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Stoichiometry of diauxic growth of a xylanase-producing *Bacillus* strain

Gerhard Schneider, Patricia Taillandier, and Pierre Strehaiano

Abstract: In this work, the establishment of material balances and stoichiometry of the growth of *Bacillus* sp. was undertaken. This strain produces high quantities of a xylanase suitable for use as bleach boost agent in chlorine-free bleaching sequences of paper pulp. As carbon dioxide plays an important role as a growth factor, bacterial growth in two fermentations, one fed with air and another fed with carbon-dioxide-enriched air, were compared. For this purpose, a method permitting the determination of the consumption of the two carbon sources, xylan and peptone, was proposed. The material balances revealed that in both cases, the bacteria first use peptone as their carbon source, and then xylan in the second part of the growth phase. The aerated culture showed diauxic growth on these two substrates, whereas carbon-dioxide-enriched air caused disappearance of the metabolic adaptation phase, and rendered biomass production more economic. The fermentation fed with air needed 30% more xylan than the fermentation fed with carbon-dioxide-enriched air for the same quantity of biomass produced.

Key words: *Bacillus* sp., carbon dioxide, material balances, diauxic growth.

Résumé : Dans ce travail, l'établissement des bilans de matière et de la stœchiométrie de la croissance de *Bacillus* sp. a été entrepris. Cette souche produit de grandes quantités de xylanase adaptée pour être utilisée comme une aide au blanchiment des pâtes à papier dans des séquences de blanchiment sans chlore. Comme le dioxyde de carbone joue un rôle important de facteur de croissance, le développement de la bactérie a été comparé pour deux fermentations : une alimentée avec de l'air et une autre alimentée avec de l'air enrichi en dioxyde de carbone. Pour cela, une méthode permettant de déterminer la consommation des deux sources carbonées, xylane et peptone, a été proposée. Les bilans de matière ont révélé que dans les deux cas, les bactéries utilisent d'abord comme source carbonée les peptones, puis le xylane dans la deuxième partie de la phase de croissance. La culture aérée a montré une croissance diauxique sur ces deux substrats, alors que l'air enrichi en dioxyde de carbone a conduit à une disparition de la phase d'adaptation du métabolisme et a rendu la production de la biomasse plus économique. La fermentation alimentée avec de l'air a nécessité 30% de xylane de plus par rapport à la fermentation alimentée avec de l'air enrichi en dioxyde de carbone pour la même quantité de biomasse produite.

Mots clés : *Bacillus* sp., dioxyde de carbone, bilans de matière, croissance diauxique.

Introduction

Recent years have seen a surge of interest in xylanolytic enzymes from microbial sources, as they are potentially valuable in the hydrolysis of xylan in various industrial applications. Currently, the most important application from an ecological and economic point of view is the prebleaching of paper pulp. This leads to savings in the consumption of expensive chlorine dioxide, which generates high molecular weight organochlorines, and other bleaching chemicals like hydrogen peroxide and ozone. Moreover, xylanase treatment allows higher target pulp brightness levels (Viikari et al.

1994; Bajpai 1999). Xylanases could also be used for enzymatic conversion of agro-industrial residues into fermentation substrates, selective degradation of xylan in food and animal feed, in fibre technology, or clarification of juices and wines (Coughlan and Hazlewood 1993).

The *Bacillus* strain chosen for this study has been reported to be an efficient producer of an endo- β (1,4)-xylanase (EC 3.2.1.8) suitable for the pulp and paper industry (Samain et al. 1992). This enzyme is devoid of cellulolytic activity, thus preventing hydrolysis of cellulose fibres, and it is stable and active at temperatures up to 60°C and pH between 5 to 7. In addition to this, it has been successfully applied in a totally chlorine-free bleaching sequence (Pham 1996). For enzyme synthesis, this *Bacillus* strain requires xylo-oligosaccharides (partially hydrolysed xylan from xylanase action) as inducer. A batch culture with xylan as substrate, where a small constitutive xylanase activity generates the inducer, is able to produce up to 2750 nkat/mL (one nanokatal (nkat) corresponds to the quantity of enzyme required to liberate one nanomole of xylose equivalent per second) in 32 h (Pham et al. 1998). Samain et al. (1997) showed that the enzyme production is also catabolically re-

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pressed when the xylo-oligosaccharides accumulate in the medium. They proposed a substrate-limited fed-batch process with continuous xylo-oligosaccharide feeding that was able to produce up to 16 666 nkat/mL.

