CHARACTERIZATION OF MICROBIAL BIOMASSES AND AMYLOLYTIC PREPARATIONS OBTAINED FROM MUSSEL PROCESSING WASTE TREATMENT

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Abstract

This work describes a treatment of glycogen-rich wastes from industrial mussel processing, involving the production of a protein fraction and a medium suitable for the culture of amylolytic microfungi. A strain of Aspergillus oryzae was chosen which allows the simultaneous production of single cell protein and a highly stable amylolytic preparation.

The characteristics of the biomass obtained (proportion and in vitro digestibility of the proteins, levels of essential amino acids and nucleic acids, types of fatty acids present) were shown to be suitable, in principle, for animal feeds. The amylolytic preparation, obtained by ultrafiltration (with cut-off at 30 kD) of the cell-free medium, was very similar to commercial α-amylase preparations, containing mainly α-amylase, together with a small proportion of glucoamylase.

Key words: Mussel-processing wastes, production, characterization, microfungal-SCP, amylases.

INTRODUCTION

Approximately 50×10^6 litres per year of highly polluting (COD≈25 g O_2 litre^{-1}), glycogen-rich wastes (mussel processing wastes: MPW) are produced during the industrial processing of mussels at the Galician Rias (northwest of Spain) estuarine systems, whose primary productivity, reaching 260 g carbon m^{-2} year^{-1} (Fraga, 1976), is among the highest in the world.

The possibility of depuration of these effluents, by using them as substrate for the culture of amylolytic yeasts and microfungi, has been studied previously, and it has been found that COD reductions greater than 90% can be reached, with biomass production yields that, depending on the species involved, vary between 66 and 88% on the basis of the glycogen consumption (Murado et al., 1986, 1989).

Moreover, several species of microfungi useful as single cell protein (SCP) sources (specially Aspergillus oryzae) leave a high level of amylolytic activity in the post-incubated media, when the pH of the cultures is maintained above 4"0 to avoid the denaturation of the α-amylase (Siso et al., 1988; Miron et al., 1988).

The microfungi are thought to be less suitable than yeasts for SCP production. However, their safety in this respect has been established in a number of cases (Barnes, 1976; Barker et al., 1981; Anderson & Solomons, 1984; Udall et al., 1984; Friedrich et al., 1987), the microfungal biomass is usually easy to separate from the culture medium (Pomeranz, 1976) and the textural characteristics of the mycelium often favour its acceptability (Worgan, 1976). Furthermore, in the specific case studied here, use of microfungi can promote diversification of bioproducts derived from MPW treatment.

Thus, in this work, the composition of four microfungal species giving high biomass yields when growing on MPW was studied, as well as the main characteristics of the amylolytic preparation obtained from the post-incubated medium of a selected strain of one of them. The basis of a system that could contribute to the financing of the depuration process is described.

METHODS

Culture methods

The species used are given in Table 1. Culture medium ('M', Table 2) was prepared (Murado et al., 1986) by means of a pretreatment by acidification of the crude wastes (original pH near neutrality) to pH 4.5 (adding ca. 0.5 ml 5N HCl litre^{-1}) and subsequent pouring off of the spontaneously clarified supernatant after 3-5 h sedimentation of a

mainly protein precipitate formed. Amounts of NH_4Cl and KH_2P_4 were added to obtain initial nitrogen and phosphorus concentrations of 1.25 and 0.45 g litre^{-1}, respectively.

Cultures for screening of the amylolytic capacity of the various, A. oryzae strains tested were carried out in 250-ml Erlenmeyer flasks containing 50 ml of M medium partially buffered with 0.05 M biphthalate/NaOH, initial pH 6.0 (Siso et al., 1988), in a rotary shaker at 200 rpm. Incubations to study the influence of aeration on amylase production were carried out similarly, but with different volumes of medium (50, 100, 150 and 200 ml) in the 250-ml Erlenmeyer flasks. In all the other cases, submerged batch cultures were performed in a 5-1itre New Brunswick fermentor at 30ºC, with aeration at 1 litre litre^{-1} min^{-1}. Control of pH at 5.5, as well as prolongation of incubation times up to 50-55 h, were used only for simultaneous production of biomass and enzyme preparation from A. oryzae cultures.

