

EFFECTS OF *Aspergillus niger* INOCULUM CONCENTRATION UPON THE KINETICS OF STARCHY WASTEWATER PRETREATMENT IN A TANKS-IN-SERIES BIOREACTOR UNDER TRANSITORY CONDITIONS

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Abstract - This paper discusses the effects of three *Aspergillus niger* inoculum concentrations (0.12, 2.3 and 3.6 g/l) upon the kinetics of starch pretreatment under aerobic and transitory conditions using a tanks-in-series reactor. A synthetic wastewater containing starch as model polysaccharide was fed into the reactor system to study this polymer transformation by *Aspergillus niger*. Starch and metabolites (oligosaccharides with a molecular weight lower than 1 kDa) in the individual reactors were quantified respectively by the starch iodine complex (SIC) and anthrone methods. Enzyme activities were characterised with API ZYM kits. Starch degradation and metabolite accumulation were both influenced by both fungal inoculum and reactor HRT. Starch degradation improved from 34 to 99% with a parallel in increase inoculum concentration from 0.12 to 3.6 g/l. An overall of 400 mg/l of metabolites accumulated in the reactor system. *A. niger* secreted both extracellular and cell-wall-bound enzymes among which amylases.

Keywords: Starch; Pretreatment; Bioreactor; Enzyme; *Aspergillus niger*.

INTRODUCTION

The organic matter contained in domestic wastewater is typically composed of 40-60% proteins, 25-50% carbohydrates and 10% lipids. In addition 50 to 60% of the dissolved organic carbon species have molecular weights higher than 1000 daltons (1 kDa) (Levine et al., 1985; Grady et al., 1984; Logan and Jiang, 1990). These polymeric compounds in sewage are polysaccharides, polypeptides (proteins), lipoproteins, lipopolysaccharides

and glycoproteins. Although indigenous bacteria utilise such polymers as a source of energy for growth, these compounds cannot be transported across the cytoplasmic membrane and directly into the cell (Maxham and Maier, 1978). Bacteria must hydrolyse the polymers into monomers by enzymes before metabolising them (Confer and Logan, 1997a and b). Starch had previously been considered as a model polysaccharide substrate (Banerji et al., 1966 1968) in order to study the mechanism of macromolecular substrate degradation in wastewater

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treatment processes. Starch contains two chemically distinct species of glucose polymers: amylose, which is essentially linear, and amylopectin, which is highly branched. In this study, we chose this macromolecular model substrate because its use in industry and everyday life is quite widespread. Starch and its derivatives are present in wastewater from the food and fermentation industries as well as in domestic sewage.

Whereas macromolecular substrate degradation in biological wastewater treatment processes is well documented (Maxham and Maier, 1978; Confer and Logan, 1991; Haldane and Logan, 1994; Confer and Longan, 1997a and b; Ubukata, 1997; Goel et al., 1997, 1998), information on the degradation of these molecules under sewer conditions is scarce (Coulibaly et al., 2002; Coulibaly and Agathos, 2003). Optimising macromolecular substrate hydrolysis in a sewer network could lead to production of assimilable molecules. The consequences of this transformation are rapid metabolism of pollutant and alleviation of the burden on existing underdimensioned treatment plants. A possible strategy to transform macromolecular substrate in sewer lines could be the bioaugmentation of industrial filamentous fungi classified as GRAS (generally regarded as safe) and known for their capacity to depolymerise starch as well as their resistance to toxic pollutants (Coulibaly et al., 2003).

The aims of this research were (i) to study under transitory conditions the degradation of starch by *Aspergillus niger*, (ii) to determine the effect of fungal inoculum concentration upon starch degradation kinetics, (iii) to measure metabolite accumulation and (iv) to measure the capacity of *A. niger* to secrete enzymes in the growth medium. The fungal strain was chosen for its capacity to excrete hydrolases while at the same time growing in domestic wastewater (Coulibaly and Agathos, 2003; Coulibaly et al., 2006).

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 seven days. Prior to use in the reactor system, *A. niger* was precultured in the medium described by Garcia et al. (1997). Preculture

of spores and fungal biomass recovery were carried out as described previously (Coulibaly et al., 2002). Three fungal dry biomass weights (0.06, 1.15 and 1.8 g) were used to inoculate reactor R₁.