Since enzyme production first requires control of biomass growth, the aim of our study was to contribute to a better understanding of metabolism and nutrient requirements of bacterial development. This information could be of importance in designing an industrial xylanase production process. Although many xylanase-producing microorganisms have been reported, and some of them already serve for industrial enzyme production, material balances and stoichiometry of cell growth have never been reported.

In earlier work, it was found that the presence of carbon dioxide at concentrations higher than 0.034% (concentration found in air) is essential for cell growth of this *Bacillus* strain (Schneider et al. 1999). Similar effects are well known in lactic acid bacteria and plant cells, but to the best of our knowledge, have never been reported in *Bacillus* species. In addition, carbon dioxide at a concentration of 1% in the inlet gas had a stimulating effect on metabolic activity. Since very little information is available about the role of carbon dioxide in the metabolism of a strictly aerobic heterotrophic *Bacillus*, we established and compared the material balances of two fermentations, one fed with air and another fed with carbon-dioxide-enriched air.

Materials and methods

Organism

The *Bacillus* sp. I-1018 used in this study was obtained from the Collection Nationale de Cultures de Micro-organismes (CNCM) of the Pasteur Institute, Paris, France. Cellular suspensions of the strain, grown for 18 h in culture medium described below, were stored at -18°C with adjunction of 10% glycerol (v/v).

