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MODELING PROCESS FOR BIOPRODUCTION OF XYLANASE BY *STREPTOMYCES* SPP. P12-137 ON LIGNOCELLULOSES AGRO-WASTES

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The production of xylanase without cellulase is required for the prebleaching of pulps, paper and food industry. The strain *Streptomyces* spp.P12-137 developed from the spores of the wild type organism was used in this work. Cultures in Erlenmeyer flasks, under shaking condition (150 rpm) at temperature and pH values (28°C, 5.0 respectively) revealed a xylanase activity of 27.77 $IU \cdot mL^{-1}$ after 120 h fermentation. This study demonstrates that *Streptomyces* spp. P12-137 is able to produce xylanase when wheat bran is used as a substrate. Fermentation was performed in a glass bioreactor with forced aeration. Data obtained have been compared to data from mathematical model obtained by numerical simulation using Matlab 7.9.0.529 (MathWorks, Inc. USA). The numerical simulation of the bioprocess could be a useful tool for adopting a control strategy to achieve increased xylanases yields under pilot or industrial conditions.

Keywords: xylanase, xylan, modeling, bioprocess, control, bioreactor

Introduction

Xylan is the main hemicellulosic polysaccharide and it represents up to 20…35% dry weight of wood and agricultural wastes (Senthilkumar *et al.*, 2008). Biodegradation of xylan is performed by xylanolytics enzymes which are produced by fungi and bacteria. Xylanases (endo-1,4-β-xylanase, E.C.3.2.1.8) are glycoside hydrolases catalyzing the hydrolysis by 1,4-β-D-xylosidic linkages of the xylan backbone (Savitha *et al.*, 2009). Xylanases have multiple applications in the pulp and paper industries, food, beverages industry, medicine and for this reason much atention was focused on them during the last 10 years (Wong *et al.*, 1988). Obtaining the fermentable sugars from lignocellulosic biomass is an important step in the biotransformation of these resources into useful products such as fuels, chemicals and polymers (Smaali *et al.*, 2009; Aristodov and Pentilla, 2000; Mielenz, 2001).

Microbial xylanases are enzymes with biotechnological potential in many industrial processes, such as pre-bleaching of kraft pulp, improvement of the digestibility of animal feed, juice clarification and bioethanol production from lignocellulosic resources. A variety of microorganisms including bacteria, yeasts and filamentous fungi were reported to produce xylanolitic enzymes using solid-state cultivation systems and submerged liquid cultivation processes. Most of the researches are focused on a submerged culture, which allows control of the pH and the temperature of the medium and several environmental factors (homogenization of the medium, aeration and sheering) required to optimize microbial growth (Assamoi *et al*., 2008).

The process models, as relations of the input, output and inner variables, though incomplete and simplified, can be effective to describe the phenomena and the influences of great importance for control, optimization and better theoretical knowledge. The function of any biological model is to describe the metabolic reactions rates and their stoichiometry on the basis of bioreactor conditions, with the main difficulties, to identify the main factors affecting cellular growth, bioproducts formation and the building up of a suitable model structure for the intracellular processes (Caramihai *et al.*, 2008). Biological systems are influenced by different process variables, which have direct influences on microorganisms metabolism. For monitoring the process variables, several sensors are used, which are attached to the bioreactor's body.

The work objective was to develop an appropriate control method for xylanase synthesis in submerged system fermentation using *Streptomyces* spp. P12-137. Actinomycetes are gram positive bacteria able to produce important industrial enzymes involved in lignocellulose degradation (Flores *et al.*, 1997; Holtz *et al.*, 1991).

Materials and methods

Microorganism and culture maintenance

Streptomyces spp P12-137 developed from the wild type strain and preserved in MIUG Collection of „Bioaliment" Platform, from „Dunărea de Jos" University of Galati, is a mesophilic actinomyces that grows best at 30^0C. *Streptomyces* spp. P12-137 was isolated from garden soil (Galati province, Romania) using Gauze-Agar medium and was used in this investigation. The hyphae of this strain are gray-purple and produce arthrospors as fruiting bodies. The strain was grown on Gauze-agar medium containing ($g \cdot L^{-1}$): starch 20, K_2HPO_4 0.5, $MgSO_4 \cdot 7H_2O$ 0.5, KNO_3 1, NaCl 0.5, $FeSO_4 \cdot 7H_2O$ 0.01, agar 25. The pH was adjusted to 7.2…7.4. The agar slants were stored at 4°C and sub-cultured once a month.