Reactor System

The sewer simulating-system was the reactor system previously described by Coulibaly et al. (2002). Briefly, it is composed of 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), which linked each reactor to its neighbouring unit. The reactors and the feeding tank were all agitated at 150 rpm 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 found in long sewer lines (Özer and Kasirga, 1995). The reactor system was inoculated and sampled in the same way as that described in our previous research (Coulibaly et al., 2002). Briefly, each subreactor (R₁ to R₅) was filled with 500 ml of synthetic wastewater, giving a total volume of 2500 ml for the reactor system. Then the first reactor was inoculated with biomass prepared as indicated above.

All reagents used were of analytical grade.

Synthetic Wastewater Composition

The synthetic wastewater composition was as follows (mg/l): KH₂PO₄, 700; K₂HPO₄, 1400; (NH₄)₂SO₄, 195; CaCl₂, 50; MgSO₄.7H₂O, 12.5; MnSO₄, 5; FeCl₃, 5; ZnSO₄.7H₂O, 5; Starch, 500. The pH of the medium was 6.8 after autoclaving.

Analysis

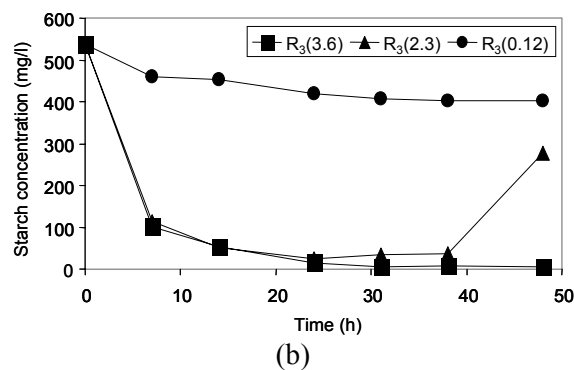
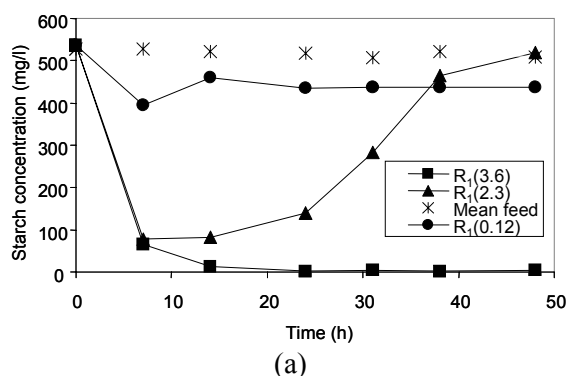
The miniaturised starch iodine complex (SIC) method of McCready et al. (1950) was used to monitor the starch concentration in the reactors. The SIC method provides information on polysaccharides having a degree of polymerisation higher than six (Swanson, 1948). Total carbohydrate concentration in the sample was determined by the anthrone method (Dreywood, 1946). Starch hydrolysis product (metabolites with a molecular weight below 1 kDa) concentrations were determined by subtraction of the starch content (SIC method) from the carbohydrate content (anthrone method) of each sample. The enzymatic activity profiles of the fungus were determined with the API ZYM kit from

BioMerieux (Marcy-l'Etoile, France) in accord with the manufacturer's instructions. The API ZYM kit is a standardised semiquantitative micromethod able to detect 19 different types of enzymes and had previously been used to screen enzyme profiles in environmental research (McKellar, 1986; Boczar et al., 1992; Morgan and Pickup, 1993; Cicek et al., 1998). Fungal biomass in the reactors was determined by dry cell weight. Filtration of mixed liquor was done on Whatman No. 4 filter paper, followed by oven drying at 105°C (Coulibaly et al., 2002).