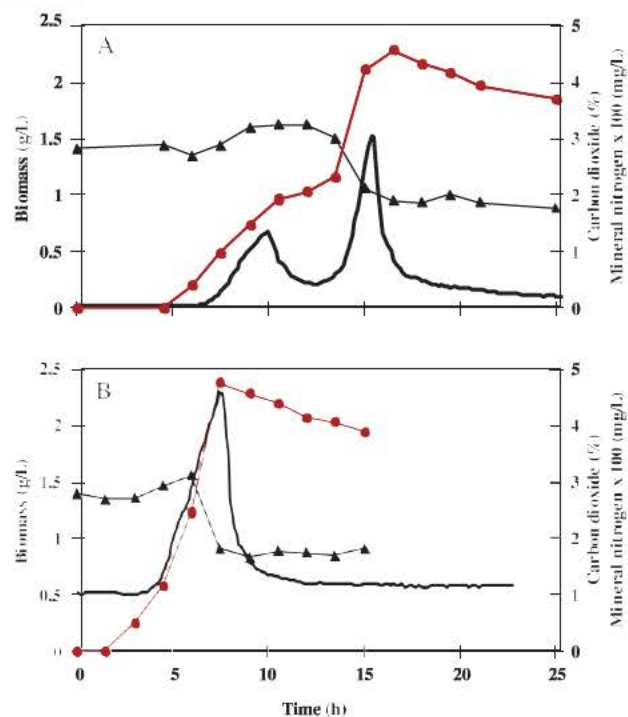
Culture medium

The culture medium described here was used for strain storage, inoculum growth, and fermenter cultures. Its composition was (in g/L): soluble xylan from oat spelts 3.16; peptone from casein pancreatically digested (Merck No. 1.02239.0500) 1.94; $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ 0.6; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.25; K_2HPO_4 2.3; NH_4Cl 0.8; and 10 mL each of trace element and vitamin solutions. Soluble xylan was prepared as follows. A suspension of xylan from oat spelts (Sigma) (10 g/L) was treated for 15 min with ultrasound (35 kHz) and then autoclaved at 121°C for 30 min. After decantation of the insoluble part (after 2 days at least), the supernatant was recovered by pumping, and the concentration of xylan was adjusted for the fermentation. The composition of the trace element solution in g/L was: nitrilotriacetic acid 12.8; $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ 1.35; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ 0.1; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ 0.024; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.1; ZnCl_2 0.1; $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ 0.025; H_3BO_3 0.01; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ 0.024; NaCl 1; $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ 0.12; $\text{Na}_2\text{SeO}_3 \cdot 5\text{H}_2\text{O}$ 0.026. The vitamin solution was prepared as follows (in mg/L): D-biotin 2; folic acid 2; pyridoxine-HCl 10; thiamine-HCl 5; riboflavin 5; nicotinic acid 5; DL-calcium pantothenate 5; vitamin B12 0.1; *p*-aminobenzoic acid 5; lipoic acid 5. The pH was adjusted to 8 with NaOH prior to autoclaving at 121°C for 20 min.

Inoculum preparation

Shake flasks (250 mL) containing 50 mL of culture medium were seeded with 4 mL of thawed stored cell suspension and incubated (50°C , 250 rpm) for 18 h before inoculation of the fermenter.

Fig. 1. Cell density (biomass) (e); mineral nitrogen concentration (Δ); and carbon dioxide concentration in the outlet gas (-). A: fermentation fed with air; B: fermentation fed with CO_2 -enriched air.



Fermenter cultures

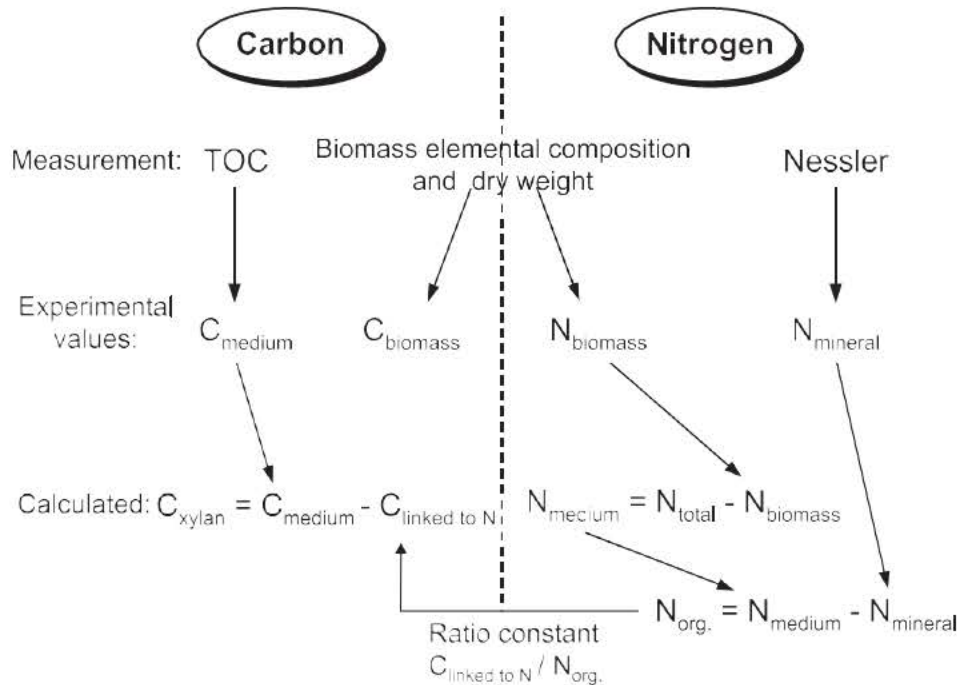
Fermentations were carried out in a 5 L Inceltech type Discovery Series 210 fermenter with a working volume of 4 L at a cultivation temperature of 50°C . The cultures were sparged with air or with air enriched with CO_2 (1%) at a flow rate of 1 L/min. Dissolved oxygen levels were measured by an oxygen probe (Ingold) and controlled (10% of saturation) by agitation speed. The outlet gas stream first passed through a drier and then through modules (Servomex) for on-line measurement of CO_2 (infrared) and O_2 (paramagnetism) contents. The pH was measured with a pH-probe (Ingold) and was also recorded on-line. The fermenter was seeded with inoculum to give an initial concentration of about 3×10^6 bacteria/mL (determined by counting on a Petit-Salumbeni haemocytometer).

