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Reuse of wastewater from pulp industry for the optimization of fungal xylanase production

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ABSTRACT. The production of enzymes using agro-industrial waste is a low cost alternative for the reuse of byproducts, with the subsequent impact decrease on the environment. Current analysis produced xylanase using fungus *Aspergillus niger*, with two types of wastewater generated during the pulp chemical bleaching phase as inducers. Xylanase was produced by submerged liquid fermentation and factorial design optimized parameters that influence production (concentration of wastewater and production period). Initial culture conditions (pH, temperature and agitation) were optimized independently. Alkaline wastewater was more effective than acidic wastewater for the induction of xylanase in optimized conditions: 50% of culture medium, 7-day production, 30°C, pH 6.0 and agitation at 160 rpm. Despite different results, acidic and alkaline wastewaters induced xylanase production by *A. niger* when employed in concentrations lower than or equal to 50% of culture medium and in the most optimal conditions described above. Alkaline wastewater is highlighted as the most efficient for such production.

Keywords: enzyme, *Aspergillus niger*, factorial design, agro-industrial waste, xylanase.

Reutilização de efluentes da indústria de celulose para otimização da produção de xilanase fúngica

RESUMO. A produção de enzimas, a partir de resíduos agroindustriais, é uma alternativa para reutilização destes subprodutos como substrato de baixo custo reduzindo seu impacto causado pelo descarte no meio ambiente. Diante disso, o objetivo deste estudo foi a produção de xilanase por *Aspergillus niger*, utilizando dois efluentes gerados nas fases de branqueamento químico de polpa de celulose como indutores. A produção de xilanase foi realizada por fermentação líquida submersa, e utilizou-se planejamento fatorial para otimização dos parâmetros influentes de produção (concentração de efluentes e período de produção) e as condições iniciais de cultivo (pH, temperatura e agitação) foram otimizadas de forma independente. O efluente alcalino se mostrou mais efetivo do que o efluente com característica ácida, na indução de xilanase em condições otimizadas: 50% em relação ao meio de cultura, sete dias de produção, 30°C, pH 6,0 e agitação de 160 rpm. Conclui-se que ambos os efluentes, ácido e alcalino, apesar de levarem a diferentes resultados, são capazes de induzir a produção de xilanase por *A. niger* quando empregues em concentrações menores ou iguais a 50% em relação ao meio de cultura e nas condições ótimas descritas acima, destacando-se o efluente alcalino como mais eficiente para produção de tal enzima.

Palavras-chave: enzima, *Aspergillus niger*, planejamento fatorial, indústria de celulose, xilanase.

Introduction

The production of wastewater by the pulp industry resulting from the chemical bleaching stage has exponentially increased due to rise in demands for the product (Muthezhilan, Ashok, & Jayalakshmi, 2007). Although the chemical composition of wastewater from the pulp industry depends on the pulping process, it is generally accepted that the chlorine-based chemical bleaching process is a great pollution generator. The process of chemically bleaching pulp generates large amounts of wastewater with low biodegradability and may contain toxic substances such as bio-accumulative organo-chlorine compounds (Kaur, Mahajan, Singh, Garg, & Sharma, 2010; Chandra & Sankhwar, 2011). Production of enzymes from agro-industrial wastewater is an alternative for the reuse of these byproducts as low-cost substrates due to the reduction of impact caused by its deposition in the environment (Sharma & Kumar, 2013).

Xylanase is a hydrolytic enzyme with potential applications in various industrial sectors, for example, in the bioconversion of plant biomass into biofuel (Jiang et al., 2015), and, consequently, the

improvement in the quality of animal feed (Bakri, Al-Jazairi, & Al-kayat, 2008). However, it has been generally studied in its application in pulp industry acting on the hydrolysis of xylan linkages, an important structural component of hemicellulolytic polysaccharide which occurs in the constitution of the wall of the plant´s cell (Gupta & Kar, 2009). Its function is the manufacture of cellulose fiber, formed by linkages between lignin and hemicellulose. It becomes more permeable and facilitates the release of lignin, reducing chemical products at the bleaching stage (Techapun, Sinsuwongwat, Poosaran, Watanabe, & Sasaki, 2001; Muthezhilan et al., 2007; Sharma et al., 2014). Although xylan is the main inducer for the production of xylanase, it is an expensive substrate and thus economically nonviable for the industry. Consequently, the use of pulp industry wastewater represents the possibility of using a low-cost substrate (Fang, Chang, Hsieh, & Fang, 2007; Bakri et al., 2008; Pérez-Rodríguez et al., 2014).