RESULTS AND DISCUSSION

The synthetic wastewater described above was used to study starch degradation in transient regime simulating sewer conditions at 20°C and pH 6.8 and with a total hydraulic retention time (HRT) of 14 h for the reactor system. Experiments were performed under aerobic conditions, and the dissolved oxygen concentration never dropped below 2 mg O₂/l. Fungal inoculum concentrations used were 0.12, 2.3 and 3.6 g/l, and the ratio between them are respectively $1 = \frac{0.12}{0.12}$, $19.2 = \frac{2.3}{0.12}$ and $30 = \frac{3.6}{0.12}$. The time course of starch transformation in reactors R₁ (HRT = 2.8 h), R₃ (HRT = 8.4 h) and R₅ (HRT = 14 h) is shown in Figure 1. These reactors gave information on what happens upstream (R₁), in the middle (R₃) and downstream (R₅) in the reactor system. With the 0.12 g/l biomass concentration, a slow starch degradation of 21% was obtained in 7 h in reactor R₁ (Figure 1 a). Afterwards, starch degradation decreased to attain 11% in 48 h, but when inoculum concentration was increased from

0.12 to 3.6 g/l, the degradation kinetics of the starch were modified. About 86% of the starch was degraded in 7 h with the 2.3 g/l biomass. Thereafter, starch degradation decreased to 0%. With the 3.6 g/l biomass concentration, about 98% of the starch had been degraded at 7 h. Beyond this period the rest of the starch was completely removed from this reactor. However in reactor R₃ (Figure 1b), 99% of the starch had been degraded after 24 h of incubation with the 3.6 g/l biomass concentration but with the 2.3 g/l biomass concentration, starch degradation varied between 80 and 96% in the time interval of 7 to 38 h. After 38 h, a decrease in starch removal was observed. In R₃, the 0.12 g/l fungal biomass concentration gave relatively constant (17%) starch degradation after 14 h. In reactor R₅, with the 0.12 g/l concentration, a maximum starch removal of 34% was reached in 31 h of incubation (Figure 1c), but starch degradation was relatively the same for the 2.3 and 3.6 g/l inocula. For these two biomass inocula, rapid starch degradation was obtained in 7 h (65-82%). Thereafter, starch removal increased slowly to reach 99% at 38 h. With the 3.6 g/l biomass concentration, starch degradation was maintained at 99%, but a decrease in starch removal was observed with the 2.3 g/l concentration. Starch degradation depended on both fungal inoculum concentration and subreactor HRT. This result could be explained by the increase in starch hydrolysis in relation to fungal biomass concentration and the period of contact (important at longer HRTs) between this biomass and starch. As a consequence, more of the starch degrades when the HRT is longer (Coulibaly et al., 2002). The observation of a decrease in starch degradation could be explained by the dilution of the reactor system by the feed and the biomass washout from reactors.



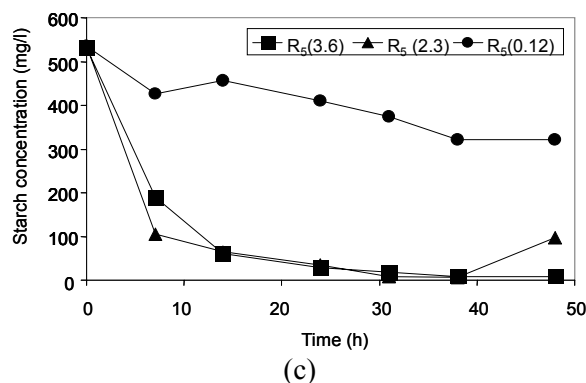
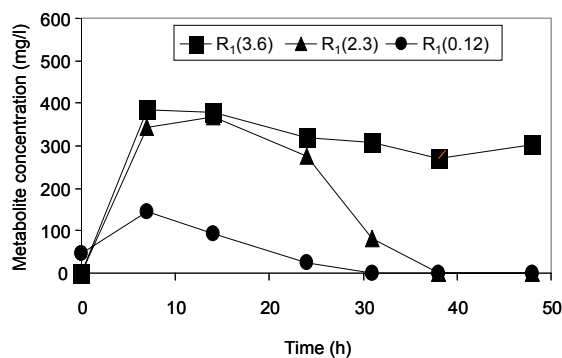


