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Full Length Research Paper

Transformation kinetics of mixed polymeric substrates under transitory conditions by *Aspergillus niger*

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A mixture of polymeric substrates (simulating a complex wastewater) was transformed under sewer conditions and aerobiosis by *Aspergillus niger* in a tanks-in-series reactor at a hydraulic retention time of 14 h. Starch was totally removed after 7 h of incubation. Removal of the protein portion with a molecular weight larger than 2 kDa followed the starch removal and the total proteins were the latest to be removed. Alkaline phosphatase, leucine aminopeptidase, valine aminopeptidase, α-βglucosidase and α-mannosidase were abundantly secreted in the growth medium. This research is the first report on mixed polymeric substrate biodegradation under sewer condition by *A. niger*, and could be considered as an open window on fungal biomass valorisation in wastewater treatment.

Keywords: Polysaccharide, wastewater, starch, bovine serum albumin, macromolecules, tank-in-series reactor, enzyme.

INTRODUCTION

The organic composition of wastewater is typically 40-60% proteins, 25-50% carbohydrates and 10% lipids (Metcalf and Eddy, 1991). 50-60% of the dissolved organic carbon has a molecular weight greater than 1 kDa (Grady et al., 1984; Levine et al., 1985; Logan and Jiang, 1990). Dissolved proteinaceous material, defined as peptides larger than 2 kDa, constitutes more than 75% of the protein contents in wastewater (Confer and Logan, 1991). Glucids are abundant in wastewater and include simple sugar, dissolved macromolecules, particulate polysaccharides and largely insoluble and heterogeneous exopolymers produced by bacteria (Pavoni et al., 1972; Tago and Aida, 1977). Macromolecules have small diffusion coefficients that limit their movement to unattached cells and aggregate in suspended growth reactors and in biofilms (Logan et al., 1987ab). Also, bacteria are unable to directly assimilate those molecules unless a first hydrolysis step occurs (Eliosov and Argaman, 1995; Salyers et al., 1996).

Relative to these difficulties, biodegradation of wastewater macromolecular substrates has been studied in conventional treatment systems in order to understand

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Abbreviations. HRT: Hydraulic retention time. BSA: Bovine serum albumin. TSA: Tryptic soy agar. Peptides ($MW_{>2000}$): Peptide with molecular weight larger than 2 kDa.

the processes mechanisms and to optimise their biodegradation (Banerji et al., 1966, 1968; Maxham and Maier, 1978; McLoughlin and Crombie-Quilty, 1983; Haldane and Logan, 1994; Confer and Logan, 1997ab; Ubukata, 1997; Hvitved-Jacobsen et al., 1998). In sewers, organic pollutants are removed by physical, chemical and biological processes that take place naturally (Koch and Zandi, 1973; Green et al., 1985; Nielsen et al., 1992; Özer and Kasirga, 1995; Raunkjaer et al., 1995; Warith et al., 1998; Vollertsen and Hvitved-Jacobsen, 1998).

Bioaugmentation has been shown as a promising alternative to remove recalcitrant organic substances from wastewater and to enhance the processes rates (Van Limbergen et al., 1998; Ro et al., 1997). Bioaugmentation of sewer networks with well-selected microorganisms under transitory conditions could be useful in partially or totally releasing assimilated molecules back into solution, and increasing their transportation to the cells. The consequences of this fractionation of macromolecules will be of benefit for enhancing substrate removal in a sewer network by suspended bacteria, biofilm and bacteria adsorbed on sediment. There are potential investments and operating cost savings by reducing the size of treatment systems, especially in environments where the build-up of large wastewater treatment facilities is impossible. The residual biomasses of fungi used in industries to produce enzymes are stocked in landfill nowadays, but they could serve as inoculums for pretreatment.

The aims of this study were (i) to determine the kinetics of the pretreatment under transitory conditions of a synthetic wastewater containing a mixture of starch and Bovine Serum Albumin (BSA) as two model macromolecular substrates, and (ii) to verify the secretion of extracellular enzymes in the growth medium by *Aspergillus niger*.

