Depuration and Valuation of Mussel-Processing Wastes.

Characterization of Amylolytic Postincubates from Different Species Grown on an Effluent

Ma. P. González, Ma. I. G. Siso, M. A. Murado,* L. Pastrana, Ma. I. Montemayor & J. Mirón

Instituto de Investigaciones Mariñas (CSIC) r/Eduardo Cabello, No. 6. 36208-Vigo, Spain

*To whom correspondence should be addressed.

Abstract

Mussel-processing wastes, which contain glycogen (≈ 10 g/litre) as their main component, can be used as a culture medium for several species of amylolytic yeasts and microfungi useful as single cell protein (SCP) sources. In addition, cell-free media from these cultures (with COD reduced to $\approx 10\%$ of its initial value) could, in principle, be used for the hydrolysis of greater volumes of the same effluent, which could then be concentrated, converting it into a more versatile microbial substrate.

With this objective in mind, the optimal reaction conditions, stability and kinetics of postincubates from several amylolytic species with distinct growth features when cultured on the effluent, were examined. Some of the mechanisms involved in the regulation of amylolytic activity are also discussed, as well as some of the methodological problems often associated with this kind of study.

Key words: Depuration, mussel-processing wastes, amyolysis, inhibition, repression, SCP.

INTRODUCTION

The first step in the industrial processing of mussels is thermal treatment, normally by steam, to open the valves and precook or cook the contents. The effluents thus produced (mussel-processing wastes, from now on MPW) are relatively rich in organic matter

(average COD $\approx$ 25 g/litre), particularly glycogen ($\approx$ 10 g/litre), and reach volumes of 300-400 litres per tonne of raw mussel treated.

In the estuarine systems known as the Rías Baixas (Galicia, NW of Spain), the annual production of mussel semicultures is approximately 250000 tonnes, 60% of which are processed in coastal plants, almost without seasonal interruption. The unchecked dumping of the corresponding effluents in coastal waters contributes to the progressive deterioration of the very medium that generates this resource, a marine ecosystem whose primary productivity is among the highest in the world: 260 g carbon/m² year¹ (Fraga, 1976).

In an effort to find a solution to this problem, this laboratory has studied the possibility of using the MPW as a culture medium for the production of SCP, and has obtained satisfactory results with amylolytic yeasts (Murado et al., 1986). And microfungi (Murado et al., 1989), as well as with mixed cultures of amylolytic yeasts or microfungi with non-amylolytic yeasts (Murado et al., 1986; Siso et al., 1988; Miron et al., 1988).

Nevertheless, evidence was soon found that the nature of the MPW might allow a more attractive economic scenario than the production of SCP. Indeed, the high amylolytic activity remaining in the filtered postincubated media of some of the microorganisms assayed suggested the possibility of developing a more complex treatment, comprising:

- Cultivation of an amylolytic microorganism on a portion of the MPW, with the production of SCP and a postincubate with a high amylase content, from which highly-active amylolytic preparations could be obtained.
- Use of the amylolytic preparations for the partial or total saccharification of another portion of the MPW (eventually concentrated), increasing its versatility as a microbial substrate.
- Use of the saccharified media as a basis for several microbial bioproductions (ethanol, gibberellic acid, gluconic acid and glucose oxidase are alternatives presently under investigation).

Thus, if the diversity and value of the products obtained were increased, it would justify the application of more sophisticated and efficient designs to the process. This study, centered on the growth features of different amylolytic species when cultured on MPW

and the enzymatic properties of their postincubates, is a first report on the results obtained in the development of this scheme.

