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Biological Treatment of Agro-Industrial Wastewater for the Production of Glucoamylase and *Rhizopus* Biomass

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Abstract: The present work concerns the biological treatment of a starch wastewater, coupled with the production of microbial biomass and glucoamylase. Every unit operation of the process was tested in a laboratory scale, in order to perform a feasibility analysis. Fermentation tests confirmed that a stirring rate of 300 rpm was the optimum (in the investigated range) as concerns biomass growth and enzyme activity. Ultrafiltration tests showed that the best operating conditions in the investigated range for enzyme recovery were: temperature 25°C, transmembrane pressure 100 kPa, membrane cut off 30 kDa. Diafiltration tests evidenced that an effective enzyme washing (about 85% reduction of chemical oxygen demand) was achieved with 3 volumes of water per volume of feed and no significant enzyme deactivation was observed.

Keywords: Wastewater, *Rhizopus*, glucoamylase, fermentation, ultrafiltration, diafiltration

INTRODUCTION

Starch wastewater is one of the most common wastewaters in the food industry (1). It has higher biochemical oxygen demand (BOD) levels than

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town sewage, is highly polluting, and can impose heavy loads on the environment or be expensive in terms of sewer disposal. Heightened environmental awareness has prompted regulatory organizations to assign real economic value to environmental factors, thereby forcing industries to consider environmental factors and a variety of waste treatment techniques to address these issues (2).

The possibility of depuration of other wastes from food industry, by using them as substrates for various bioproductions of potential economic interest has been reported before in the case of mussel processing (3), brewery (4), whey (5), and olive processing (6). In particular, starch waste (2, 7, 8) materials offer the advantages of availability and consistent quality, being a readily convertible material at competitively low cost, from which a wide variety of products can be prepared.

In the present paper, a wastewater coming from potatoes manufacturing has been considered. Considering the high BOD content due to starch, this reflue represents an optimal medium for the growth of heterotroph microorganisms, such as fungi and yeasts. In particular, *Rhizopus oligosporus* has been tested for its well known ability to use this kind of medium as a carbon source (7). Furthermore the produced biomass represents a valuable product to be used as animals food for its high content in proteins. A further advantage in the use of *Rhizopus* is the production during fermentation of glucoamylase, an extracellular enzyme, commercially interesting for its ability to hydrolize starch and glucose (9, 10).

The aim of the present work is to establish the main process operating conditions in the biotreatment of starch wastewater. Both bioreaction and subsequent ultrafiltration for enzyme recovery were studied. The novelty with respect to other work in the literature on the same subject (7, 11) is mainly in the investigation of each unit operation of the whole treatment process, both fermentation and downstream aimed at the enzyme recovery. Fermentation tests have been performed under different operating conditions in order to investigate the effect of stirring rate and aeration on the biomass growth and on the enzyme production. Enzyme recovery by ultrafiltration was then tested for different membrane cut off and temperatures. Subsequent diafiltration was also performed aimed at glucoamylase washing. The obtained results showed the potential of the process technical feasibility.

MATERIALS AND METHODS

Microorganism

Rhizopus oligosporus Saito ATCC n. 22959 was used in this investigation. Tables 1 and 2 show solid and liquid media composition. Petri plates and shaken flasks were used for microorganism maintenance and inoculum, respectively.

Table 1. Solid medium (PDA: Potato Dextrose Agar)

Component	Concentration (g/L)
Potato extract	4
Dextrose	20
Agar	15

Fermentation Tests

A 5 L Biostat B Braun Biotech International was used for fermentation tests, using the liquid broth PSYb (Table 2) as growth medium. Fermentation operating conditions were chosen as optimised by other authors (12): temperature 30°C, pH 4, air 0.2 vvm. Stirring was 100, 300, or 500 rpm. At the end of the fermentation tests, biomass was recovered by filtration (pressure filter *MILLIPORE* 142 MM 1.4 μm) and broth was stored for enzyme recovery. Periodically, aliquot amounts were sampled for biomass, starch, glucose, and glucoamylase determinations.

Fermentation tests were performed in two replicates with an average 10% standard error.