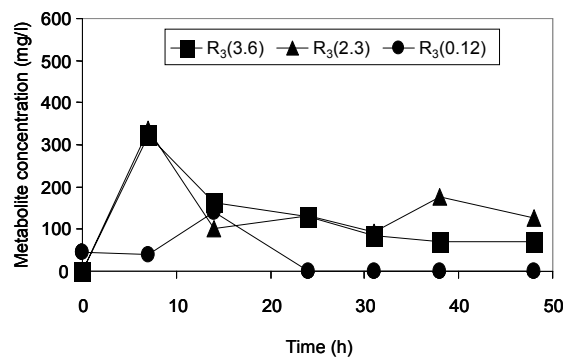
Figure 1: Kinetics of starch degradation under transitory conditions in the reactor system. (a): R_1 ; (b): R_3 and (c): R_5

Analysis of starch degradation products (metabolites with a molecular weight below 1 kDa) allowed showing that they accumulated in the reactor system (Figure 2). In reactor R_1 (Figure 2a), metabolites accumulation increased with fungal inoculum concentration. Maximum concentrations of metabolites were observed at 7 h, and those were 144, 342 and 387 mg/l respectively for the 0.12, 2.3 and 3.6 g/l biomass concentrations. Beyond 7 h, these concentrations decreased to 0 mg/l at 40 h for the 0.12 and 2.3 g/l inocula, but with the 3.6 g/l concentration, metabolite concentrations were remained relatively constant at 300 mg/l. In reactor R_3 (Figure 2b), metabolite profiles were more or less the same for the 2.3 and 3.6 g/l inocula. At these

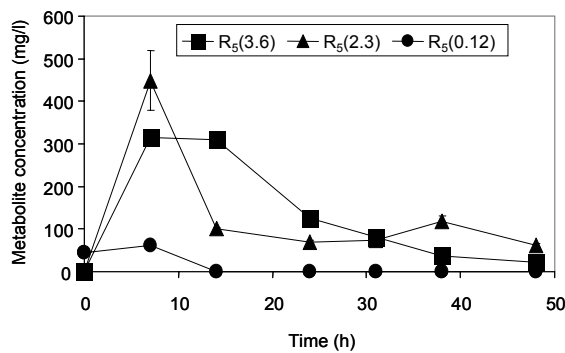
biomass concentrations, maximum metabolite concentrations (322 and 336 mg/l) were also observed at 7 h. Thereafter, metabolite accumulation decreased to 70 mg/l for the 3.6 g/l and near 120 mg/l for the 2.3 g/l concentration, but with the 0.12 g/l biomass concentration, the maximum metabolite concentrations (141 mg/l) was obtained in 14 h and then decreased to 0 mg/l in 24 h. Metabolite profiles in R_5 were relatively the same as those in R_3 (Figure 2c), but with the 0.12 g/l inoculum, the maximum metabolite concentration was lower (63 mg/l) than in R_3 and became 0 mg/l at 14 h. The decrease in metabolite accumulation could result from the combined action of their metabolisation, dilution of the reactor system and biomass washout from the reactors.



(a)



(b)



(c)

Figure 2: Accumulation of starch degradation products (metabolites with a molecular weight below 1 kDa) under transitory conditions in the reactor system. (a): R_1 ; (b): R_3 and (c): R_5

In Figure 3 the time course of biomass in reactors R_1 , R_3 and R_5 is shown for the different inoculum concentrations. For the 0.12 g/l concentration, biomass was rapidly washed out of reactor R_1 and dispersed in R_3 and R_5 (Figure 3a). Maximum biomass concentrations in R_3 (43 mg/l) and R_5 (8 mg/l) were observed respectively at 7 h and 14 h. Biomass profiles for the 2.3 g/l concentration (Figure 3b) are relatively the same as those for the 0.12 g/l. After 24 h of incubation, the biomass had been completely washed out of R_1 . Reactor R_3 was progressively filled with biomass and reached a maximum biomass concentration (435 mg/l) at 14 h. The biomass concentration in reactor R_5 reached its maximum (400 mg/l) at 37 h. For the 3.6 g/l inoculum, biomass was washed out of reactor R_1 , but a constant concentration (600 mg/l) remained beyond 31 h (Figure 3c). Reactor R_3 was progressively enriched with the biomass and its maximum concentration (800 mg/l) was observed after 24 h. In this reactor, a biomass concentration of about 400 mg/l remained. In R_5 , the biomass profile was more or less the same as that in R_3 . Biomass concentration increased in this reactor to reach a maximum of 437