METHODS

General

Species used were *Endomyces fibuliger*, *Aspergillus niger*, *Aspergillus oryzae*, *Aspergillus awamori* and *Fusarium semitectum* (CBS 2521, 554-65, 125-56, 139-52 and 479-83, respectively, abbreviated in this paper as E1, A1, A2, A3 and F1). Culture medium was prepared as Murado et al. (1986 composition in Table 1) by acidification of the MPW, with pH near neutrality, to pH = 4.0-4.5 (addition of 0.5-1.0 ml 5M HCl per litre MPW) and pouring off the clarified supernatant after 3-5 h sedimentation of a precipitate (≈ 2 g/litre) composed mainly of protein.

Incubations were achieved in 250 ml Erlenmeyer flasks containing 50 ml of medium, in an orbital shaker at 30°C/200 rpm. As inocula, spore (microfungi) or cell (yeasts) suspensions in sterile distilled water were adjusted (after calibration between OD measurements and direct haemocytometric counting) so that by adding 0.5-1.0 ml to each flask, the initial populations of the cultures were 0.5×10^6 spores (or cells)/ml. At different times, biomasses were harvested by paper filtration (A1, A2, A3) or centrifugation (E1, F1), washed with distilled water and dried to constant weight over P₂O₅ at 55°C and under reduced pressure.

Paper-filtered media and supernatants were used to prepare cell-free media (postincubates) by further membrane (0.45 μ m) filtration. Powdered enzymatic preparations were obtained by diafiltration of cell-free media, in a Minitan cell (Millipore) with cutoff at 30 000 daltons, followed by lyophilization of the corresponding retentates.

In order to distinguish between the inhibitory effect (of amylolytic activity) and the repressive effect (of amylase synthesis) by glucose in *E. fibuliger*, 24 cultures started on MPW were incubated for 18 h, a sample of 2.5 ml was taken from each culture at the

end of this period, and the whole subsequently distributed in 4 series dosed with the following sterile solutions:

Cultures 1-6 (control):	2 ml distilled water.
Cultures 7-12:	2 ml glucose solution (12.5 mg/ml).
Cultures 12-18:	2 ml glucose solution (50 mg/ml).
Cultures 18-24:	2 ml of a 50 mg/ml glucose and 1.65 µg/ ml cyclic AMP solution.

After additional 3.5 and 7 h incubation periods, another sample (3 ml) was taken from each culture. In every case the samples from each treatment were joined in pairs (therefore each treatment was studied in triplicate) and filtered (0.45 µm). The biomass retained in the filter was washed with distilled water and dried to constant weight over P₂O₅ at 55°C and under reduced pressure. A portion, 1.5 ml, of the filtrate was used to determine total amylolytic activity and glucoamylase by means of DNS and p-NPGP reactions, respectively (see below). Another portion (2 ml) was put in a Centricon microconcentrator (30 000 daltons) and centrifuged at 5 000 g, the former determinations being carried out on the ultrafiltrates (which should not show activity) and the retentates, collected by inverse microconcentrator centrifugation with 2 ml of distilled water.

Analytical methods

Total sugars

Phenol-sulphuric acid method, according to Strickland and Parsons (1968), with glucose as standard.

Reducing sugars

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

Glucose and oligosaccharides

Reversed-phase HPLC with water as eluent and refractive index detection, according to Franco and Garrido (1987).

Amylolytic activity

Four alternative methods were used.

Determination of reducing sugars production from starch (Bernfeld, 1951). In this method, useful for the evaluation of total amylolytic activity (TAA: glucoamylase plus α -amylase), 80 μ l of cell-free medium (suitably diluted, if necessary) were added to 400 μ l of a solution composed of 0.15M citrate-phosphate buffer, pH = 5.0 (1 volume) and 2% soluble starch (1.5 volumes), previously maintained at 40°C for 15 min. The mixture was incubated for 10 min at the same temperature, and the reaction stopped by addition of 480 μ l of DNS reagent. When an increase of 1 mg/ml of reducing sugars (glucose equivalents) was obtained in these assay conditions, the cellfree medium contained 1 EU/ml.