Enzyme Recovery

In order to test the glucoamylase recovery step, the fermentation broth was fed through a plane membrane module (Millipore MINITAN S) by a peristaltic pump (tangential velocity 0.3 m/s). Three different polysulfone membranes were tested with 10,000 30,000 and 100,000 Da Molecular Weight Cut Off (MWCO) and area of 36 cm^2. The mixing in the reactor was ensured both by a magnetic stirrer placed into the reactor and by the recirculation flow of the retentate stream. The temperature was either 25°C or 40°C, according to experimental conditions. Permeability ultrafiltration tests were performed by recirculating both permeate and retentate (13).

Table 2. Liquid medium (PSYb: Peptone Starch Yeast)

Component	Concentration (g/L)
Soluble starch	10
Peptone	5
Yeast extract	5
$MgSO_4 \cdot 7H_2O$	0.2
K_2HPO_4	0.2

Diafiltration tests were performed in a continuous operation (13) at 100 kPa tranmembrane pressure, feeding distilled water at a flowrate equal to the permeate one, in order to maintain a constant volume. After diafiltration, the enzyme was then freeze dried in a Flexi-Dry lyophiliser (FTS Systems, Inc., Stone Ridge, New York).

Experimental Determinations

Microbial growth was monitored by dry weight. The collected broth samples were filtered under vacuum on a 0.45 μm filter (Millipore filtration system); the solid phase was washed three times with distilled water and dried at 80°C for 24–48 h. Starch and glucose were both measured by the enzymatic colorimetric GOD-PAP (glucose oxidase-phenol 4-aminophenazone peroxidase) test. Glucoamylase activity was determined by a method based on the determination of liberated glucose by the glucose oxidase-peroxidase coupled reaction. All details on procedures and reactions can be found elsewhere (11).

Data Analysis

The following equation was fitted to experimental permeability data: (13)

$$J_P = \frac{TMP}{R'_m + \phi \cdot TMP} \tag{1}$$

where J_p (L h^{-1} m^{-2}) is the permeate flux, TMP (kPa) is the transmembrane pressure, R'_m and ϕ are adjustable parameters, estimated through non linear regression techniques.

The enzyme retention coefficient (σ) was calculated as follows (13):

$$\sigma = 1 - \frac{C_P}{C_R} \tag{2}$$

where C_P and C_R were glucoamylase concentration experimentally determined in the permeate and in the retentate, respectively.

In diafiltration tests, a component concentration in the retentate during the process can be mathematically described by the following Equation (13)

$$C_R = C_0 e^{-V_D(1-\sigma)} \tag{3}$$

where C_R and C_0 are concentration in the retentate and at the beginning of diafiltration test, respectively, and V_D represents the permeated volumes referred to feed volume.

RESULTS

Fermentation

Rhizopus fermentation tests were performed under different stirring rates (100, 300, and 500 rpm) in order to find the best condition for both biomass and enzyme production. In fact stirring influences both broth aeration and shear rate: the first one might positively act on the microorganism's growth but the shear rate might lead to a partial enzyme deactivation. The range 100–500 rpm is in a typical range of fermentation operation (14). The liquid medium PSYb (Table 2) was used considering its similarity with real potatoes manufacturing reflues. Preliminary experiments have also been performed with a real reflue coming from a Central Italy potatoes manufacturing industry which showed a similar behavior to medium PSYb, as concerns biomass production and glucoamylase activity. Figure 1 shows starch, glucose, biomass, and glucoamylase vs. time profiles during the batch fermentation performed at 300 rpm. Figure 2 shows starch concentration and glucoamylase activity profiles observed during fermentation tests carried out with different stirring rates: 100, 300, and 500 rpm.