Analytical methods

Microbial growth was followed by dry cell weight measurement. For that purpose, a 50 mL sample of the culture broth was centrifuged at 14 000 rev/min for 15 min. The pellet was dried in the centrifugation tube and weighed (the coefficient of variation of this method is 0.3%). The supernatant liquid was collected for measurement of ammonia (mineral nitrogen) concentration by the Nessler method, and total organic carbon measurement with a Dohrmann analyzer (oxidation of the sample in the liquid phase). The composition of the biomass, xylan, and peptone were established by the Service Interuniversitaire de Microanalyse at the Ecole Nationale Supérieure de Chimie de Toulouse (combustion of the sample and gas analysis).

Xylanase activity in the culture medium was assayed by measuring the reducing sugars liberated from birchwood xylan at 60°C and pH 5.8 using a Robinson-Britton universal buffer. Liberated reducing sugars were determined by DNS method with xylose as

Fig. 2. Analysis of the culture media for the carbon and nitrogen balances.



standard (Miller 1959) (the coefficient of variation of this method is 1.4%).

culture, the pH range was between 6.8 and 7.1 (data not shown).

Results

Kinetics of batch fermentation fed with air

Biomass growth in the fermenter fed with air showed a lag phase of about 6 h followed by a first growth phase that lasted 4 h (Fig. 1A). There was then a slowdown of growth and metabolic activity over 3 h, which was matched by decrease in CO_2 production. Subsequently, a short second growth phase took place, and after reaching maximal biomass (15.5 h), the culture entered the stationary phase where cells began to sporulate. Mineral nitrogen (ammonia) concentration increased slightly during the first growth phase, probably due to deamination of amino acids in the peptone. Mineral nitrogen consumption was observed in the second growth phase only. The pH ranged between 7 and 7.8 (data not shown).

Kinetics of batch fermentation fed with CO_2 -enriched air

This fermentation was carried out exactly as the first fermentation, except that the inlet gas stream was enriched with 1% of CO_2 (Fig. 1B). In this case, the lag phase lasted only 2 h and there was only one growth phase. The culture attained maximal biomass after 7.5 h with a final level identical to the first fermentation. Obviously, the presence of carbon dioxide had a stimulant effect on metabolic activity and cell development, as biomass production was shortened for more than a half compared with the fermentation without carbon dioxide feeding. Mineral nitrogen concentration increased during the first 6 h and was only consumed in the last 1.5 h of the growth phase. The consumed amount was nevertheless equal to the fermentation fed with air. In this

Carbon and nitrogen balances

The main difficulty consisted in the impossibility of direct quantification of the complex substrates, xylan and peptone, consumed during fermentation. Xylan from oat spelts used in this study is a heterogeneous polysaccharide mainly composed of xylose, glucose, and arabinose monomers. Quantitative analysis of this substrate needs complete hydrolysis of the polysaccharide and analysis of the monomers, for instance by high performance liquid chromatography. Unfortunately, the classic methods for acid hydrolysis partially destroy the monomers (Buchert et al. 1993).

For that reason, we proposed a method for analysis of the culture medium in order to determine indirectly xylan and peptone consumption as shown in Fig. 2. The decomposition of the culture medium was based on four measurements: total organic carbon, biomass elementary composition, dry weight of biomass, and ammonia analysis (Nessler method). These measurements led to (i) the carbon content of the supernatant obtained after sample centrifugation, (ii) carbon and nitrogen in biomass according to its elemental composition (established before), and (iii) mineral nitrogen in the supernatant. From these results we calculated the nitrogen concentration of the culture medium by the difference between total nitrogen at the beginning and nitrogen in biomass. It has been verified that there was no loss of ammonia during the fermentations, consequently the sum of nitrogen in biomass and the culture medium is identical to total nitrogen at the beginning. Organic nitrogen was calculated from the difference between nitrogen in the culture medium and mineral nitrogen. The hypothesis applied here was that the elemental composition of xylan and peptone remained constant during their consumption by the microorganisms. Con-

Fig. 3. Carbon balance of fermentation fed with air.