Determination of glucose released from starch, by the glucose oxidase-peroxidase method (Lee et al., 1970). Although this procedure is, in principle, specific for glucoamylase, significant variations of the response can be detected (Miranda et al., 1987) when α -amylase is also present.

Specific determination of glucoamylase by measuring p-nitrophenol released from p-nitrophenyl- α , D-glucopyranoside (p-NPGP), according to Hiromi et al. (1969).

Determination of unchanged substrate (starch-iodine reaction) after incubation of cellfree medium with starch, by the method of Spencer-Martins and Van Uden (1979). Although not strictly specific for α -amylase, the sensitivity of this assay is significantly lower against glucoamylase.

RESULTS AND DISCUSSION

Growth features of different amylolytic species

The main characteristics of the different cultures grown on MPW are given in Fig. 1, which shows that:

- The evolution of the pH throughout the incubation period seems to have a strong effect on the profile of total amylolytic activity (TAA), which falls when the pH descends below 4.0 (A1, A2, A3).
- The development of the species follows two different models, which can be distinguished by the production of a high transient reducing sugars surplus probably involved in the evolution of the pH (A1, A2, A3), or its absence (E1, F1).

The first effect can be explained considering that α -amylase is more sensitive to acid pH than glucoamylase (Miranda et al., 1987), and that α -amylase predominates in A2, whereas glucoamylase predominates in E1 and A1 (Fogarty & Kelly, 1980; Gonzalez, 1987; Franco et al., 1989), as was repeatedly confirmed in this laboratory by differential thermal deactivation (Miranda et al., 1987). Therefore (Fig. 2), when media partially buffered with 0.05M biphthalate/NaOH at initial pH = 6.0 are used, the TAA increases from 2 to 10 EU/ml in A1 (glucoamylase predominant) and from 15 to 60 EU/ml in A2 (α -amylase predominant), there being no effect whatsoever in the case of E1 (glucoamylase predominant and pH profile always above 4.0, even in non-buffered media). The behaviour of the buffered cultures was repeated when pH was controlled by alkali addition, preventing its decreasing below 5.0, a result that negates a possible specific effect of biphthalate.

The nature of the second characteristic is more complex, and could be due to either a strong (E1, F1) or a weak (A1, A2, A3) regulation of amylolysis by the products of hydrolysis, through the inhibition of amylolytic activity and/or repression of amylase synthesis. This will be discussed later.

Amylolytic capacity of the different postincubates

Figures 3 and 4 show the basic features of the postincubates from the species which showed the highest TAA (E1, A1, and A2) in cultures on MPW. It can be pointed out that despite the different relative proportions and sensitivities to acid pH of α -amylase and glucoamylase (e.g., Saha & Ueda, 1983; Siso et al., 1988), TAA shows very similar pH and temperature optima in the three postincubates (Fig. 3). In suboptimal conditions,

the least marked falls in activity take place in A2. As expected from the relative proportions of α -amylase and glucoamylase in A1 and A2 (Fogarty & Kelly, 1980; Miranda et al., 1987), the postincubates from A2 are more thermostable than those from A1 (Fig. 4). Those from E1, although glucoamylase predominates, are more sensitive to acid pH values than those from both microfungi.

Figure 5 shows the kinetics of hydrolysis of glycogen in vacuum-concentrated MPW treated with different volumes of cell-free media from 48-h cultures of E1, A1 and A2, on biphthalate-buffered MPW (TAA = 3, 10 and 50 EU/ml, respectively). It was proved in all cases, by HPLC, that glucose constituted at least 95% of the final concentration of the reducing sugars.

Once again, A2 (with the highest TAA) appears as the best species for our objective: only in this case can exhaustive hydrolysis be reached even with the lowest enzyme/substrate ratio. With postincubates from A1, hydrolysis is only complete at the highest enzyme/substrate ratio. However, since TAA is five times lower in A1 than in A2, the results suggest either that there is an excess of enzyme in the former situation, or a more favourable α -amylase/glucoamylase ratio in the latter. With postincubates from the yeast, hydrolysis is never complete, stabilizing at about 86% even in the most favourable situation.