As inocula, spore suspensions in sterile distilled water were adjusted (after a previous calibration between 750 nm OD measurement and direct haemocytometric counting) so that the initial populations of the cultures were 0.5 x 10^6 spores ml^{-1} (flask cultures) and 0.5 x 10^5 spores ml^{-1} (starting batches of the fermentor cultures; in further runs, 5-10% of culture volume was used as recurrent inoculum).

Biomass was harvested by paper filtration (except for centrifuge decantation with F. semitectum) at the time of production of maximum biomass, washed with distilled water, lyophilized and, finally, homogenized for analytical determinations.

Enzyme recovery from mycelium-free media

Mycelium-free medium from paper-filtered A. oryzae cultures was ultrafiltered using hollow-fibre Amicon (DC 10LA) cartridges with cut-off at 30 kD, to obtain a retentate volume equivalent to 1/15-1/20 of the initial volume. Then, a phase of diafiltration was started during which the permeate drainage was compensated for by the continuous addition of a volume of tap water equivalent to three times the retentate volume reached in the first phase. Finally, the process was prolonged into a new phase of ultrafiltration, to reach a final retentate volume equivalent to no more than 1/100 of the starting

volume. This final retentate was lyopbilized and the resulting powdered material stored over silica gel at 4ºC.

Analytical methods

Ash: Calcination (550ºC/6 h) in a muffle furnace.

Dissolved oxygen: Direct measure, as percentage of saturation, by means of an oxygen electrode.

Chemical oxygen demand (COD): APHA (1980).

Reducing sugars: 3,5-Dinitrosalicylic acid reaction (Bernfeld, 1951) with glucose as standard.

Total sugars: Phenol-sulphuric acid method (Dubois et al., 1956) according to Strickland and Parsons (1968), with glucose as standard.

Proteins: Method of Lowry et al. (1951), applied to samples previously incubated with an equal volume of 1N NaOH at 30ºC for 20 h. Bovine serum albumin, subjected to the same treatment, was used as standard.

Protein amino acids: Proteins were first precipitated with an equal volume of 10% TCA and the pellet resuspended in distilled water. An aliquot of this suspension was put into an ampoule and HCI added to produce 1 mg of protein per ml of 6 N HCI. The ampoule was vacuum-sealed and treated at 121ºC for 24 h. Aliquots of the hydrolysed sample were taken to dryness and resuspended in 0.1 N HCI for chromatographic analysis, according to the methods of Burbach et al. (1982) and Qureshi et al. (1984).

This hydrolysis procedure destroys the tryptophan and produces small quantities of cysteic acid by partial degradation of cystine and cysteine. The chromatographic method does not allow of the detection of proline.

Fatty acids: GC determination of methyl esters (Miller & Berger, 1985). A quantitative mixture of 32 fatty acid methyl esters (GL 85-NU Check Prep. Inc., Elsyian, Minnesota) was used as standard.

Nucleic acids: In extracts obtained according to the method of Croes (1967), RNA and DNA were determined through ribose (Ogur & Rosen, 1950; Schneider, 1957) and deoxyribose (Burton, 1956; Giles & Myers, 1965) measurements, respectively.

Elemental analysis (C:N:H): In a Perkin Elmer 240 autoanalyser.

In vitro digestibility: Method of Hsu et al. (1977).

Total amylolytic activity (TAA): A sample of 80 µl of cell-free medium (suitably diluted, if necessary) was added to 400 µl of a solution composed of citratephosphate buffer 0-15 M; pH 5.0 (1 volume) and 4% soluble starch (1.5 volumes), previously maintained at 40°C for 15 min. This mixture was incubated for 10 min at the same temperature and the reaction stopped by addition of 480 µl of dinitrosalicylic acid. 1 EU ml^{-1} is equivalent to an increase of 1 mg ml^{-1} of reducing sugars (glucose equivalents) in this assay.