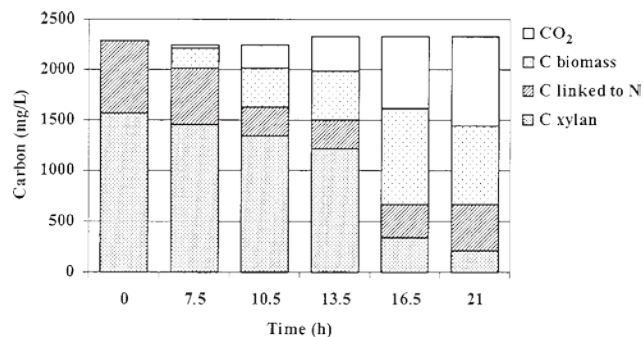
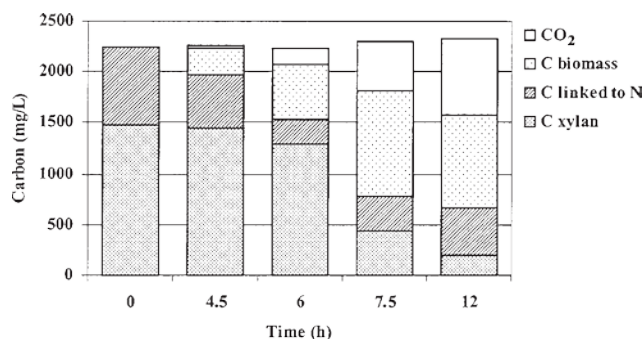


Fig. 4. Carbon balance of fermentation fed with CO₂-enriched air.



sequently we considered the ratio carbon linked to nitrogen / organic nitrogen, as constant. This assumption allowed the calculation of carbon linked to nitrogen. The final value allowed us to separately determine the carbon from xylan, allowing us to calculate xylan consumption.

Figure 3 shows the carbon balance of the fermentation fed with air versus fermentation time. During the first growth phase, which ends after 10.5 h, mostly peptone was transformed into biomass and carbon dioxide. Xylan consumption became important only during the second growth phase between 13.5 and 16.5 h of the culture. When the culture entered the stationary phase (after 16.5 h), some residual xylan not assimilable by the microorganisms, and carbon linked to nitrogen from produced compounds, were found. These included xylanase, and probably other secreted proteins.

The carbon balance of the fermentation fed with CO₂-enriched air is shown on Fig. 4. The same phenomena as in the fermentation fed with air could be observed, but in a shorter time. At the beginning of growth, there was also consumption of peptone, whereas xylan was only used in the last 1.5 h of the growth phase. During the stationary phase, residual xylan and secreted proteins were detected.

The nitrogen balance of the fermentation fed with air showed the liberation of mineral nitrogen during the first growth phase (Fig. 5). This is probably due to deamination of amino acids from peptone, which served partly as carbon source in this phase. The concentration of mineral nitrogen diminished only during the second growth phase, where it was incorporated in biomass. Organic nitrogen developed likewise as carbon linked to nitrogen (Fig. 3), because they both belong to peptone. In the stationary phase, the decrease of biomass can clearly be seen because of the sporulation

Fig. 5. Nitrogen balance of fermentation fed with air.