mg/l after 24 h, before decreasing to a constant value of 300 mg/l. Biomass washout of the reactor system for the different inoculum concentrations illustrated the transitory aspect of its functioning. Maintenance of residual biomass in reactors R_1 , R_3 and R_5 when the 3.6 g/l fungal biomass concentration was used could be explained by the morphological transformation of the fungal biomass from small pellets to filaments and gross pellets as observed in the reactor system. This fungal morphological transformation is well documented and is attributed to inoculum concentration, pH and sheer stress (Tung et al., 2004; Pazouki and Panda, 2000). Biomass in filament and gross pellet forms could not be transferred to the adjacent reactor because of its size or adsorption on reactor constituents. This residual biomass could hydrolyse starch; this explains the high accumulation of metabolites in the reactor system inoculated with the 3.6 g/l biomass concentration. On the other hand, this biomass could metabolise metabolites when starch had been completely removed; this explains the difference between the metabolite concentrations in R_5 for 31 to 48 h for the 2.3 and 3.6 g/l inocula (Figure 2).

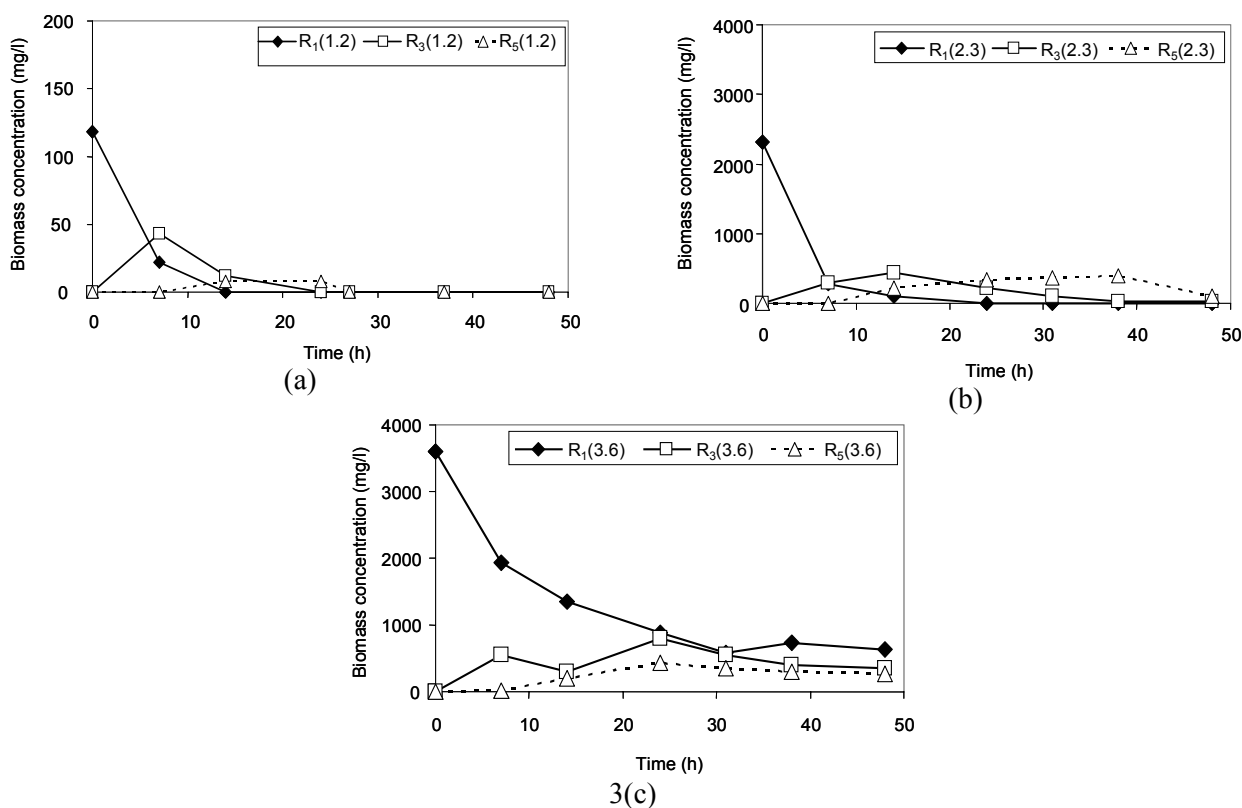
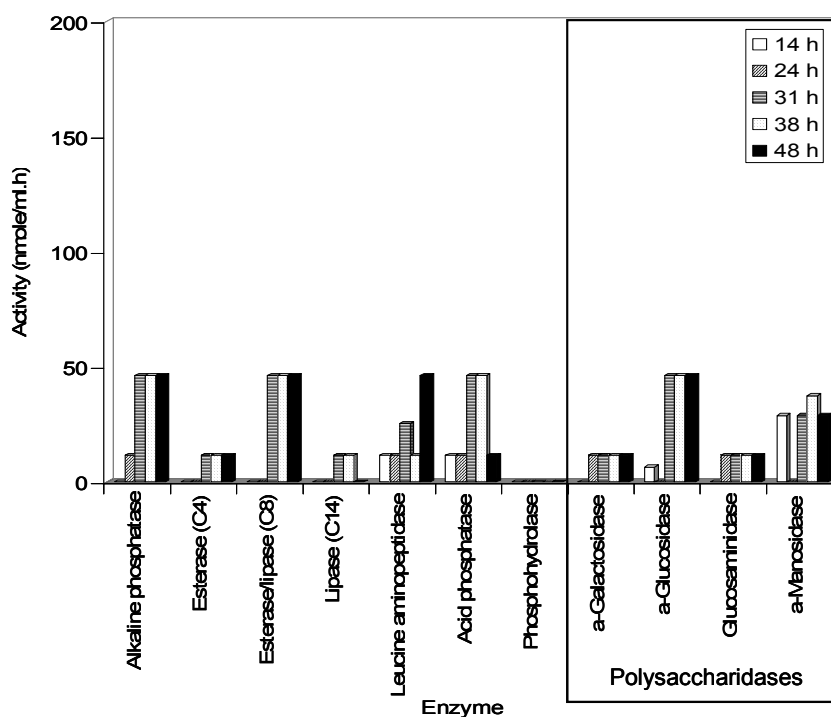