Glucoamylase: Specifically determined by measuring p-nitrophenol released from p-nitrophenyl-α, Dglucopyranoside. For conversion into glucose equivalents of the activities obtained in this way, the method proposed by Miranda et al. (1987) was used.

Proteolytic activity: Measured by determination of tyrosine released from casein solutions, by means of the following procedure: 0.5 ml of cell-free medium (suitably diluted, if necessary) was added to a solution composed of 0.5 ml of 1% (w/v) casein in 0.02 M NaOH and 1.0 ml of 0.4 M phosphate buffer, pH 6.0. This mixture was incubated for 10 min at 30ºC and the reaction stopped by addition of 3 ml of 5% (w/v) trichloroacetic acid. The mixture was shaken and, after 5 min, centrifuged.

A portion (0.5 ml) of the supernatant was then treated with 2.5 ml of 0.1 M NaOH in 2% (w/v) Na_2CO_3 and, after 10 min, with 0.25 ml of Folin phenolic reagent (commercial solution diluted 1:1 in distilled water) and immediately shaken. After 30 min at room temperature, the absorbency was measured at 750 nm. The calibration was carried out against tyrosine solutions in 0.01 N HCl treated in the same way as the supernatant.

Statistical methods

To study the combined influence of pH and temperature on the activity of enzymatic preparations from A. oryzae, a second-order rotatable design was used (Box et al., 1989; Akhnazarova & Kafarov, 1982).

RESULTS AND DISCUSSION

Figure 1 shows a diagram of the suggested MPW treatment which would exploit SCP production from A. oryzae, as well as the amylase content of the post-incubate medium after biornass separation. The amylolytic activity remaining in the post-incubates of A. niger, A. awamori and Fusarium semitectum was significantly lower than in the culture fluid of A. oryzae; so A. oryzae was selected for further experiments on amylase production. The growth features of all these species of fungi when cultured on the M medium were described in previous papers (Murado et al., 1989; Gonzalez et al., 1992), so here only the biomass composition is described.

Screening of suitable strains of Aspergillus oryzae and effect of aeration on growth and amylase production

Despite the relatively qualitative homogeneity (e.g. α-amylase clearly predominant in all cases) that is shown in Figs 2 and 3, the strains tested revealed significant differences from the point of view of compatibility with the different objectives proposed, i.e. reduction of COD and production of biomass and amylolytic enzymes in reasonably short times. Under this criterion, the strain CBS 125-59 represented the best option and was selected for subsequent cultures.

The results of experiments with different volumes of medium in Erlenmeyer (Figs 4-6) reflect the effects of the limitation to oxygen transfer (and probably to mass transfer in general) imposed by the volumes of medium and the rise in viscosity due to the growth of the biomass. The rate of decrease in dissolved oxygen, that reduces when the culture commences lysis, can be interpreted as the principal cause of the differences between microbial activities: biomass and enzyme production, nutrient consumption, pH evolution. Values comparable to case A (50 ml per 250-ml Erlenmeyer) were obtained in the 5-1itre fermentor used in the next part of this work, with aeration rates of 1.0-1.5 litres litre^{-1} rain^{-1}.

Of particular interest is the pronounced effect on amylase production that shows how TAA and glucoamylase levels increase in correlation with the increase in percentage oxygen saturation (Fig. 6) and how the lack of the latter depresses the progressive increase, with incubation time, of the contribution of the glucoamylase to the TAA (Fig. 5).

Various authors (e.g. Fogarty & Kelly, 1980; Paszczynski et al., 1985) have indicated the susceptibility of amylases, and more particularly the glucoamylase from A. niger, to proteases, suggesting that a stepwise degradation of the native forms during the course of culture could be the reason for the multiplicity of forms with regard to molecular weight and activity against different types of dextrins.

Since the reduction in the production rate of amylases after 45 h coincides with a rise in the level of proteases (Fig. 5), the cell-free media from the four types of cultures involving different volumes per Erlenmeyer, after 45 and 68 h incubation periods, were ultrafiltered using Centricon microconcentrators with cut-off at 100, 30 and 10 kD. TAA and glucoamylases determinations in the corresponding permeate and retentate fractions did not show any differences dependent on percentage oxygen saturation or the level of proteases. In every case the majority fraction (>94%) of TAA and glucoamylase was found in the 30-kD retentate, with a small proportion (<5%) of glucoamylase in the 100-kD retentate and with no trace of activity in the 30-kD permeate nor in the 10-kD retentate.