Current study aims at optimizing xylanase production by *Aspergillus niger* using pulp industry wastewater, with independent variables analyzed by factorial design.

Material and methods

Xylanase production by submerged liquid fermentation

The microorganism *Aspergillus niger* (IOC/CCFF 3998) has been used for xylanase production. Inoculum standardization was adjusted by counting 10^7 spores mL^{-1}. Production of xylanase was carried out by submerged liquid fermentation, in triplicate, using culture medium described by Nair, Sindhu, and Shankar (2008) containing 0.5 g KCl; 0.5 g $MgSO_4.7H_2O$; 2.5 g $(NH_4)_2HPO_4$; 0.5 g NaH_2PO_4; 0.01 g $CaCl_2.2H_2O$; 0.01 g $FeSO_4.7H_2O$; 0.002 g $ZnSO_4.7H_2O$, with modification in concentration between 1.0 g and 0.5 g xylan L^{-1}, without loss of enzymatic production by xylanase defined in previous experiments on production evaluation. The initial culture conditions were also described by Nair et al. (2008), or rather, pH 5.0, 30°C and shaking at 100 rpm. Optimization was carried out by factorial design.

The central composite rotatable design

Optimization of xylanase production was outlined by factorial design (2^2) with central (0) and axial (-1.41 and 1.41) points from the analysis of data obtained in the initial controls, experimental variables and their corresponding levels (Table 1). The results underwent variance analysis (ANOVA) with p rates 0.05 and 0.1, by Statistix 9.0.

Table 1 Specification codes and true rates of independent variables (wastewater concentration and production period) valuated in the factorial design for production of xylanase.

Variables	Codes	- 1.41	- 1	0	+ 1	+ 1.41
Wastewater concentration (%)	y	0	14	50	86	100
Production period (days)	x	4	5	7	9	10

Effect of pH, temperature and agitation in xylanase production

The best conditions for pH, temperature and agitation for xylanase production were independently evaluated, while conditions determined by factorial design (wastewater concentration and production period) were kept constant. Different pH rates evaluated were pH 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 for the two types of wastewater. Three different temperatures (30, 45 and 60°C) were used to determine the optimal temperatures for production. Further, 100 rpm, 160 rpm and 200 rpm were assessed to determine optimal agitation.

Analytic methods to determine xylanase activity

After recovering the enzyme extract by vacuum filtration, the filtrates were analyzed for protein concentration following method by Bradford (Bradford, 1976). Xylanase activity was determined following the method described by Nair et al. (2008), using xylan as substrate. The reaction, comprising 900 μL^{-1} 1% xylan solution in sodium citrate (50 mM, pH 5.3) added to 100 μL^{-1} of enzymatic extract, was incubated at 50°C for 5 min. After this period, the reaction was stopped, 1.0 mL 3,5-dinitrosalicylic acid (DNS) was added and boiled at 100°C for 5 min. It was then measured by spectrophotometry at 540 nm for specific activity of xylanase. Specific activity of xylanase (U mg^{-1}) was determined by the ratio of protein concentration (mg mL^{-1}) and xylanase activity (U min^{-1} mL^{-1}). One unit of xylanase has been defined as the quantity of enzyme that releases 1 μmol of xylose per minute, according to experimental conditions.