Medium for seed culture and enzyme production

The spore suspension obtained from a fresh slant of *Streptomyces* spp.P12-137 was transferred to 100 mL of seed medium containing ($g \cdot L^{-1}$): wheat bran 20, K_2HPO_4 0.075, KH_2PO_4 1.5, KNO_3 2, xylose 0.1, trace element solution 2.7 $mL \cdot L^{-1}$ in 500 mL Erlenmeyers flasks after pH was adjusted to 7…7.2. The trace element solution

contained ($g \cdot L^{-1}$): $ZnSO_4 \cdot 7H_2O$ 0.14, $MnSO_4 \cdot H_2O$ 0.16, $FeSO_4 \cdot 7H_2O$ 0.5, $CoCl_2 \cdot 2H_2O$ 0.2. Cultivation was performed for 72 h at 28°C in a rotary shaker at 150 rpm. Five milliliters of this seed culture were used to inoculate 95 mL of enzyme production medium in 500 mL flasks.

Cultivation conditions for xylanase production in a bioreactor

Cultivation of *Streptomyces* spp P12-137 strain for xylanase production was carried out in a standard 1L stirred tank bioreactor (Applikon Biobundle 1L, USA). The autoclaved bioreactor with 700 mL production medium, the same volume as that of the seed medium, was inoculated aseptically with 5% (vvm) seed culture. Cell concentration can not be ascertained because actinomycetes are filamentous microorganisms.

The bioreactor was equipped with sensors for pH, dissolved oxygen (DO), temperature and agitation. The fermentation was carried out at pH 6, temperature of 30°C, 1vvm air flow rate and agitation speed of 200 rpm. When the bioreactor was running at constant pH, the pH of the medium was continuously monitored and corrected upon need with 6.5 or 7.0. During the process samples were harvested under sterile conditions every 24 hours for xylanase activity assay and evaluation of growth dynamic by DO_{610nm} measuring. Data from the bioreactor were acquired and processed with bioreactors´ software "BioXpert Lite 1.10".

Enzyme extraction

A suitable volume of fermentation broth was centrifuged (10 000 x g for 10 min at 4°C), the supernatant was considered equivalent to crude enzyme and it was used for assaying xylanase activity after appropriate dilution.

Assays

One unit (U) of enzyme activity was defined as the amount of enzyme required to release 1 μmol of product per min, at 50°C, in 0,1M sodium-acetate buffer, at pH 5.5. Xylanase activity was determined by incubating the enzyme with 1%, birchwood xylan (Roth, Germany), according to the method of Bailey *et al.*, 1992. The amount of reducing sugars was determined by 3,5 dinitrosalicylic acid (DNS) method (Miller, 1959) using xylose solution as a standard reference. The reaction was stopped by addition of DNS reagent (Lide, 1998).

The mathematical modelling of the bioprocess for xylanase biosynthesis

The mathematical model for biological systems has the most important properties according to Edwards and Wilke postulates (Caramihai *et al.*, 2008): (a) it is capable to represent all the culture phases; (b) it is flexible enough to approximate different data types without the insertions of significant distorsions; (c) it must be continuously derivable; (d) each model parameter has to have a physical significance and should be easy to evaluate (Bellgardt, 2000).

The equation for specific growth rate which expresses the cell growth, substrate consumption and product formation is presented below:

$$\mu = \frac{1}{x}\frac{dx}{dt} \quad (1)$$

The specific growth rate is dependent on substrate concentration – S, cells concentration – X, product concentration – P , pH, temperature – T, dissolved oxygen concentration – C , inhibitors – I:

$$\mu = f(S, X, P, pH, C, I, t) \quad (2)$$

Starting from relation (2) more types of growth model have been obtained which take into account the influence of these process parameters.

The objective of this work was to develop an appropriate control method for obtaining xylanase in submerged systems fermentation (SmF) using the strain *Streptomyces* spp.P12-137.