MATERIALS AND METHODS

Microorganisms and culture conditions

A. niger MUCL 28817 was obtained from the fungal collection of the Catholic University of Louvain (MUCL). *A. niger* was cultivated on tryptic soy agar (TSA) from Difco laboratories (Detroit, Mich., USA) in a 260-ml flat bottle (Nunc, Roskilde, Denmark) at 28°C for 7 days. Prior to use in the reactor system, *A. niger* was precultured in the medium described by (Garcia et al., 1997). The preculture of spores and the recovery of the fungal biomass were proceeded as described previously (Coulibaly et al., 2002). An aliquot of 50 ml of fungal biomass suspended in 100 ml of sterile distilled water was filtered as above and the biomass was used to inoculate the reactor.

Reactor system

The sewer simulating system was the reactor system previously described (Coulibaly et al., 2002). Briefly it is composed by a set of five stirred tanks in series. The system included one membrane pump (Prominent, CfG, Heidelberg, Germany), which fed the first reactor, and four peristaltic pumps (Gilson, Manupilus 2, Namur, Belgium) linking each reactor to its neighbouring unit. The reactors and the feeding reservoir were agitated with magnetic stirrers (Ika-Combimag RCO, Namur, Belgium). The reactor system was operated at an overall hydraulic residence time (HRT) of 14 h, which is encountered in long sewer lines (Özer and Kasirga, 1995).

Reactor inoculation and sampling

The reactor system was inoculated and sampled in the same way as described in our previous research (Coulibaly et al., 2002). Briefly, it was filled with 500 ml of synthetic wastewater. Then, the first reactor was inoculated with biomass prepared as indicated above.

Synthetic wastewater composition

The synthetic wastewater was composed of (in mg l^{-1}) KH_2PO_4, 700; K_2HPO_4, 1400; $(NH_4)_2SO_4$, 195; $CaCl_2$, 50; $MgSO_4.7H_2O$, 12.5; $MnSO_4$, 5; $FeCl_3$, 5; $ZnSO_4.7H_2O$, 5; BSA, 250; and Starch, 250. The pH of the medium was 6.8 after autoclaving. The starch was autoclaved separately to avoid precipitation. BSA was added to the medium after dissolution in sterile water and filtration through a 0.2 µm filter.

Biomass (SS) determination

Fungal biomass in the reactors was determined by dry cell weight. Filtration of mixed liquor was done on Whatman N° 4 filter paper, followed by oven drying at 105°C.

Protein determination

Total protein was determined using the Bicinchoninic acid (BCA) method (Smith et al., 1985). While peptides with molecular weight larger than 2 kDa ($MW_{>2000}$) were determined using the Coomassie Blue (CB) method (Confer and Logan, 1997a).

Starch Determination

The miniaturised starch iodine complex method of McCready et al. (1950) was used to monitor the starch concentration in the reactors.

Substrate removal calculation

The concentration of substrate removed (ΔS) in a time point was calculated by subtracting the substrate concentration from that of the feeding.

The initial specific substrate uptake rate ($\frac{\Delta S}{\Delta t * SS_o}$) in the reactor R_5 was calculated by dividing the concentration of substrate removed (ΔS) by the time spent (Δt) and the initial biomass concentration (SS_o).

Enzyme profiles

Enzyme activities were determined with the API ZYM kit from bioMerieux (Marcy-l'Etoile, France) and the manufacturer's

instructions were followed throughout. The API ZYM kit is a standardised semiquantitative micromethod able to detect 19 different types of enzymes. It has previously been used to screen enzymatic profiles in environmental research (McKellar, 1986; Boczar et al., 1992; Cicek et al., 1998; Morgan and Pickup, 1993). All reagents used were of analytical grade.