Results

The central composite rotatable design

Wastewater concentration and production period were the two independent variables evaluated by factorial design to obtain xylanase, Codified axial points were calculated with (2k) ¼, where k is the number of independent variables in the study. The factorial design developed for acidic wastewater, referring to the specific activity (Figure 1), showed $R^2 = 0.95$, and proved to be significant for variables wastewater type and production period, at the linear and quadratic level, with $p \leq 0.1$. The equation that describes the model for this design is: $z = 39.15-0.98x-5.71.x^2-20.70.y-5.40.y^2+4.10.x.y$.

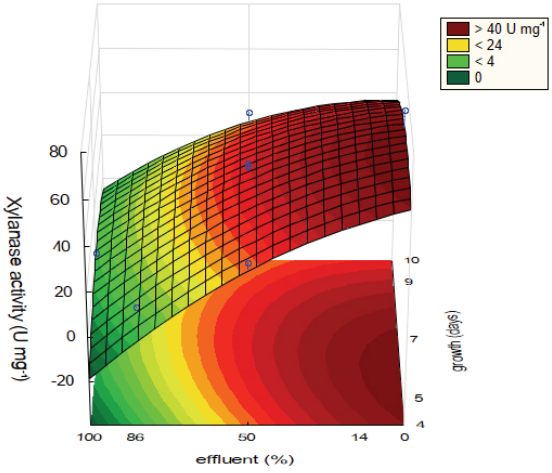

Figure 1. Response surface graph shows influence of variables concentration of effluent and production period in the specific activity obtained from factorial design in the production of xylanase by acid effluent.

The factorial design developed for alkaline wastewater, referring to specific activity (Figure 2), showed $R^2 = 0.84$, with the influence of analyzed variables which were significant at $p \leq 0.05$. The equation for the model in this design is: $z = 346.26 - 6.18.x - 136.62.x^2 - 30.55.y - 141.97 .y^2 + 12.61.x.y$.

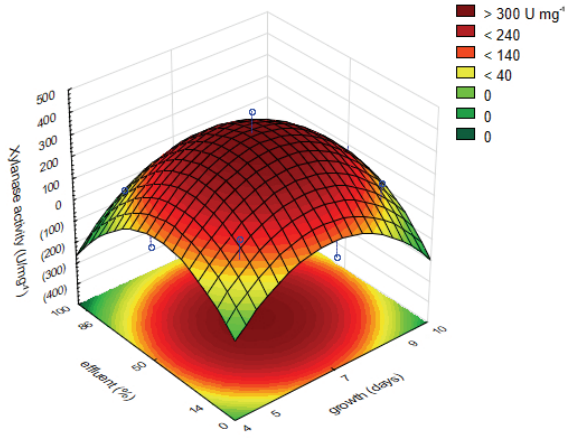

Figure 2 Response surface graph shows influence of variables concentration of effluent and production period in the specific activity obtained from the factorial design in the production of xylanase by alkaline effluent.

The optimal conditions obtained with factorial design, 50% concentration of wastewater and a 7-day-production period, were fixed as production parameter and validated. Since validation rates are shown close to or greater than the predicted rates found by equations that describe the model in the factorial design (Table 2), they prove the efficacy of these conditions.

Table 2. Xylanase activity and specific activity in productions with acid and alkaline wastewater related to the validation parameters set by factorial design.

Wastewater	Xylanase activity ($U\ min^{-1}\ mL^{-1}$)★		Specific activity ($U\ mg^{-1}$)★	
	Predicted rates	Found rates	Predicted rates	Found rates
acid	5.99	6.28 ± 0.79	39.15	38.65 ± 3.88
alkaline	4.52	4.29 ± 0.32	346.26	382.15 ± 98.7

★ rates from average in triplicates.