Composition of the sediment from MPW pretreatment and biomass from cultures on pretreated MPW

Table 3 shows the composition of the sediment obtained in the acidification-decantation pretreatment of MPW, and its suitability as a feed supplement is obvious. These data correspond to the sediment 2 in Fig. 1. This results from the resuspension in distilled water and decantation of liquid from the precipitate formed after a second acidification to pH 4.5. Although the volume of this co-product is small, it should be pointed out that its production, which is not difficult, does not add significant cost to the principal process.

The gross composition of the biomass obtained (in all cases 8 g litre^{-1}, approximately, in 40 h of incubation) is given in Table 4. Proteins constitute the main component, except in the case of A. niger, in which the carbohydrate fraction seems to be predominant, although only if the results of the protein determinations by the Lowry method are

considered. The in vitro digestibility values are acceptable and comparable to those found in other similar products (Worgan, 1976; Hsu et al., 1977).

The nucleic acids content is reasonably low in the four species harvested near stationary phase. Taking into account that the Protein Advisory Group of FAO recommends diets with less than 2 g day^{-1} of nucleic acids from SCP origin (for prevention of gout-like diseases) these contents should even permit the use of this SCP as a human food supplement.

Amino acid analysis (Table 5) shows that the four species contain appreciable quantities of essential amino acids, with a total clearly superior to that of the FAO reference protein (FAO, 1974). As is usual in the composition of SCP, the main problem is caused by the absence of methionine (although this is present in F. semitectum). The main benefit is the high proportion of lysine, which makes the product interesting as a potential supplement for cereal-based diets, which tend to be deficient in lysine and threonine.

Total fatty acid content (Table 6) is also low, with linoleic acid (18:2) as the most abundant in the four species and there was no evidence of molecules with a chain length greater than C20. Unusual components in the biomass acids were not detected.

On the basis of these results, it can be concluded that the four species tested represent an interesting potential source of protein for animal feeding, whose safety is now deserving of being studied in detail.

Amylase preparation from A. oryzae cultures

In a typical operation, 8 litres of a post-incubate from A. oryzae (54 h; 55.2 EU ml^{-1}; COD=7.8% of the initial value in the effluent), obtained by paper filtration, were submitted to further clarification in a continuous decanter centrifuge; a process which took 4 min and caused negligible losses in volume and enzymatic activity.

The supernatant was immediately concentrated to 2-5 litres by ultrafiltration with cut-off at 30 kD. Then a diafiltration phase (constant volume) was initiated with continuous

addition of 5 litres of tap water, followed by another concentration step reducing the volume to 0.5 litre. The process was completed in 9.3 min and produced an activity of 846.1 EU ml^{-1} in the final retentate; a recovery of 95.8% of the initial activity.

Finally, this retentate was lyophilized to give 6.49 g of a dry powder with a TAA of 60.85 EU mg^{-1}, which represented a production of 0.811 g litre^{-1}, with a recovery of 89.48% of the activity in the whole process.

The effects of pH and temperature on TAA, considering both variables separately, show identical results (Fig. 7) using both the post-incubated media or powdered preparations. The combined effect of these variables on the powdered preparations was studied by a complete second-order factorial plan, using a rotatable design whose experimental domain and coding criteria are shown in Table 7. The observed and expected responses, as well as the standardized residuals are shown in Table 8.

After contrasting the significance of the model coefficients by Student's t-test (a = 0.05) and the global one of the model itself by the Fisher F-test (similarly a=0.05), the results allow a description of the effects of pH and temperature by the following empirical equation:

$$R = 96.90 + 6.92 \text{ pH} + 6.31 \text{ T} + 17.19 \text{ pH T}$$
$$- 33.84 \text{ pH}^2 - 23.55 \text{ T}^2$$

This presents a maximum at the point (pH=0.15 (natural value 5.7); T= 0.195 (natural value=47.8ºC)) and in which, using coded values for the independent variables according to Table 7, the response (R) is obtained in terms of TAA as a percentage of the maximum. In the general behaviour of the enzymatic system (Fig. 8), the decrease in activity determined by the conjunction of superoptimal pH with suboptimal temperatures is particularly noteworthy.