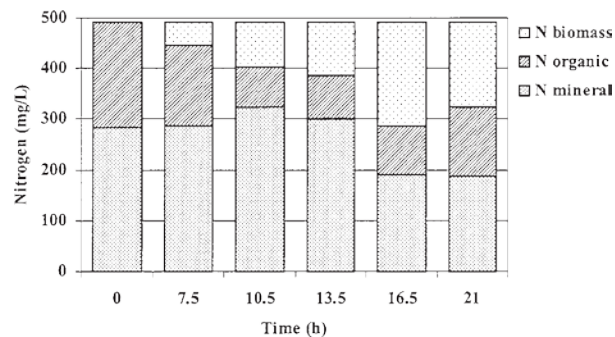
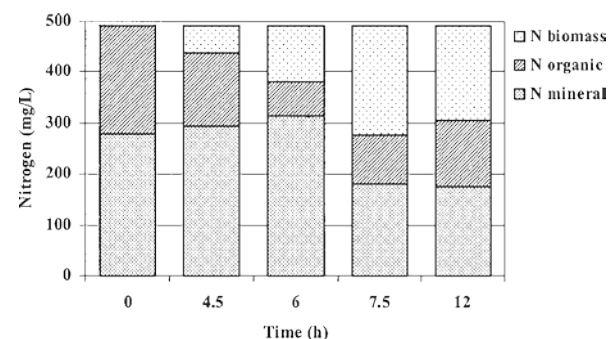


Fig. 6. Nitrogen balance of fermentation fed with CO₂-enriched air.



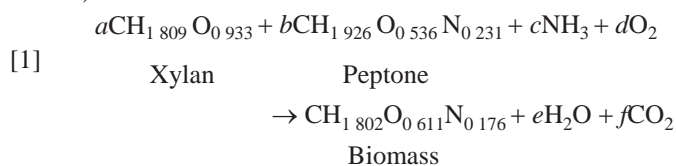
and the enhancement of organic nitrogen due to the secretion of proteins.

It can be seen from the nitrogen balance of the fermentation fed with CO₂-enriched air (Fig. 6) that the concentration of mineral nitrogen increased until the 6th hour. It was incorporated in biomass towards the end of the growth. In the stationary phase the observations were identical to those of the fermentation fed with air.

Stoichiometry of the bioreaction

To detect other metabolites, we analysed the culture supernatant by high performance liquid chromatography, after centrifugation of samples taken at different times. The column used was Biorad Aminex HPX-87H for sugars, organic acids, and alcohols, allowing the detection of the most common metabolites found in *Bacillus* species (the threshold of detection for both acetic acid and ethanol is 0.01 g/L).

Since no other compounds were detected, the only metabolite to take into account for the establishment of stoichiometric eq. [1] was carbon dioxide. The coefficients of this equation are calculated for one C-mole biomass produced (C-mole = quantity of biomass containing 1 mole of carbon).



To determine the coefficients of eq. [1], a balance on each element carbon, hydrogen, oxygen and nitrogen was established.

Table 1. Stoichiometric coefficients for the two cultures for one C-mole biomass produced.

Culture	Time (h)	<i>a</i> Xylan	<i>b</i> Peptone	<i>c</i> NH ₃	<i>d</i> O ₂	<i>e</i> H ₂ O	<i>f</i> CO ₂
Air, 1 st phase	0 to 10.5	0.463(0.569)	1.116(1.107)	-0.082	0.604	0.470	0.579
Air, 2 nd phase	13.5 to 16.5	1.856(2.314)	-0.073(-0.090)	0.193	0.741	0.997	0.783
Air	0 to 16.5	1.352(1.305)	0.417(0.414)	0.080	0.754	0.843	0.769
Air + CO ₂	0 to 7.5	1.049(0.997)	0.408(0.406)	0.082	0.446	0.564	0.457

The two supplementary equations necessary to resolve the system of linear equations with 6 unknowns were the ratio of carbon dioxide/biomass and ammonia/biomass issued from experimental data.

The stoichiometric coefficients for the two fermentations are given in Table 1. The coefficients *a* (xylan) and *b* (peptone) from the material balances of the previous paragraph are given in parentheses for comparison.