The analysis of fermentation results suggested that fermentation courses were successful in any case as concerns the reflue treatment. In fact starch concentration was equal to 0 after one day processing in the case of 300 and 500 rpm stirring rate, while about 36 hours were needed in the case of a 100 rpm stirring rate. It can also be observed that biomass production and starch consumption increased with the stirring rate. This behavior was expected, considering that *Rhizopus* is an aerobic microorganism and the

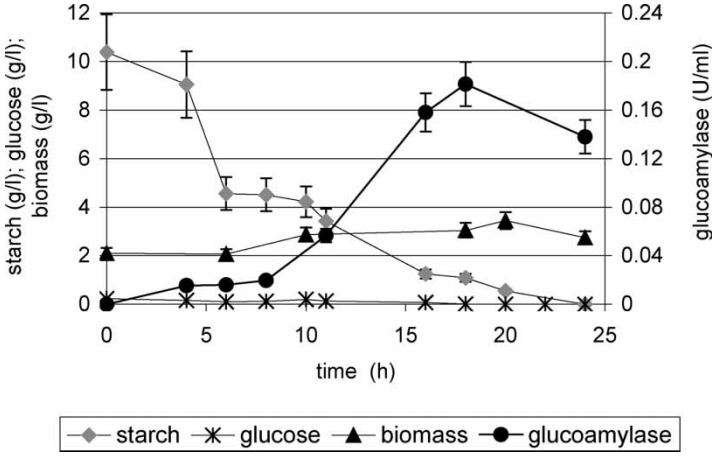

Figure 1. *Rhizopus* fermentation vs. time profiles (stirring rate of 300 rpm).

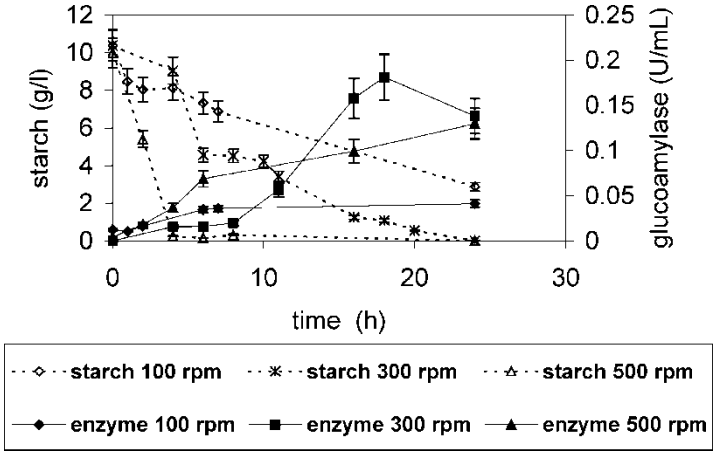

Figure 2. Effect of stirring rate on starch and glucoamylase vs. time profiles during fermentation.

oxygen transfer rate is enhanced by high stirring rate. As concerning the enzyme activity, Fig. 2 shows that there is a maximum for a stirring rate of 300 rpm. This might be associated to a probable enzyme deactivation due to shear stress which might become important at the end of fermentation (24 h) at 300 rpm and after the first 12 hours processing at 500 rpm. Substrate consumption, biomass and enzyme production profiles were also used for the development of a kinetic model for microbial growth which was successfully fitted to experimental data. Further details can be found elsewhere (11).

Ultrafiltration

After cells separation, the fermentation broth was treated by ultrafiltration in order to recover the produced glucoamylase. Factors investigated were membrane cut off (10, 30, and 100 kDa) and temperature (25 and 40°C). Three membrane cut off were tested: 10, 30, and 100 kDa. These values were around the enzyme average dimension (48–112 kDa) (15–17) and were tested in order to find the optimal cut off, as concerns permeate fluxes, enzyme retention and enzyme activity. Two levels of temperature were chosen: 25 and 40°C. Figures 3, 4, and 5 show the experimental results obtained in all broth ultrafiltration tests.

Figure 3 in particular, shows permeate fluxes vs. transmembrane pressure obtained in permeability tests. Lines in Fig. 3 have been calculated by Equation (1), whose parameters have been estimated by non linear regression techniques