Although this kind of statistical design seems not to be much used in the type of experimental work described here, the situation constitutes a typical example in which the common strategy of 'one variable at a time' is inadequate, and may give false estimations of the optima.

Finally, Table 9 shows the essential characteristics of the powdered amylolytic preparation (PAP) compared with those of Sigma a-amylase (A-0273). The stability of PAP is high, and no loss of activity was detected after 26 months' storage at 4°C. Its activity is similar to the Sigma product in terms of enzyme units per milligram of powder, but appreciably less in terms of enzyme units per milligram of protein. This is due to the presence in PAP of peptide materials from the MPW.

It should be noted that if the 30-kD ultrafiltration step is carried out on the permeate of a previous cut-off at 100 kD, the specific activity rises to the levels of the commercial preparation as a consequence of the exclusion of a great part of the proteic materials coming from the MPW. Nevertheless, ultrafiltration at 100 kD also excludes the small amount of glycogen remaining in the post-incubated media, which, on concentrating in the 30-kD retentate, increases the stability of PAE

From Fig. 9 it can be seen how the stability of PAP (Table 9) in distilled water solution at 50°C is slightly less than that of the commercial product. However, when PAP is supplemented with glycogen to the same total sugars concentration as the commercial product both stabilities become equal.

The basis of a design for the valuation of MPW

Figure 1 constitutes a schematic diagram of the treatment of MPW described in the preceding sections. Although operations such as the pretreatment of the effluent and the ultrafiltration of post-incubated media from A. oryzae cultures for the production of enzymatic preparations have already been carried out on scales of 500 and 50 litres respectively, at the moment the complete system functions only on a scale of 5 litres, with discharge every 48-55 h.

On the other hand, in a preliminary ultrafiltration experiment (100 kD) with pretreated (acidified and clarified) effluents, it was found that the process leads to the total retention of the glycogen, easily permitting the obtaining of media with a concentration of 100 g litre^{-1} of polysaccharide. The total or partial saccharification of these concentrated media with a part of the amylolytic preparations here described (a subject

we are at present working on) could thus lead to a more versatile microbial substrate, suitable for bioproductions of greater economic interest than SCE

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Table 1. Microfungal species used

Species	Source
Fusarium semitectum	CBS 479-83
Aspergillus awamori	CBS 139-52
Aspergillus niger	CBS 554-65
Aspergillus oryzae	CBS 125-59
A. oryzae	CBS 110-47
A. oryzae	CBS 112-51
A. oryzae	CBS 115-33
A. oryzae	CBS 125-49
A. oryzae	CBS 201-75
A. oryzae	CBS 570-65
A. oryzae	CBS 816-72
A. oryzae	CBS 819-72

CBS: Centraalbureau voor Schimmelcultures, Baarn, the Netherlands.

Table 2. Average composition (g litre^{-1}) of the culture media obtained from acidified MPW, and their range of variation

Glycogen	10.00 (7.00-12.50)
Reducing sugars	0.15 (0.10-0.80)
Proteins	3.50 (2.00-4.00)
Taurine	2.50 (2.00-3.00)
Total nitrogen	1.60 (1.00-2.00)
Total phosphorus	0.09 (0-08-0.11)
NaCI	18.00 (17.00-22.00)
COD (0_2)	25.00 (19.00-26.00)

Table 3. Composition a of the sediment 2 ($\approx$ 2 g per litre of wastes, see Fig. 1) obtained in the pretreatment of the MPW

Organic matter	91.49

Ash	8.51
Crude protein (N x 6.25)	77.82
Lowry protein	70.64
Total carbohydrates	2.91
In vitro digestibility (%)	82.67
Cysteic acid	1.35
Aspartic acid	13.05
Glutamic acid	19.16
Serine	4.97
Histidine	4.65
Glycine	8.64
Threonine	3.34
Arginine	7.09
Alanine	7.01
Tyrosine	2.85
Valine	5.05
Phenylalanine	4.24
lsoleucine	4.81
Leucine	5.87
Lysine	7.91

[a]Values of amino acids are given as % (w/w) with respect to the protein fraction. The other values are given as % (w/w) with respect to the whole sediment 2 or, in digestibilities, the total N.