The biosynthesis of xylanases is an aerobic process which takes place in the submerged system under controlled conditions and can be expressed using the material balance, the energy and the kinetic relations. To find the differential equations of each component of the process the following fundamental equation of balance can be used:

$$\text{accumulation} = \text{input} +/- \text{reaction} - \text{output} \quad (3)$$

The balance equation product of the reaction appear with the sign + or - it contribute to the accumulation and use of appropriate size status. Biosynthesis of xylanase in the bioreactor in batch system is characterized by four sizes status: biomass concentration, organic substrate, dissolved oxygen and concentration in the product. Input, output and reaction values are listed below:

- $X(t)$ – Total biomass:

input: $D(t)X_r(t)$ is the amount of biomass made to inoculum;

reaction: $\mu(t)X(t)$ is the amount of biomass made in bioreactor thus the reaction appears with a sign „+”; the amount of biomass produced is given by the growth rate of microorganisms which depends on the substrate concentration and dissolved oxygen.

- $S(t)$- Substrate:

input: $D(t)S_{in}$ is the substrate brought in the bioreactor along with the inoculum;

reaction: - $\mu(t)/Y \cdot X(t)$ reaction to the substrate occurs with sign „-” because it expresses the substrate used for biomass growth; the reaction of the balance equations is given (Eq.4) by the growth rate of microorganisms and production coefficient;

- $DO(t)$ – Dissolved Oxygen concentration:

input: $\alpha W(DO_{max} - DO(t))$ is the dissolved oxygen brought in the bioreactor by aeration and should not exceed the maximum quantity DO_{max};

reaction: - $k_0\mu(t)X(t)/Y$ in the reaction of aerobic metabolism, biomass is growing by consuming the dissolved oxygen on the bioreactor and it will appear with sign „ –”;

- $P(t)$ – Product concentration (xylanase):

reaction: $q_p(t)X(t)$ as a result of the biosynthesis process, the products accumulated in the medium and are represented by extracellular or intracellular xylanase (in biomass). Accumulation is evidenced by the sign „+".

Thus, the mathematical model of enzyme biosynthesis process is described by the following equations:

$$\frac{dX}{dt} = \mu(t)X(t) + D(t)X_r(t) \quad (4)$$

$$\frac{dS}{dt} = -\frac{\mu(t)}{Y}X(t) + D(t)S_{in} \quad (5)$$

$$\frac{dDO}{dt} = -\frac{k_o\mu(t)X(t)}{Y} + \alpha W(DO_{\max} - DO(t)) \quad (6)$$

$$\frac{dP}{dt} = q_p(t)X(t) \quad (7)$$

$$\mu(t) = \mu_{\max}\frac{S(t)}{k_S + S(t)} \cdot \frac{DO(t)}{k_{DO} + DO(t)} \quad (8)$$

where: $X(t)$ – biomass; $S(t)$ – substrate; $DO(t)$ – dissolved oxygen; DO_{max} - maximum amount by $DO(t)$; $X_r(t)$ – biomass add by inoculum; Y - production coefficient; $D(t)$ – dilution rate; μ - specific growth rate of microorganisms; μ_{max} – maximum growth rate; k_s – saturation constant; k_{DO} – saturation constant for oxygen; α – transfer rate for oxygen; k_o – model constant; W – air flow rate; S_{in} – inoculum substrate concentration; q_p – product rate (Caraman *et al.*, 2007).

The model shown in equations (4)-(8) has been simulated on considering the following parameters and initialization data for the process: $DO(0)$=5mg/L; P(0)=0; μ_{max} = 0.15 mg/L; k_{DO} = 2 mg/L; α = 0.018; DO_{max} = 10 mg/L; k_o = 0.5; q_p = 0.00675; $S(0)$ = 20 g/L; S_{in} = 0.005 g/L; $X(0)$ = 0.07 g/L; Y = 0.70; D = 0.025; W = 60.

Results and discussion

For the strain *Streptomyces* spp P12-137 torque dynamic growth with the xylanase production, shown in Figure 1, the biosynthesis maximum is reached during the exponential growth phase, when the substrate is used as carbon source with plastic and energetic part. Xylanase production curve shows two peaks, one after 96 hours of cultivation and another after 168 hours, suggesting that it is possible to synthesize a complex of enzymes with sequential action. Another interpretation would be that after 96 hours, when maximum of biosynthesis is reached, xylose released has a catabolic repression of xylanase action. Between 96 to 168 hours, xylose released in excess is consumed for biomass growth after the end of the exponential phase. As the xylose consumption, the catabolic repression is reduced and microbial cells restart biosynthesis proved by the second maximum in the graph. The decreased activity after 192 hours of cultivation is explained by the decrease of cells number in the decline phase and throughout the process by

catabolic repression subsequent by enzyme biosynthesis in the second maximum point.