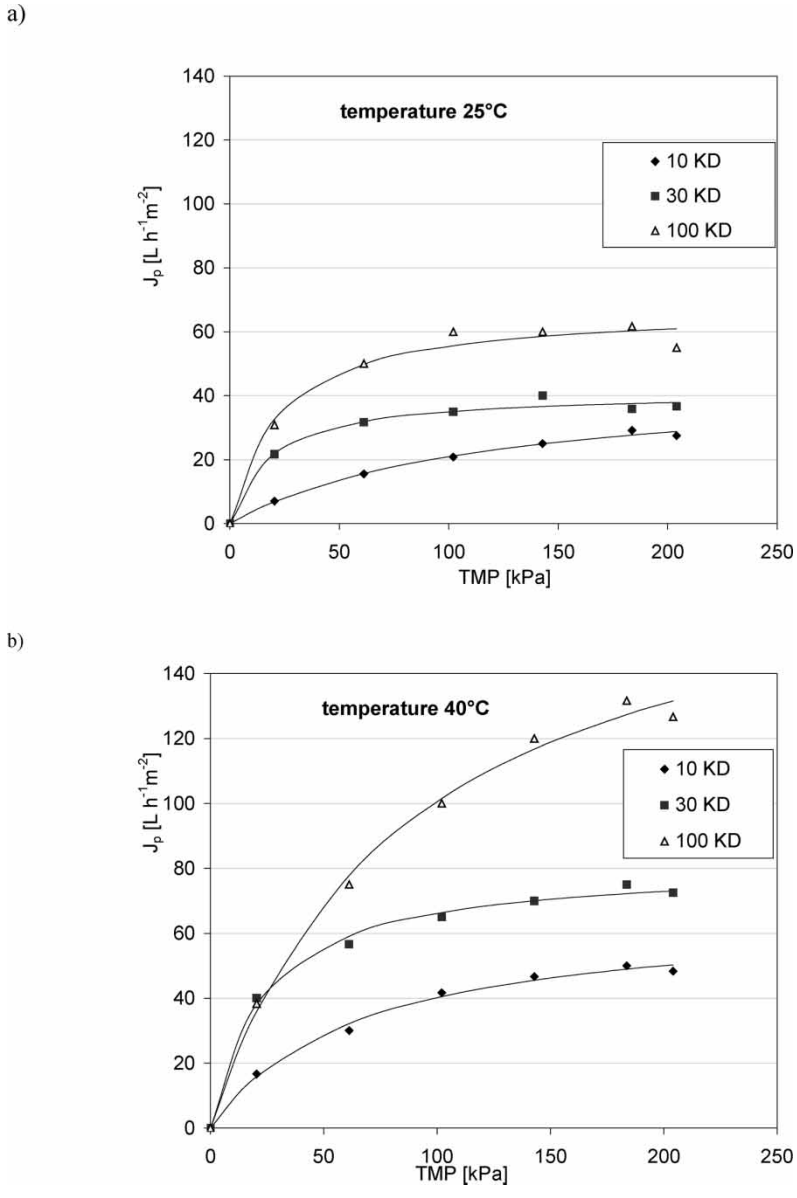

Figure 3. Permeate flux vs. transmembrane pressure for different membrane cut-off at 25°C (a) and 40°C (b).

(Table 3). Permeability data in Fig. 3 suggest that, as expected (13), permeate fluxes increase both with membrane cut off and with temperature. In fact, limit values for permeate flux were 25, 33, and 58 $L/h/m^2$ for 10, 30, and 100 kDa membrane cut off, respectively, at 25°C, and they increased to 40, 63, and

