC)	**AI**	23.93±1.8	35.42±1.7	9.33±1.8	90.92±0.9	80.72±3.8	19.28±0.5	41.25±2.5
	AS	24.41±1.6	26.21±1.6	31.22±0.5	–	50.43±3.6	49.56±0.2	24.97±3.2
CW (70 ºC)	**AI**	6.64±1.1	6.96±1.6	1.33±6.9	68.19±1.1	91.35±2.2	8.65±2.6	78.95±0.6
	AS	41.20±1.1	43.60±5.4	35.56±0.3	–	34.14±2.2	65.85±3.2	7.67±0.6
(C) Purification treatments at the selected temperatures								

CW (4 ºC)	**AIAI**	19.54 ±4.2	21.90 ±2.1	17.55±2.9	84.07 ±0.9	85.40 ±8.7	14.60 ±7.7	35.35±2.6
	AIAS	3.30 ±4.9	4.58 ±1.5	2.24±2.8	–	87.24±6.0	12.76±2.1	11.88±0.8
	ASpH	2.93 ±5.0	0.44 ±1.5	6.99±1.6	–	31.97±5.7	68.03±6.5	5.02±1.6
	ASET	17.27 ±2.3	26.14 ±1.1	7.26±3.1	–	65.51±2.3	34.49±2.2	12.79±0.9
CW (70 ºC)	**AIAI**	5.64 ±6.4	6.67 ±2.7	3.87±2.6	68.40 ±0.5	96.45 ±2.8	3.55±2.7	77.05±6.3
	AIAS	0.77 ±4.2	1.11 ±2.0	0.34±2.9	–	85.25 ±6.5	14.75±4.5	7.37±1.1
	ASpH	9.40 ±3.9	5.48 ±2.6	17.57±1.9	–	21.97±1.4	78.03±7.7	3.05±0.3
	ASET	26.43 ±1.4	37.74 ±0.4	12.84±2.6	–	59.20 ±1.4	40.80 ±5.8	7.31±0.4

An approach to the treatment of the mussel wastewater at an industrial scale

The process above described in a lab scale using 250 mL flasks with 50 mL of MPW medium was pre-scaled at industrial scale on a 70 L fermenter (BIOSTAT) with 50 L of MPW. In all cases, higher yields than those obtained in a lab scale were found. Based on these results an industrial process for the production of byproducts from wastewater mussel processing can be formulated. The process is dimensioned according to the size of the fermenter (R=10.000 L). The wastewater effluents from mussel processing industry (~4.7 g/L of glycogen) were deposited in a sedimentation tank (with a 5R dimension), stabilized at pH=4.5 and allowed to stand for ~12 h at room temperature. After ~48 h of a fermentation process a ~140 Kg of CW/R were obtained. The CW pellet obtained can be divided in two ways: (a) Simple process for the direct preparation of cell walls, a product that would be an outlet for feed additive rich in β-glucan, and (b) Process more refined and expensive to obtain a product with pharmacological uses, the process would include an alkaline extraction (KOH or NaOH 1 M at 70 °C) and an acid (1 M H_3PO_4 at 70 °C) with successive centrifugation steps. Obtaining the AIAI fraction formed by more than ~70 % in polysaccharides 1,3-β-D-Glucose. Note that both processes (a) and (b) may be combined. A part of the supernatant from the alkaline and acidic treatment could be used to set the pH of the fermentation and decanter tanks of subsequent fermentation process. While another part of the supernatants can go to the neutralization tank and later recalculated to the settling tank to reuse the content in polysaccharides and proteins (~20 Kg/R). However, the incorporation of *n* fermentation and sedimentation tanks would be multiplied by *n* times to the capability of the process without increasing excessively the initial investment in other industrial equipment such as autoclaves, ultrafiltration plant, centrifuges and extraction tanks, which would need to be properly sized. Thus, considering 100 possible complete processes annually and annual concentration average of ~5 g/L glycogen, an industrial plant with a *n*=20 would be enough to achieve a full recovery of the wastewaters effluents from the mussel processing, generating a range of products that could make the process cost profitable.

CONCLUSIONS

The present paper addresses an effort to find a solution to this problem, based on a laboratory optimization, presenting an industrial management solution, by using MPW effluents as a culture medium for the production of single cell protein (SCP), β-Glucans and mannoproteins, providing local authorities, amongst others, with an environmentally friendly, eco-designed and profitable solution that allows to integrate the mussel industry into the ecosystem in a sustainable way.

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