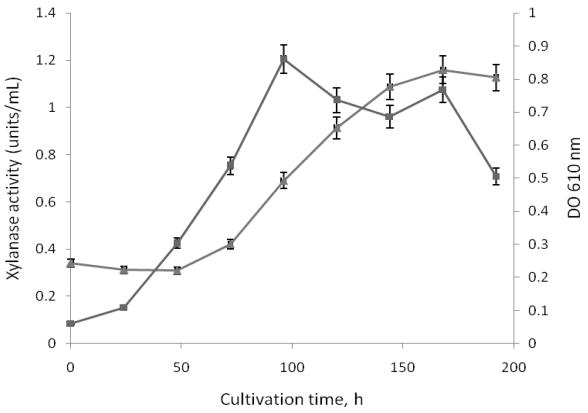

Figure 1. Dynamics of growth and production of xylanases by *Streptomyces* spp. P12-137 strain in SmF system after 196 hours of cultivation at 28°C, 150 rpm on enzyme production medium with birchwood xylan and ammonium sulphate. ■ xylanase activity (U/mL); ▲Optical density for cells DO 610 nm. The points represent average determinations in triplicate

We have also analyzed the behaviour of the selected strain *Streptomyces* spp P12-137 in laboratory by Applikon BioBundle bioreactor with 1 liter capacity. This allows continuous recording of parameters fermentative process (temperature, pH, dissolved oxygen concentration). The analysis of dynamic of growth shows the lack of lag period, a high slope of exponential phase, stationary phase with down appearance and a declining phase after 120 hours of cultivation. Dynamics of the biosynthesis of xylanases shows two peaks, one after 20 hours of cultivation and the other after 80 hours (Figure 2).

The aspect for growth curve in bioreactor is similar to that obtained in Erlenmeyer vessels with stirring, pointing the aspect of „camel-back". Xylanase activity values are lower than those obtained in shaker cultivation due to the different biotechnological parameters, namely: increased volume of aerated medium and different agitation conditions.

Consumption of dissolved oxygen dO_2 shows after 20 hours of cultivation an ascending aspect due to the decrease of oxygen consumption by measure of stationary phase installation followed by the decline in the biomass development. Biosynthesis yield was 2.64 UI·mL^{-1} and is comparable to the potential of other strains described in the literature (Table 1).

The relations between the specific growth rate (μ) of a microbial population and the substrate concentration (*s*), is an important tool in microbiology and biotechnology. The simulation results show the control capacity of the systems; this was possible by computing the substrate feeding needed for cell growth according to the biomass concentration increase.

Biomass reaches a maximum after 50…60 hours of cultivation in bioreactor, a value similar to the one given by simulation. Dissolved oxygen consumption remains in plateau along the period of cultivation as the model presents. In the case of xylanase concentration, the model presents a significant increase and continues to grow beyond the 120 hours of cultivation. However, experimental data obtained in bioreactor shows inhibition of biosynthesis or a decrease level due to the action of proteolytic systems developed in the culture medium (Figure 3).

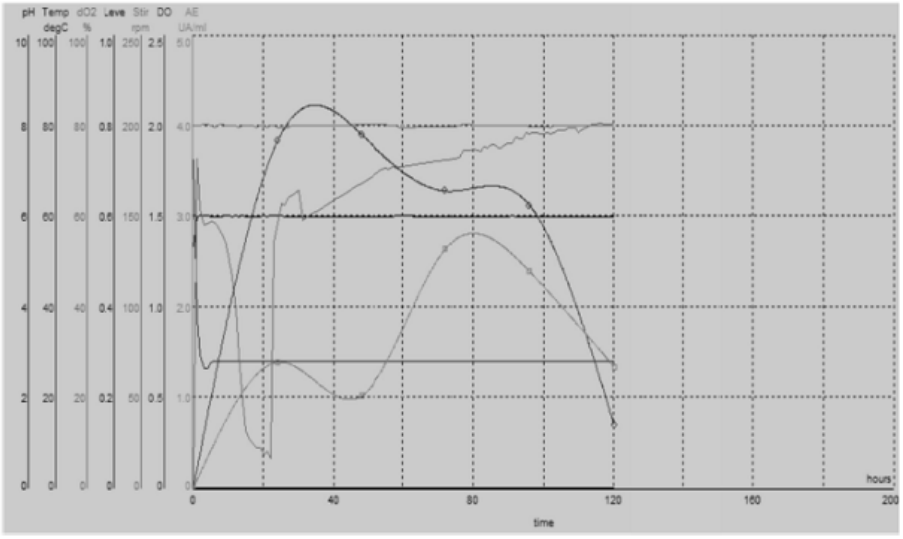

Figure 2. Biotechnological parameters of cultivation registered in bioreactor by fermentative optimized culture medium at 30°C temperature, pH 6.0, agitation at 200rpm, aeration of 1 vol.air /vol.medium for 120 hours. The process was controlled with software „BioXpert Lite"