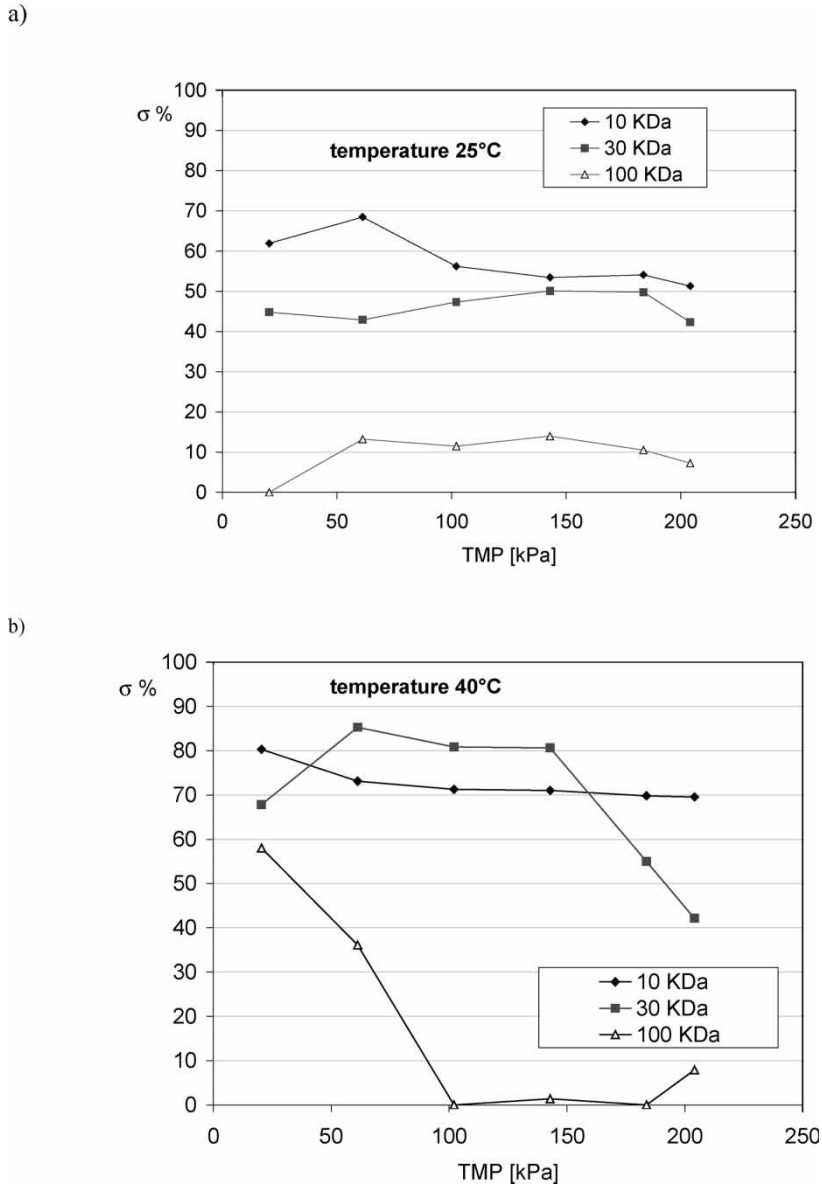

Figure 4. Glucoamylase retention coefficient for different membrane cut-off at 25°C (a) and 40°C (b).

130 L/h/m^2, respectively, at 40°C. Therefore the positive effects of membrane cut off and temperature on permeate flux are evident: permeate flux almost doubled passing from 25°C to 40°C, and it increased about three times passing from 10,000 to 100,000 Da membrane cut off. Figures 4 and 5 report