At this point it would be interesting to consider all the differences found between the behaviour of the yeast and the microfungi. The low level of hydrolysis obtained with the yeast postincubates could be due to the capacity of the yeast enzymatic system to promote the inversion of amylolysis (condensation), combined or not with an inhibitory effect of the glucose. The inhibitory effect could likewise explain the particular profile shown by the reducing sugars in yeast cultures. In fact, although among the assayed species E1 is one of the producers of high levels of TAA, in its cultures it is impossible to appreciate a well defined maximum in the level of reducing sugars and one repeatedly encounters a period of oscillating concentrations (see Fig. 1) that decrease two or three times after reaching values between 1.0 and 1.5 g/litre.

However, particularly if the inhibitory effect of the glucose is not much more intense in the yeast than in the microfungi, the reducing sugars profile in E1 cultures could also

suggest the presence, in this case, of a regulating mechanism of a different quality, which could consist in the repression of synthesis of its amylolytic system by glucose.

Condensing activity was assayed in equivalent conditions to those used for the hydrolysis of glycogen, in incubation mixtures with 20 g/litre of glucose. In microfungi postincubates activity was not detected. With yeast postincubates (Fig. 6), the appearance of small quantities of maltose was stabilized between 70 and 90 h at a maximum level of 0.36 g/litre, without traces of oligosaccharides of higher polymerization degree. Therefore, condensation does not seem to be a sufficiently important effect to explain the peculiarities of E1 amylolysis.

This eventually leads us to the subject of amylolysis regulation through inhibition or repression by glucose, in microorganisms that have this capacity. Apart from the particular interest that these mechanisms present regarding the selection of the most suitable species for the intended treatment of the MPW, it should be indicated that divergent conclusions have frequently been reached in this matter, probably due in part to differences in methodological approach.

Inhibition or repression?

In the bibliography on the relative contributions of inhibition and catabolite repression to the regulation of extracellular amylolysis in different microorganisms, the most contentious aspect is whether or not a repressive process takes place. In general, this process is recognized (Stepanov et al., 1975; Sills & Stewart, 1982; Wilson et al., 1982; Pasari et al., 1987) in amylolytic yeasts such as *Endomycopsis fibuligera*, *Schwanniomyces alluvius*, *Schwanniomyces castelli*, although there are discrepancies concerning the enzyme involved (α -amylase or glucoamylase), the glucose concentration required to detect the effect, its reversibility by cyclic AMP and even the suitability of some of the experimental methods used to show the mechanism.

Nevertheless Reddy and Abouzied (1986) deny the existence of repression in *Aspergillus niger*, *Aspergillus awamori* and *Aspergillus foetidus*. Their opinion is based on the fact that, in cultures of these species, TAA, which falls in the presence of high levels of reducing sugars in the medium, is recovered on dialysis and disappears again

when various concentrations of glucose are added to the dialysed medium. This suggests that the apparent demonstrations of the repressive effect carried out with undialysed media only describe the inhibitory effect. Vallat (1983), on the other hand, did not detect glucose inhibition with commercial glucoamylases from *A. niger* and *Rhizopus* sp., contrary to the results of other authors.

In order to evaluate this problem in our own particular context, crude enzyme preparations from species with the most distant properties (E1 and A2) were obtained by ultrafiltration of the cell-free media with cutoff at 30 000 daltons and further lyophilization of the retentates, and the inhibition by glucose subsequently studied.

It must be taken into account that, in microorganisms with glucomylase predominant, the usual procedures for measuring the increase in hydrolysis products (DNS and glucose oxidase-peroxidase methods) are not suitable to characterize inhibition by glucose, which requires the detection of small variations in the level of monosaccharide against a large concentration of the same compound, present as inhibitor. Nor are the methods, useful for α -amylase, based on the detection of the remaining substrate (starch-iodine reaction), since the glucoamylase mechanism produces only small reductions per time unit in the length of the starch chains.