The first two lines of Table 1 show the coefficients for the first and second growth phases of the aerated fermentation, established with the quantities of compounds produced or consumed during these phases. This in turn determines the diauxic growth of *Bacillus*. The sole carbon source during the second growth phase is xylan, which is transformed into biomass and carbon dioxide, whereas nitrogen for biomass comes from ammonia. On the other hand, the possibility cannot be excluded that some amino acids from peptone are still consumed; but the secretion of proteins by the bacteria is more important.

Comparing the stoichiometric coefficients of the two fermentations at the end of the growth phase at 16.5 and 7.5 h (the last two lines of Table 1), it can be seen that growth under carbon-dioxide-fed conditions was more efficient. The consumption of the two nitrogen sources was equal, but consumption of xylan was 30% higher for the fermentation fed with air per C-mole biomass produced. This enhanced consumption of xylan provoked an enhanced production of water and carbon dioxide and a higher consumption of oxygen.

The good correlation of coefficient *b* with the material balance is because biomass can be measured with high precision, and that in both cases we assume that the global formula remained constant during the fermentation. The coefficient *a* depended on the margin of error on total organic carbon measurement.

Discussion

In this work, we attempted a more detailed analysis of the metabolism of biomass growth of a *Bacillus* strain that is able to produce high quantities of xylanase.

Aerated fermentations exhibited a diauxic growth; *Bacillus* sp. first used the organic nitrogen component (peptone) as carbon source, and in a second growth phase, used only the polysaccharide (xylan). It should be specified here that xylan first had to be hydrolyzed to xylo-oligosaccharides by extracellular endo- β (1,4)-xylanase in the culture broth. Only these oligomers can be assimilated by the cells. The transition from peptone- to xylan-consumption took 3 h, where cells had to synthesize intracellular enzymes to metabolize these xylo-oligosaccharides. However, the material balance on carbon revealed slight consumption of xylan in the first growth phase. This could be due to a basic level of

intracellular constitutive enzymes capable of hydrolyzing some xylo-oligosaccharides.

The fermentation fed with air and 1% carbon dioxide also showed peptone consumption at the beginning, and xylan consumption towards the end of the sole growth phase. The transition stage, where bacteria adapted their metabolism for assimilation of xylo-oligosaccharides, disappeared as shown by biomass and carbon dioxide production. Since the presence of 1% carbon dioxide also shortened the lag phase of bacterial growth, it can be concluded that the whole intracellular enzyme production profits from this compound.

Calculation of the stoichiometric coefficients of the biochemical reaction with 3 experimental values gave coherent results for the consumption of xylan and peptone compared with the material balances on carbon and nitrogen. The stoichiometric equation also revealed that biomass was produced more efficiently under carbon-dioxide-fed conditions. The fermentation fed with air needed 30% more xylan, which was transformed into water and carbon dioxide for the same quantity of biomass produced. It seems that this *Bacillus* species is obliged to take metabolic pathways that require more energy when there is no supplemental carbon dioxide available. We can suggest the hypothesis that in presence of carbon dioxide, anaplerotic pathways are used for intermediate production, thus less energy is lost for biosynthetic purposes (Doelle 1975). Since global material balances cannot reveal simultaneous production and fixation of carbon dioxide, further experiments would be necessary. A radiotracer experiment with carbon-14 labelled carbon dioxide, and comparison of the radioactivity levels between the two biomasses could unequivocally answer this question.

The experiments showed that sparging of the fermenter with carbon-dioxide-enriched air led to the same final cell density compared with the aerated culture. In further experiments we also proved that carbon dioxide does not influence xylanase production (unpublished results). These batch cultures reached an enzymatic activity of around 2500 nkat/mL.

To economize costs in an industrial process, one might cease carbon dioxide supply when the cell density reaches a level at which endogenous carbon dioxide can supply the needs of the cells. Another possibility consists of the use of a large inoculum size and/or the adjunction of carbonate for carbon dioxide supply at the beginning.

Acknowledgements

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