Figure 3: Biomass profiles in reactors R_1 , R_3 and R_5 for three fungal inoculum concentrations under transitory conditions in the reactor system. Biomass(a): 0.12 g/l; (b): 2.3 g/l and (c): 3.6 g/l

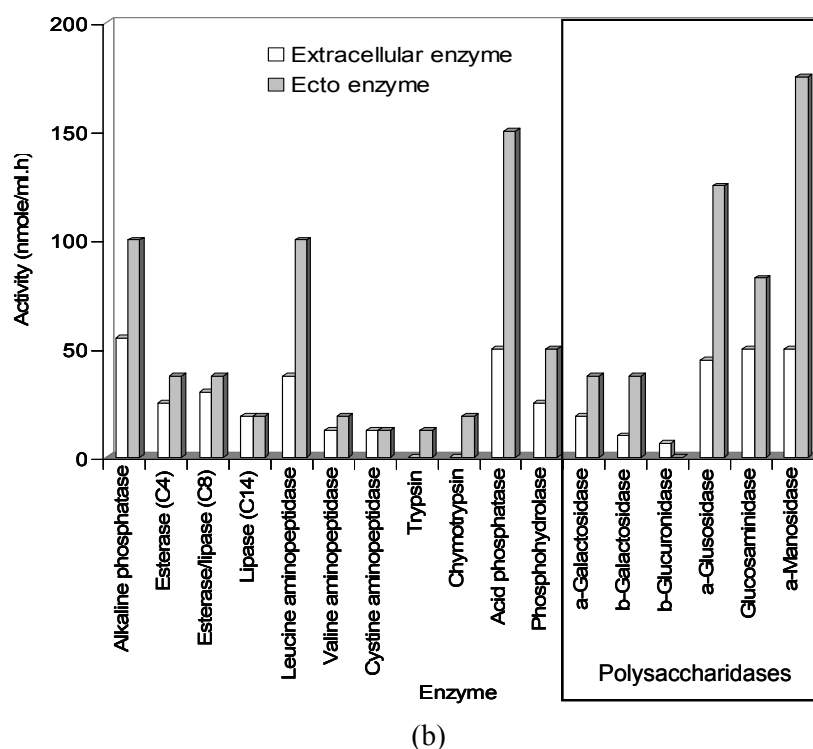
An abundant accumulation of metabolites in the reactor system was observed at 7 h (Figure 2). This result could be explained by the high concentration of biomass in the reactor at 7 h (Figure 3). This biomass could contribute to starch hydrolysis, as demonstrated below, by the presence of cell-wall-bound enzyme on fungal biomass.