In the case of the yeast, the inhibitory effect of glucose could only be characterized using the glucoamylase specific substrate p-NPGP. The process proved to be competitive (Fig. 7, E1), with kinetics inhibition constant, $K_i = 1.51$ mg/ml. Nevertheless, it must be pointed out that the different affinities of the enzyme for p-NPGP and starch (Miranda et al., 1987) prevent the comparative use of such K_i in starch-glucose systems. The impossibility of detecting the inhibition by glucose, even at levels of 20 g/litre, through the starch-iodine reaction, can be attributed to the fact that the yeast's α -amylase is not inhibited by glucose.

In the case of *A. oryzae* preparations, in which the α -amylase level is sufficient to allow inhibition assays by methods based on the determination of the remaining substrate, the results showed that α -amylase was not inhibited at concentrations of 15 g/litre of glucose, nor 30 g/litre of maltose, whereas glucoamylase was inhibited by glucose (Fig. 7, A2: p-NPGP as substrate, competitive process, $K_i = 1.10$ mg/ml).

The difference between the K_i values does not justify the different behaviour registered for the E1 and A2 cultures with regard to the reducing sugars profile. Certainly, A2 produces a significantly superior level of TAA than E1, with α -amylase predominant, but A1 cultures, with a lower level of TAA than the yeast and with similarly predominant glucoamylase, showed a clear maximum level of reducing sugars. This leads again to the hypothesis of repression in E1 cultures.

In order to verify this hypothesis, a series of yeast cultures was dosed with increasing concentrations of glucose after 18 h incubation, and the amylolytic activities present in the media were determined by different procedures after supplementary incubation periods of 3.5 and 7.0 h (see Methods). To differentiate between the possible amylase level fall (repression) and the inhibitory effects of the amylolytic activity due to glucose, triplicate determinations were carried out in parallel experiments using filtered (0.45 μ m) media and retentates from ultrafiltered (Centricon, 30 000 daltons) media.

When amylolytic activity was evaluated by the increased production of reducing sugars (DNS reaction), results were once again inconclusive. When, on the other hand, the determination was centred specifically on glucoamylase (p-NPGP as substrate, Fig. 8), the following observation was noted. The activities detected in the ultrafiltration retentates were in all cases clearly superior to those of the corresponding filtered media, thus proving the existence of an inhibitory effect which disappears on eliminating the glucose. For each type of culture and incubation period, the inhibitory effect can be quantified from the difference in enzymatic activity between the retentate (E_r , non-inhibited) and the filtrate (E_f , inhibited) tests. This can be expressed by eqn (1) as a percentage decrease in activity due to inhibition (%I) by:

$$\%I = [1 - (E_f/E_r)] \times 100 \quad (1)$$

On the other hand, the differences between the activities detected in culture retentates with differing doses of glucose can only be attributed to the effect of repression. For each incubation period, the percentage decrease in activity with respect to the control (%R) attributable to the repression caused by the different doses of glucose, can be expressed by eqn (2):

$$\%R = [1 - (E_g/E_0)] \times 100 \quad (2)$$

where

E_g = enzymatic activity in ultrafiltration retentate from cultures dosed with glucose at level g .

E_0 = enzymatic activity in ultrafiltration retentate from control culture at the same incubation time.

As shown in Fig. 8 (particularly to the right, where the results from the previous calculations are plotted), the inhibitory effect predominates over the repressive effect at short periods (3.5 h) after the addition of glucose, decreasing at 7.0 h. This seems logical, since inhibition should immediately reflect the level of reducing sugars in the medium. The repressive effect, on the contrary, as corresponds to a physiological process of intracellular location and more complex than inhibition, has a certain delay in its response to the environmental level of reducing sugars, being more intense at 7.0 than at 3.5 h.