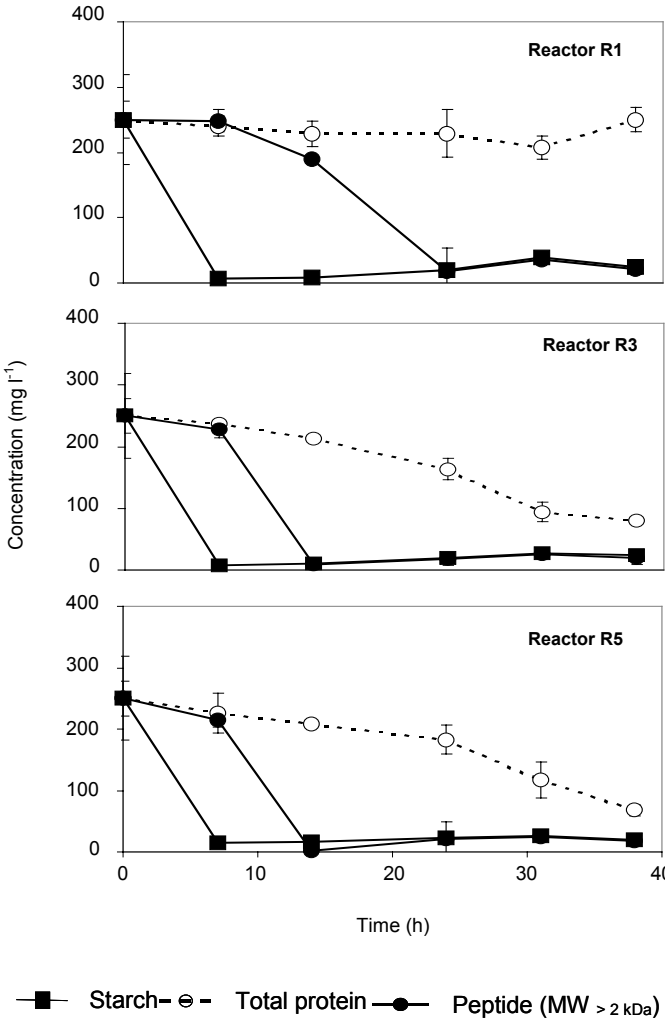

Figure 1. Kinetics of starch and BSA removal by *A. niger* under transitory conditions in reactors R_1, R_3 and R_5. Average initial synthetic wastewater composition: Starch, 250 mg l^{-1}; BSA, 260 mg l^{-1}. Initial biomass concentration in the reactor R_1 was 1185 mg l^{-1}.

RESULTS AND DISCUSSION

Triplicate tests were performed for each assay. The biotransformation of a mixture of polymeric substrates made of starch and Bovine Serum Albumin (BSA) in an equal ratio was performed under transitory conditions. The HRT was maintained to 14 h, at pH 6.8 and the dissolved oxygen concentration was kept above 2 mg O_2 l^{-1}, with starch initial specific biodegradation and BSA of 0.03 g (g SS h)$^{-1}$ and 0.01 g (g SS h)$^{-1}$, respectively.. The biomass concentration in the reactor R_1 at the beginning of the experiment was 1185 mg l^{-1}. The initial substrates concentrations in the feeding were 250 mg l^{-1} for starch and 260 mg l^{-1} for BSA. Figure 1 shows the kinetics of polymeric substrates transformation in the reactors R_1, R_3 and R_5. In R_1 (Figure 2), one could observe the transitory behaviour of the biomass that was washed out. In this reactor, starch was first completely removed after 7 h of incubation. The starch degradation rate was maintained until 14 h, after what it decreased. The dwindling of the starch degradation rate observed above 14 h, could be explained by the combined effect of the enrichment of the reactor R_1 in starch (by dilution) and the biomass washout. In R_1, peptides ($MW_{>2000}$) and total protein were fairly removed. Peptides ($MW_{>2000}$) were about 20% removed while about 10% of the total protein were removed. These substrates removal rate decreased rapidly due to the washout of the biomass and the enrichment of the reactor liquor by dilution with fresh medium.

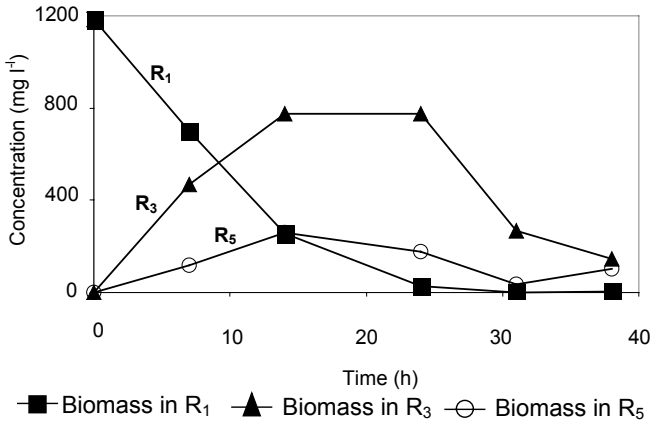

Figure 2. Kinetics of *A. niger* biomass migration trough the reactors R_1, R_3 et R_5 . Initial biomass concentration in the reactor R_1 was 1185 mg l^{-1}.