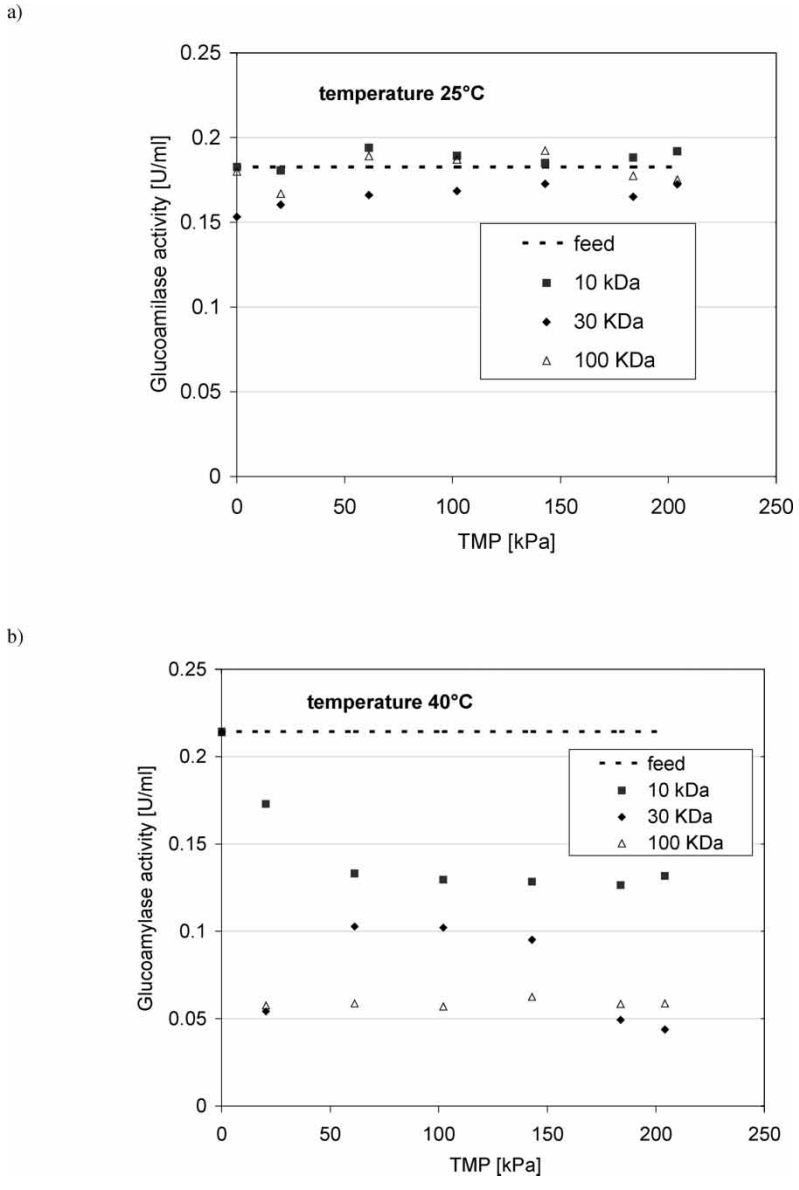

Figure 5. Glucoamylase activity for different membrane cut-off at 25°C (a) and 40°C (b).

glucoamylase retention coefficient (calculated according to equation 3) and its activity as a function of transmembrane pressure for different membrane cut off and temperatures. These data were obtained during the same permeability tests of Fig. 3. Figure 4 in particular, shows how the retention coefficient

Table 3. Experimental treatments and equation (1) parameters ($\pm$ standard error 95%)

Treatment	Membrane cut off (Da)	Temperature (°C)	Equation (1) parameters	
			R'_m	ϕ
1	10,000	25	2.5 ± 0.1	0.022 ± 0.004
2	30,000	25	0.43 ± 0.03	0.024 ± 0.002
3	100,000	25	0.32 ± 0.02	0.015 ± 0.002
4	10,000	40	0.98 ± 0.09	0.015 ± 0.002
5	30,000	40	0.28 ± 0.08	0.012 ± 0.001
6	100,000	40	0.46 ± 0.09	0.0054 ± 0.001

decreases with the membrane cut off. At 25°C (Fig. 4 a) it passes from about 50%, in the case of 10 and 30 kDa cut off, to about 10% for the 100 kDa membrane. This result was partially expected, considering the relatively high dimension of this last membrane with respect to the enzyme. At 40°C (Fig. 4b) different profiles were observed, which are very probably associated to enzyme deactivation (see further considerations). Figure 5 shows that no enzyme deactivation takes place at 25°C (Fig. 5a): the glucoamylase activity in the retentate is equal to the feed one, at any pressure. On the contrary, a significant deactivation takes place at 40°C (a loss higher than 50%, Fig. 5b), with any membrane cut off. Furthermore at 25°C there is no significant dependence on pressure, neither of retention coefficient nor of activity; on the other hand, at 40°C they both decrease with transmembrane pressure. This aspect might be attributed to the partial deactivation of glucoamylase which takes place at 40°C and at relatively high values of transmembrane pressure. In fact, a decrease of enzyme activity is associated with damage of the protein tertiary structure, and it can pass through the membrane more easily. In conclusion, these results suggest that the enzyme recovery necessarily has to take place at 25°C, in order to preserve the enzyme activity. Furthermore a membrane cut off of 100,000 Da cannot be used, considering that most of the enzyme can pass through the membrane (retention coefficient of about 10%). The analysis of data shows that the best operating conditions in the investigated range seem to be temperature 25°C, transmembrane pressure around 100 kPa, membrane cut off 30 kDa. These would give a permeate flux of about $35\,L/h/m^2$ (Fig. 3a) and a glucoamylase retention coefficient of about 50% (Fig. 4a), with no enzyme deactivation (Fig. 5a). A lower cut off (10 kDa) would give a higher enzyme retention (about 60%), but lower permeate fluxes. An optimization might be performed, by minimizing the whole process cost. Besides, further tests with a membrane cut off between 30 and 100 kDa would be necessary to truly determine the optimum membrane cut off for this process.