Finally, it should be pointed out that the addition of cyclic AMP (at least in the conditions assayed: 0.066 mg/litre cyclic AMP added simultaneously with a dose of 2 g/litre glucose) does not cause any diminution in the repressive effect.

CONCLUSIONS

The amylolytic yeast *Endomyces fibuliger* is, since the development of the Symba yeast process (Jarl, 1969; Skogman, 1976), the preferred microorganism in the treatment of amylaceous effluents for depuration with SCP production. However, apart from the COD reduction and the biomass obtained, the amylolytic activity remaining in the postincubate media represents an interesting additional possibility for those kinds of treatment. The postincubates can, in fact, be used to obtain the corresponding enzymes or for the hydrolysis of effluents clarified and concentrated by ultrafiltration (100000 daltons), transforming them into a substrate suitable for other bioproductions (Pastrana, 1991).

In this last respect, the results obtained with diverse microfungi, principally *Aspergillus oryzae*, whose biomass is also suitable for the formulation of fodder (Barker et al., 1981), are clearly superior to those of yeast. Although in both microorganisms,

and with a very similar inhibition constant, glucoamylase is inhibited by glucose, various factors seem to favour *A. oryzae*: (a) the high α -amylase/glucoamylase proportion in the microfungus enzymatic system, (b) the capacity (although very moderate) of the yeast enzymatic system to promote the inverse reaction of hydrolysis and (c) despite previous divergent results in this respect, the repression by glucose (non reversible with cyclic AMP) of glucoamylase synthesis in the yeast.

ACKNOWLEDGEMENTS

To CICYT for its financial support (project ALI 90-0789).

REFERENCES

- Barker, T. W., Drouliscos, N. J. & Worgan, J. T. (1981). Composition and nutritional evaluation of *Aspergillus oryzae* biomass grown on palm oil processing effluents. *J. Sci. Food Agric.*, 32, 1014-20.
- Bernfeld, P. (1951). Enzymes of starch degradation and synthesis. *Advances in Enzymology*, 12, 379-427.
- Fogarty, W. M. & Kelly, C. T. (1980). Amylases, amyloglucosidases and related glucanases. In *Microbial enzymes and Bioconversions, Economic Microbiology*, Vol. 5, ed. A. H. Rose. Academic Press, London, UK, pp. 115-70.
- Fraga, E (1976). Fotosíntesis en la Ría de Vigo. *Inv. Pesquera*, 40, 151-7.
- Franco, J. M. & Garrido, J. L. (1987). Determination of oligosaccharides (DP1-DP8) in samples with high content of salt and organic matter by reversed phase HPLC. *Chromatographia*, 23(8), 557-60.
- Franco, J. M., Murado, M. A., Siso, M. I. G., Miron, J. & Gonzalez, M. P. (1989). An HPLC method for specific determination of α -amylase and glucoamylase in complex enzymatic preparations. *Chromatographia*, 27(7/8), 328-32.
- Gonzalez, M. P. (1987). Depuración y aprovechamiento de los efluentes del procesado industrial del mejillón. Thesis, University of Santiago de Compostela, Spain.
- Hiromi, K., Shibaoka, H., Fukube, H. & Ono, S. (1969). Quantitative determination of anomeric forms of sugar produced by amylases. IV: Anomeric form of maltose produced from phenyl α -maltoside catalyzed by saccharifying α -amylase from *Bacillus subtilis*. *J. Biochem.*, 66(1), 63-7.