To better understand starch degradation by *A. niger*, enzymatic activities of the growth medium (extracellular enzymes) and the cell wall (ecto-enzymes) were measured in reactor R₅ when the inoculum concentration 3.6 g/l was used for starch degradation. Figure 4a shows the time courses of extracellular enzyme profiles in reactor R₅. No enzyme activity was characterised at 7 h, probably because of the API ZYM test sensitivity. But after this period, *A. niger* secreted a pool of enzymes, among which polysaccharidases (α -galactosidase, α -glucosidase, glucosaminidase and α -mannosidase) (Figure 4a). This capacity of *A. niger* to secrete different enzymes into its growth medium had previously been observed (Coulibaly and Agathos, 2003). It is well known that these enzymes

contribute to polysaccharide hydrolysis. Amylase (α -glucosidase and α -mannosidase) activities were already present at 14 h. The coincidence between amylase activities at 14 h and reactor system HRT suggest that these enzymes were spontaneously secreted by *A. niger*. For α -glucosidase, this activity increased with time to attain a maximum of 50 nmole/ml h at 31 h. The maximum activity of the α -mannosidase (37.5 nmole/ml h) was observed at 38 h. Extracellular enzyme and ecto-enzyme activities in reactor R₅ at 48 h are shown in Figure 4b. Enzymatic activities of ecto-enzymes (37.5 - 175 nmole/ml h) were generally higher than those of extracellular enzymes (12.5 - 50 nmole/ml h). These results suggest that both fungal biomass and its growth medium could contribute to hydrolysis of starch to provide metabolites. The presence of ecto-enzymes and extracellular enzymes for *Aspergillus* is well documented (Iwashita et al., 1998; Nagamine et al., 2003). The mechanism of starch degradation by *A. niger* under transient conditions appears to be similar to that proposed for bacterial degradation of macromolecular substrates (Confer and Logan, 1997 a and b).



(a)



(b)
Figure 4: Enzymatic activities in reactor R_5 when the reactor system was inoculated with a 3.6 g/l concentration of fungal biomass. (a): Time course of extracellular enzymes activities; (b): Comparison of extracellular enzyme and cell-wall-bound enzyme activities at 48 h

CONCLUSIONS

A continuous flow reactor was used to simulate transient sewer conditions. At the hydraulic residence time (HRT) of 14 h, starch degradation by *A. niger* in the system depended on inoculum concentration and subreactor HRT. In the effluent of the final reactor, 99% of the starch was degraded at fungal biomass concentrations of 2.3 and 3.6 g/l, but starch degradation was low (34%) at 0.12 g/l. An important accumulation of hydrolysis products with a molecular weight lower than 1 kDa (400 mg/l) had already been observed at 7 h, but metabolite concentrations in the reactor system depended on fungal inoculum concentration. The mechanism of starch degradation by *A. niger* appeared to be the same as that by bacteria. Fungal biomass secreted extracellular and cell wall-bound-enzymes which may contribute to starch hydrolysis. Given the mode of starch degradation by *A. niger* under transient conditions, pretreatment of starch-containing water with this fungi may lead to the release of hydrolytic fragments into solution. This may have considerable implications for treatment systems because the daughter molecules are easily biodegradable. The

fungal biomass could be obtained as industrial fermentation waste.

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