The biomass profile in R_3 (Figure 2) could be divided into two steps. In the First instance, the biomass accumulates in the reactor, and thereafter is washed out. The biomass accumulation reached a maximum of 800 mg l^{-1} after 14 h before being washed out. As observed for R_1 (Figure 1), starch was first removed. About 95% of the starch was removed after 7 h of incubation and this rate of degradation was maintained until the end of the experiment. The disappearance of total protein and peptides ($MW_{>2000}$) increased with time. About 98.5% of the peptides ($MW_{>2000}$) were removed after 24 h, whereas within the same period, about 48.5% of total protein portion were removed. The removal rate of the peptides ($MW_{>2000}$) was superior at all time than the total protein. This difference could be explained by the conversion of

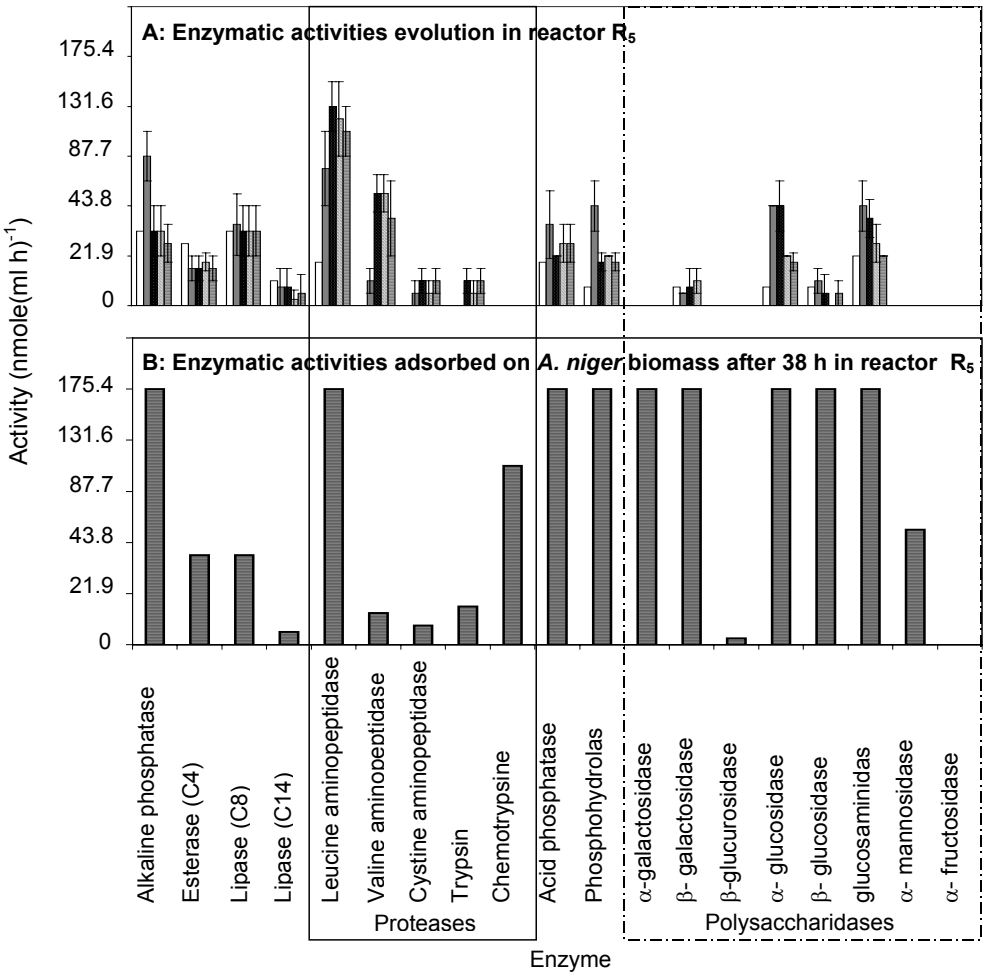

Figure 3. Enzymatics activities accumulated in reactor R_5 by *A. niger* under transitory conditions, when degrading a synthetic wastewater containing starch (250 mg l^{-1}) and BSA (260 mg l^{-1}). The initial biomass concentration in the reactor R_1 was 1185 mg l^{-1}.

before being washed out. The macromolecules degradation rate in R_5 (Figure 1) was comparable to that of R_3. In R_5, about 90% of starch was degraded after 7 h of incubation in comparison with 95% in R_3. Peptides ($MW_{>2000}$) were totally removed after 24 h whereas about 38% of total protein was removed at the same period. The starch initial specific biodegradation (0.03 g (g SS h)$^{-1}$) was three times higher than the BSA (0.01 g (g SS h)$^{-1}$). The difference observed between the biodegradation rate of starch and protein has previously been observed in natural environment and in laboratory set-up (Confer and Logan, 1997ab; Raunkjaer et al., 1995); suggesting the difficulty to degrade protein in wastewater.

The enzymatic activities in the supernatant of R_5 and on the biomass were checked in order to further understand the biotransformation of starch and BSA under transitory conditions by *A. niger*. Figures 3A and 3B show the enzymatic profiles in the supernatant and on the biomass. Fourteen of the nineteen tested enzymes were secreted in the growth medium by *A. niger*. For all of the enzymatic activities measured, that adsorbed on the fungal biomass are in general greater than the activities in the growth medium. These enzymes could be grouped into phosphatases (alkaline phosphatase, acid phosphatase, phosphohydrolase), lipases (esterase, esterase-lipase, lipase), proteases (leucine, valine and cystine aminopeptidases, trypsin) and polysaccharide hydrolases (β-galactosidase, α-glucosidase, α-mannosidase, β-glucosaminidase). The most abundantly produced enzymes were alkaline phosphatase, acid phosphatase, phosphohydrolase, esterase-lipase, leucine and valine aminopeptidases, α-glucosidase and β-glucosaminidase. Leucine and valine aminopeptidases were abundantly secreted in the medium and their

highest activity were 131.6 nmole (ml h)$^{-1}$ and 52.6 nmole (ml h)$^{-1}$ after 24 h. The period (24 h) required to reach the maximum activity of these enzymes was greater than the HRT (14 h) of the reactor system, indicating that they were secreted within a 10 h lag period. This lag period required for *A. niger* to secrete proteases could be an explanation of the shift in the BSA removal by the fungi. As shown in figure 3, secretion of α-glucosidase was rapid. α-glucosidase had a maximum activity of 43.8 nmole (ml h)$^{-1}$ at 14 h, which coincides with the HRT of the reactor system (14 h). The coincidence between the HRT of the reactor and the period required to get the maximum activity of α-glucosidase could explain the rapid removal of starch in the reactor system. Henze and Mladenovski (1991) and Raunkjaer et al. (1995) had previously observed some differences between starch removal and protein degradation in a real sewage. But these authors did not explain the differences observed by enzymatic activities. The decrease in protease and amylase activities was concomitant with the biomass washout of the reactor system. The enzymatic profiles observed in this research were similar to those found in wastewater and activated sludge matrices (Morgan and Pickup, 1993; Frolund et al., 1995).

The mixed polymeric substrates degradation mechanism could implicate both the biomass and the extracellular enzymes. The capability of *A. niger* to degrade in a realistic period (14 h) and condition (biomass, 1.2 g l^{-1}; HRT, 14 h) a mixture of polymeric substrates under transitory conditions is an interesting finding highlighted in this study. Bioaugmentation with *A. niger* under transitory condition could be considered for pretreatment wastewater containing polymeric substrates without any enzymes addition. This could result in a cost savings in wastewater treatment and the management of *A. niger* waste biomass issuing from fermentation industries.

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