Diafiltration

Previous results observed in ultrafiltration tests were used in order to fix operating conditions for diafiltration, aimed at enzyme washing after a previous concentration by ultrafiltration. In fact, diafiltration tests were performed at 25°C, by using a membrane of 30 kDa cut off, after a VCR 10 (13) ultrafiltration step. Figure 6 shows chemical oxygen demand (COD) and enzyme activity during diafiltration as a function of permeated volumes (i.e. permeate volume over feed volume). COD was chosen as an indicator of enzyme washing degree, considering that the main components of fermentation broth are organic carbon and salts (also conductivity profiles were monitored during diafiltration and they were similar to COD profiles). The continuous line in Fig. 6 was calculated by means of equation (3) where σ_{COD} was fixed at 0.15, as experimentally determined from COD concentration in the permeate and in the retentate, by applying equation (2). Results in Fig. 6 suggest that COD concentration in the retentate (and conductivity too, data not shown) is reduced of about 85% after $3\,V_D$ diafiltration. Furthermore predicted data by means of equation (3) agree with experimental data. This means that diafiltration performances might be predicted by means of only retention coefficient experimental determination. An other interesting aspect concerns the

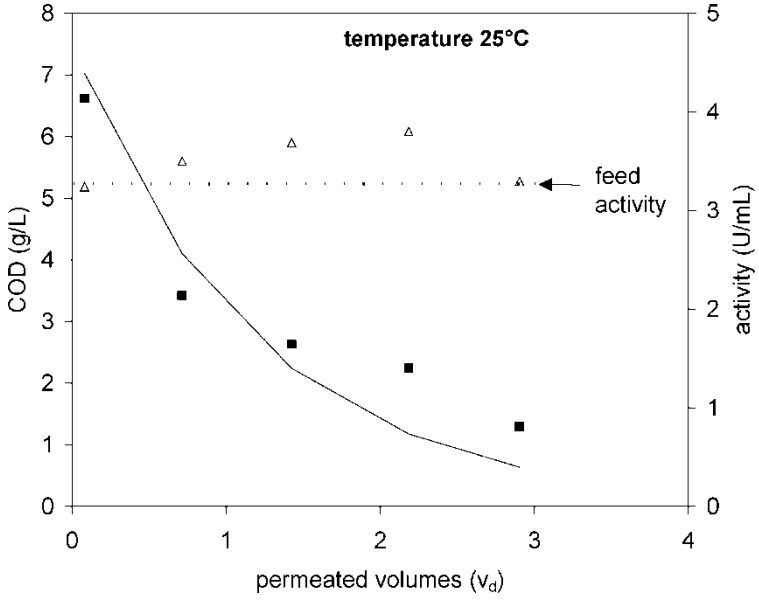

Figure 6. COD and enzyme activity in the retentate as a function of permeated volumes, during diafiltration tests (membrane 30 kDa, temperature 25°C).

enzyme activity, which remains unchanged during diafiltration: this is fundamental for further applications of the produced enzyme. As concerns permeate flux, it was constant and equal to about 40 L h^{-1} m^{-2} during the whole diafiltration test.

Activity tests performed on the lyophilised enzyme, evidenced that glucoamylase activity was not significantly reduced even after the lyophilisation step.

CONCLUSIONS

Rhizopus oligosporus fermentation on potatoes manufacturing wastewater was demonstrated to successfully reduce the reflue chemical oxygen demand (COD) and the treatment was coupled with the production of both fungal biomass and glucoamylase. Experimental tests performed both for fermentation and ultrafiltration (enzyme recovery) allowed to find the main process parameters. Fermentation tests confirmed that a stirring rate of 300 rpm was the best one (in the investigated range) for biomass growth and enzyme activity. Ultrafiltration tests showed that the best operating conditions in the investigated range for enzyme recovery were temperature 25°C, transmembrane pressure 100 kPa, membrane cut off 30 kDa. Further tests of diafiltration were also performed for enzyme purification. An effective enzyme washing (about 85% reduction of chemical oxygen demand) was achieved with no significant enzyme deactivation. These results validate the technical feasibility of the considered process.