Table 4. Gross composition (% w/w) of the microbial biomasses

	A. niger	A. oryzae	A. awamori	F. semitectum
C:N:H	100:18:15	100:21:15	100:18:15	100:21:16
Ash	9.66	11.29	7.22	9.49
Reducing sugars	3.09	2.73	4.26	1.61
Total sugars	32.25	21.60	30.53	23.84
Total N	7.15	8.37	7.54	8.55
Crude protein	44.69	52.31	47.13	53.44

(N x 6.25)				
Protein (Lowry)	28.01	42.73	34.61	44.23
Non-protein nitrogen	2.67	1.53	2.00	1.47
Total fatty acids	0.90	1.29	1.56	2.24
DNA	0.65	0.62	0.72	0.99
RNA	2.43	2.67	3.50	4.59
In vitro digestibility (%)	75.17	79.60	82.98	80.84

Table 5. Amino acid composition (g amino acid per 16 g nitrogen) of the microbial biomasses (classification according to FAO)

	A. niger	A. oryzae	A. awamori	F. semitectum	FAO standard
Essential					
Phenylalanine	7.07	10.07	7.91	8.01	2.8
Tyrosine	0.56	0.86	0.67	5.53	2.8
Histidine	4.94	7.26	4.65	11.65	-
Isoleucine	6.85	6.01	6.08	5.60	4.2
Leucine	6.77	6.29	6.04	7.22	4.8
Lysine	15.38	13.73	16.15	11.78	4.2
Methionine	-	-	-	1.62	2.0
Cysteic acid	0.79	0.90	0.80	0.30	2.2
Threonine	5.63	2.98	3.62	2.45	2.8
Valine	5.81	3.21	1.91	2.27	4.2
Arginine	7.73	8.13	6.01	11.01	-
Tryptophan	nd	nd	nd	nd	1.4
Total essential	61.53	59.44	53.84	67.44	31.4
Non-essential					
Aspartic acid	6.28	5.65	5.66	7.60	
Serine	6.40	7.67	5.97	5.25	
Glutamic acid	5.22	8.63	6.91	9.39	
Proline	nd	nd	nd	nd	
Glycine	9.39	8.05	9.73	6.01	

Alanine	8.79	4.53	6.67	4.63

nd, not determined.

- not detected.

Table 6. Fatty acid composition (% w/w) of the microbial biomasses

	A. niger	A. oryzae	A. awamori	F. semitectum
Palmitic [16:0]	0.042	0.191	0.208	0.307
Stearic [18:0]	-	tr	-	tr
Oleic [18:1]	0.348	0.130	0.139	0.268
Linoleic [18:2]	0.513	0.972	1.216	1.644
Arachidic [20:0]	tr	tr	tr	0.020

tr, traces.

Table 7. Joint effect of pH and temperature on the TAA. Experimental domain and codification of the variables used in the factorial design

Coded values	Natural values	
	T	pH
- 1.41	24	3.39
- 1.00	30	4.00
0.00	45	5.50
1.00	60	7.00
1.41	66	7.62

Codification: Decodification:

$V_c = (V_n - V_O)/\Delta V_n$ $V_n = V_0 + (\Delta V^*_n V_c)$

V_c: coded value

V_n: natural value

V_0: natural value in the centre of the domain

ΔV_n: increment of V_n corresponding to one unit of V_c.