Table 1. Biotechnological cultivation parameters obtained in the 1L Biobundle Applikon bioreactor, USA

Cultivation time (h)	Degree of biomass development, D/D_0*	Xylanase activity, UI
0	0	0
24	1.92	1.39 ± 0.39
48	1.95	1.02 ± 0.39
72	1.64	2.64 ± 0.31
96	1.56	2.39 ± 0.62
120	0.53	1.34 ± 0.56

* optical density of culture at 610 nm

The differential model expresses how several of the state variables vary with variation in one or more independent variables involved in obtaining the xylanases in batch system on agro-wastes substrates. The model can contribute to understanding the functioning of bacterial cells and to predict about how a pilot or

industrial scale bioreactor must be designed and operated in order to give optimal performance.

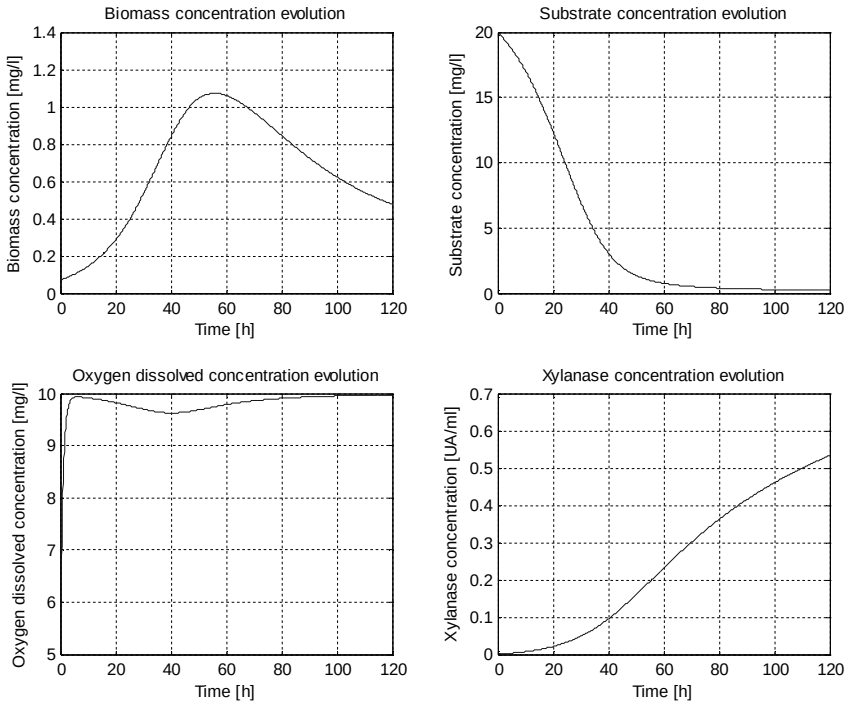

Figure 3. Simulation results of the control loop with MATLAB, version 7.9.0.529 for biomass, substrate, oxygen dissolved and xylanase concentration

Conclusions

The batch aerobic cultivation was successfully used for the xylanases production from lignocellulosic agro-wastes using the filamentous microorganism from *Streptomyces* genus. The objective of this research was the development of a mathematical model using MATLAB software version 7.9.0.529 based on experimental data obtained from the submerged cultivation performed in the laboratory bioreactor Biobundle Applikon, USA. For control loop simulation, we proposed a mathematical model whose results were compared to experimental data. The experimental data and the predicted results are in good agreement. Some little deviations from experimental data in the later stages of fermentation were observed. The model needs improving on the dynamic of xylanase biosynthesis taking into account the appearance („camel-back"). This will represent the objective of a new research.

The process models, as relations of the input, output and inner variables, though incomplete and simplified, can be effective to describe the phenomena and the

influences of great importance for control, optimization and better theoretical knowledge.

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