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REFERENCES

1. Deng, S.B., Bai, R.B., Hu, X.M., and Luo, Q. (2003) Characteristics of a bioflocculant produced by *Bacillus mucilaginosus* and its use in starch wastewater treatment. *Appl. Microbiol. Biotechnol.*, 60: 588–593.
2. Jin, B., Yan, X.Q., Yu, Q., and van Leeuwen, J.H. (2002) A comprehensive pilot plant system for fungal biomass protein production and wastewater reclamation. *Advances in Environmental Research*, 6: 179–189.
3. Murado, M.A., Siso, M.I.G., Gonzalez, M.P., et al. (1993) Characterization of microbial biomasses and amylolytic preparations obtained from mussel processing waste treatment. *Bioresource Technology*, 43: 117–125.
4. Roukas, T. (1999) Pullulan production from brewery wastes by *Aureobasidium pullulans*. *World Journal and Biotechnology*, 15: 447–450.

5. Barba, D., Beolchini, F., and Del Re, G., et al. (2001) Kinetic analysis of *K. Lactis* fermentation on whey: Batch and fed-batch operations. *Process Biochemistry*, 36: 531–536.
6. Di Giacomo, G., Brandani, V., and Del Re, G. (1991) Evaporation of olive oil mill vegetation waters. *Desalination*, 81: 249–259.
7. Jin, B., van Leeuwen, H.J., Patel, B., et al. (1999) Production of fungal protein and glucoamylase by *Rhizopus oligosporus* from starch processing wastewater. *Process Biochemistry*, 34: 59–65.
8. Jin, B., van Leeuwen, H.J., Patel, B., et al. (1998) Utilisation of starch processing wastewater for production of microbial biomass protein and fungal a-amylase by *Aspergillus oryzae*. *Bioresource Technology*, 66: 201–206.
9. Van, der Maarel, Marc, J.E.C., van der Veen, Bar., Uitdehaag, Joost, C.M., et al. (2002) Properties and applications of starch converting enzymes of the α-amylase family. *Journal of Biotechnology*, 94: 137–155.
10. Yetta, M., Nazamid, S., Zaiton, H., et al. (2000) Purification and characterisation of sago starch-degrading glucoamylase from *Acremonium* sp. endophytic fungus. *Food Chemistry*, 71: 221–227.
11. Del Re, G., Di Giacomo, G., Spera, L., and Vegliò, F. (2003) Integrated approach in the biotreatment of starch wastes by *Rhizopus oligosporus*: kinetic analysis. *Desalination*, 156: 389–396.
12. Sparringa, R.A., Kendall, M., Westby, A., and Owens, J.D. (2002) Effect of temperature, pH, water activity and CO_2 concentration on growth of *Rhizopus oligosporus* NRRL 2710. *Journal of Applied Microbiology*, 92: 329–337.
13. Cheryan, M. (1998) *Ultrafiltration and Microfiltration Handbook*; Technomic Publishing Company, Lancaster, Pennsylvania, USA.
14. Paul, G.C., Priede, M.A., and Thomas, C.R. (1999) Relationship between morphology and citric acid production in submerged *Aspergillus niger* fermentations. *Biochemical Engineering Journal*, 3 (2): 121–129.
15. Aalbæk, T., Morten Reeslev, M., Jensen, B., and Eriksen, S.H. (2002) Acid protease and formation of multiple forms of glucoamylase in batch and continuous cultures of *Aspergillus niger*. *Enzyme and Microbial Technology*, 30 (3): 410–415.
16. Minami, N.M., Lucarini, A.C., and Kilikian, B.V. (1999) Characterization of clarified medium from submerse and semisolid cultivation of *Aspergillus awamori* NRRL3112 by size-exclusion chromatography. *Brazilian Journal of Chemical Engineering*, 16 (2): 193–198.
17. Yu, R. and Hang, Y.D. (1991) Purification and characterization of a glucoamylase from *Rhizopus oryzae*. *Food Chemistry*, 40 (3): 301–308.