Table 8. Joint effect of pH and temperature on the TAA. Experimental matrix (coded values), responses obtained (R), estimated (R_e) and standardized residuals [$(R-R_c)/s$]

pH	T	R	R_e	$(R-R_c)/s$
1	1	70.29	69.92	0.14
1	- 1	19.28	22.92	− 1.41
- 1	1	24.62	21.72	1.13
-1	- 1	42.35	43.46	-0.43
$\sqrt{2}$	0	41.15	38.99	0.84
- $\sqrt{2}$	0	18.01	19.43	-0.55
0	$\sqrt{2}$	56.26	58.73	− 0.96
0	$-\sqrt{2}$	44.09	40.88	1.24
0	0	96.70	96.89	− 0.08
0	0	92.86	96.89	− 1.57
0	0	97.41	96.89	0.20
0	0	100.00	96.89	1.20
0	0	97.52	96.89	0.24

Table 9. Comparison between main characteristics of powdered enzymatic preparation (PAP) obtained from A. oryzae cultures on M medium and Sigma A-0273 α-amylase (S)

	PAP	S
Protein (Lowry) (%)	66.57	34.33
Total sugars (%)	19.72	64.69
Total amylolytic activity (EU mg^{-1})	60.85	56.13
Glucoamylase activity (EU mg^{-1})	0.43	0.27

Fig. 1. Flow diagram of the proposed process for the treatment of the MPW. MPW, Mussel processing wastes; A&D, acidification of the effluent and spontaneous decantation of the proteic precipitate formed; SD, sediments (1: crude, 2: purified) obtained from pretreatment of MPW; M, pretreated wastes (M medium); AoC, A.

oryzae culture; SCP, microbial biomass; PI, cell-free medium (post-incubate) from A. oryzae culture; UF, ultrafiltration (cut-off 30 kD); MPWd, depurated wastes; L, lyophilization; PAP, powdered amylolytic preparation

Fig. 2. Growth of nine strains of A. oryzae (key in Table 1) on M medium. B, biomass; TS, total sugars. Means of three culture replications, each with two analytical replications: 1-5% < SE < 3-0%.

Fig. 3. Growth of nine strains of A. oryzae (key in Table 1) on M medium. TAA, total amylolytic activity; GA, glucoamylase activity. Means of three culture, two analytical replications: 1.5% < SE < 3.0%.

Fig. 4. Effect of the Erlenmeyer load on the essential parameters of a culture of A. oryzae. O_2, % oxygen saturation; B, biomass; TS, total sugars; Nc, nitrogen consumption; Pc, phosphorus consumption, a, b, c and d: 50, 100, 150 and 200 ml of M medium per 250-ml Erlenmeyer flask. Means of two culture, two analytical replications: 1.5% < SE < 2-9% (in O_2: 1.5% < SE < 3.8%).

Fig. 5. Effect of the Erlenmeyer load on the essential parameters of a culture of A. oryzae. TAA, total amylolytic activity; GA, glucoamylase activity; PA, proteolytic activity. a, b, c and d as in Fig. 4. Means of two culture, two analytical replications: 1.7% < SE < 3.0%.

Fig. 6. Relations between percentage oxygen saturation and amylolytic activities in A. oryzae cultures (double reciprocal plot). Assuming lineal regression models with 1/% O_2 as independent variable, the determination coefficients (r^2) are 0.900 (TAA) and 0.929 (GA) at 45 h, and 0.956 (TAA) and 0.938 (GA) at 68 h.

Fig. 7. Effects of pH and temperature (both variables separately considered) on total amyiolytic activity of cell-free media post-incubates and powdered amylase

preparations (the same results in both cases) from A. oryzae. TAA as percentage of the maximum value. Means of three culture, two analytical replications in each case: 1.5% < SE < 2.2%.

Fig. 8. Response surface of TAA in powdered amyiolytic preparations from A. oryzae cultures, as a second-order function of pH and temperature.

Fig. 9. Stability of amylolytic preparations in distilled water solution at 50ºC. PAP, powdered amyiolytic preparation (characteristics in Table 9); S, a-amylase from Sigma; PAP + G, PAP supplemented with glycogen to a level of total sugars equivalent to that of the Sigma product. Initial TAA= 30 EU ml^{-1} in all cases. Means of two incubation, two analytical replications: r^2=0.991; 0.961 and 0.967 in PAP, S and PAP + G respectively.