- Jarl, K. (1969). Production of microbial food from low-cost starch materials and purification of industry's waste starch effluents through the Symba Yeast Process. *Food Technol.*, 23, 23-6.
- Lee, E. Y. C., Carter, J. H., Nielsen, L. D. & Fisher, E. H. (1970). Purification and properties of yeast amylo-1, 6-glucosidase-oligo- 1,4-1,4-glucoamylase. *Biochemistry*, 9(11), 2347-55.
- Miranda, M., Siso, M. I. G., Gonzalez, M. P., Murado, M. A. & Miron, J. (1987). Amyloysis in systems with α -amylase and glucoamylase. A comparative study of six procedures of evaluation. *Biotechnol. Tech.*, 1(3), 195- 200.
- Miron, J., Siso, M. I. G., Murado, M. A. & Gonzalez, M. P. (1988). Microfungus-yeast mixed cultures in the degradation of amylaceous wastes. II: an experimental design for optimization of yeast production. *Biotechnol. Lett. (Techn.)*, 2(3), 171-6.
- Murado, M. A., Gonzalez, Ma. P., Montemayor, Ma. I., Reiriz, Ma. J. F. & Franco, J. M. (1986). Producción de proteína unicelular en efluentes del procesado de mejillón. I: Utilización de levaduras en cultivos axónicos y mixtos. *Inv. Pesquera*, 50(4), 571-605.
- Murado, M. A., Siso, Ma. I. G., Gonzalez, Ma. P., Franco, J. M. & Miron, J. (1989). Producción de proteína unicelular a partir de efluentes de la elaboración del mejillón. II: Utilización de microhongos. *Rev. Agroquim. Tecnol. Aliment.*, 29(2), 209-20.
- Pasari, A. B., Korus, R. A., Willians, R. A. & Heimsch, R. C. (1987). Catabolite repression of amylase synthesis in yeast. *Biotechnol. Bioeng.*, 30, 363-7.
- Pastrana, L. M. (1991). Producción de ácido giberélico a partir de efluentes de la industria mejillonera en cultivo sumergido y en estado sólido. Thesis, University of Santiago de Compostela, Spain.
- Reddy, C. A. & Abouzied, M. M. (1986). Glucose feedback inhibition of amylase activity in *Aspergillus* sp. and release of this inhibition when cocultured with *Saccharomyces cerevisiae*. *Enzyme Microb. Technol.*, 8(11), 659-64.
- Saha, B. C. & Ueda, S. (1983). Alcoholic fermentation of raw sweet potato by a non-conventional method using *Endomycopsis fibuligera* glucoamylase preparation. *Biotechnol. Bioeng.*, 25, 1181-6.
- Siso, Ma. I. G., Murado, M. A., Franco, J. M., Miron, J. & Gonzalez, Ma. P. (1988). Microfungus-yeast mixed cultures in the degradation of amylaceous wastes. I: Interactions affecting amylolytic activity. *Biotechnol. Lett.*, 10(6), 431-6.

- Sills, A. M. & Stewart, G. G. (1982). Production of amylolytic enzymes by several yeast species. *J. Inst. Brew.*, 88, 313-16.
- Skogman, H. (1976). Production of Symba-yeast from potato wastes. In *Food from Waste*, ed. G. G. Birch, K. J. Parker & J. T. Worgan. Applied Science Publishers, London, UK, pp. 167-79.
- Spencer-Martins, I. & van Uden, N. (1979). Extracellular amylolytic system of the yeast *Lipomyces kononenkoae*. *Eur. J. Appl. Microbiol. Biotechnol.*, 6, 241-50.
- Stepanov, A. I., Afanasieva, V. P., Zaitseva, G. V. & Mednikova, A. P. (1975). Regulation of biosynthesis of amylolytic enzymes from *Endomycopsis fibuligera* STR. 20-9. *Prikl. Biokhim. Mikrobiol.*, 11(5), 682-5.
- Strickland, J. D. H. & Parsons, T. R. (1968). A practical handbook of sea water analysis. *J. Fish. Res. Board Canada*, 167, 173-4.
- Vallat, I. (1983). Mise en oeuvre de la glucoamylase immobilisée sur raffles de maïs en couche fluidisée, thesis Institut National Polytechnique Toulouse, France.
- Wilson, J. J., Khachatourians, G. G. & Ingledew, W. M. (1982). *Schwanniomyces*: SCP and ethanol production from starch. *Biotechnol. Lea.*, 4(5), 333-8.

Table 1. Average composition (g/litre) of the culture media obtained from acidified MPW and 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)
NaCl	18.00	(17.00-22.00)
COD(0 ₂)	25.00	(19.00-26.00)

Fig. 1. Cultures of five amyolytic microorganisms on MPW. B, Biomass; RS, reducing sugars; G, glycogen; TAA, total amyolytic activity. E1, *E. fibuliger*; A1, *A. niger*; A2, *A. oryzae*; A3, *A. awamori*; F1, *F. semitectum*. E1, A1, A2 are means of 5 genuine replications (in B and pH), each with 2 analytical replications (in G, RS and TAA). In B, G, RS and pH $1.2\% < SE < 3\%$; in TAA, $1.5\% < SE < 3, 4\%$. A3, F1 are as in E1, A1, A2, with 3 genuine replications, with B, G, RS and pH having $1.4\% < SE < 3\%$ and TAA $1.4\% < SE < 3.6\%$.

Fig. 2. Evolution of pH and total amyolytic activity (TAA) in cultures of *Aspergillus niger* (A1) and *A. oryzae* (A2) on MPW. Open symbols, non-buffered medium; closed symbols, partially buffered medium (0.05M biphthalate/NaOH; initial pH = 6.0). Means of 2 genuine, 2 analytical replications ($1.8\% < SE < 3.2\%$).

Fig. 3. Total amyolytic activity (TAA, as % of maximum value) of 48 h postincubates of the three most active species, as a function of pH, at 40°C (open symbols), and of temperature, at pH = 5.0 (closed symbols). Means of 2 genuine, 2 analytical replications ($1.3\% < SE < 3.2\%$).

Fig. 4. Stability in absence of substrate of 48 h postincubates of the most active species in the specified conditions of temperature and pH. Means of 2 genuine, 2 analytical replications ($1\% < SE < 3\%$).

Fig. 5. Glycogen hydrolysis (%) in concentrated MPW treated with 48 h postincubates of E1, A1, and A2, at 30°C and two volumetric relations between postincubate (P) and MPW. Open symbols, P/MPW=0.25/1; closed symbols, P/MPW= 1.5/1. Initial concentration of glycogen, in all cases was 20 g/litre. Means of 2 genuine, 2 analytical replications ($0.9\% < SE < 2.8\%$).

Fig.6. Condensing activity of the amylolytic system of E1. A postincubate (P), with a TAA of 3 EU/ml, is incubated, at 30°C and two volumetric proportions, with a glucose solution. Left, P/S = 0.25/1; right, P/S = 1.5/1. In both cases, the initial concentration of glucose in the incubation mixture was 20 g/litre. Means of 2 genuine, 2 analytical replications ($1.5\% < SE < 3.4\%$).

Fig. 7. Lineweaver-Burk plots for inhibition by glucose, with p-NPGP as substrate, in amylolytic preparations (TAA = 1.30 EU/ml each) from E1 and A2. S, substrate concentration ($\mu\text{g/ml}$); V, reaction rate ($\mu\text{g/ml min}$). In all cases $0.996 < r^2 \leq 1.000$.

Fig. 8. (a) Evolution of glucoamylase activity/biomass ratio in E1 cultures dosed with different concentrations of glucose after 18 h incubation. E_f , enzymatic activity in filtered media; E_r , enzymatic activity in ultrafiltration retentates; C, control cultures; 0.5, 2.0, glucose-dosed cultures; 2.0*, 2 g/litre glucose and 0.066 mg/litre cyclic AMP cultures. (b) Estimates of percentage decrease in enzymatic activity attributable to the inhibitory (%I) and repressive (%R) effects in the previously specified cultures. See text for details (in all cases $1.1\% < SE < 3.6\%$).