Effect of pH, temperature and agitation in the production of xylanase

Figure 3 shows the effects of variations in production conditions pH, temperature and agitation on xylanase production. The optimization of pH for acid wastewater revealed an ideal condition at pH 5.0 enhancing considerably the production of xylanase (183.3 U mg^{-1}), whereas pH 6.0 was the optimum condition for alkaline wastewater. Optimization of temperature variation demonstrated that enzymatic production was significantly affected by temperatures above 30°C, the optimal temperature for production of the two types of wastewater. In other words, temperatures at 45 and 60°C did not have a positive effect on xylanase production. Tests for agitation assessment evaluated optimization of xylanase production with wastewater, but failed to be a significant factor for production. Whereas the best xylanase production with acid wastewater was constant agitation at 200 rpm (172.5 U mg^{-1}), in the case of alkaline wastewater the best enzyme production was constant agitation at 160 rpm (221.1 U mg^{-1}).

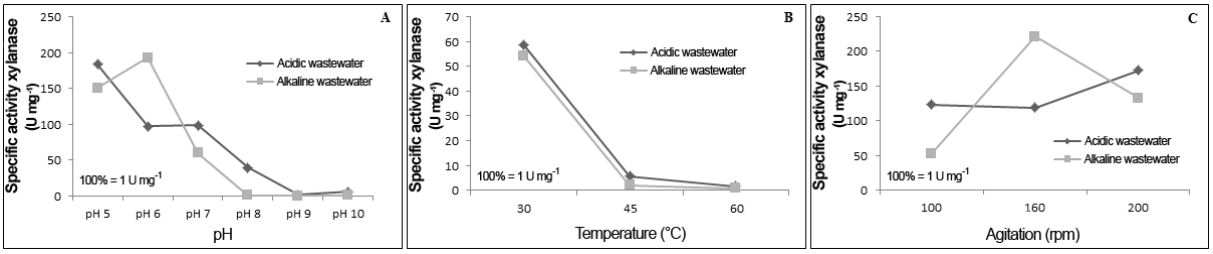

Figure 3. (A) Effects of pH on xylanase activity. (B) Effects of temperature on xylanase activity. (C) Effects of agitation on xylanase activity. Relative activities are given as percentages of specific activity (100%)

Discussion

Some byproducts, in particular lignocellulosic substrates, such as wheat bran, sugar cane bagasse, corn residue and rice straw are being employed as substrates in fermentation processes for the production of xylanase (Okafor, Okchi, Onyegeme-Okerenta, & Chinedu, 2007; Izidoro & Knob, 2014). In current study, the authors obtained significant results using pulp industry wastewater.

Corroborating results obtained, it has been observed that large volumes of wastewater and long periods of production do not enhance the production of enzyme with the highest catalytic capacity. In fact, the production of xylanase with the highest specific activity employing the two types of wastewater occurred at levels 25-50% of the medium described by Nair et al. (2008) with modification in xylan concentration (0.5 g), an interesting point for costs reduction in production, and between 5 and 10 days of production period. The above flexibility in the levels of xylanase production is important since the processes in the industrial sector are different and adaptation is required according to the situation in which it will be used.

Lakshmi, Rao, Rao, Hobbs, and Prakasham (2009) showed that the production period had the highest impact on the obtainment of xylanase. However, increasing the production period failed to increase the levels of enzymatic activity. On the contrary, they would rather be reduced, suggesting the hypothesis that decrease was caused by depletion of nutrients or proteolysis due to secretion of non-specific proteases (Sepahy, Ghazi, & Sepahy, 2011; Nasr, Soudi, Salmanian, & Ghadam, 2013).

The microbial production of xylanase is influenced by culture factors employed such as culture medium composition, pH, temperature, incubation and agitation which have a considerable impact on the efficiency of enzymatic production and must be analyzed for the optimal yield of the microorganism (Chipeta, Du Preez, & Christopher, 2008; Thomas, Sindhu, Binod, & Pandey, 2015). The parameters with the greatest influence on xylanase production for the two types of wastewater were production period, pH and agitation. The influence of production parameters varied according to the species of the microorganism. Consequently, search for the optimization of enzymatic production is extremely important (Lakshmi et al., 2009).

According to Coelho and Carmona (2003), the production of xylanase by different species of the genus *Aspergillus* showed distinct optimal pH. However, the literature shows that the ideal pH range for xylanase production lies between 4.0 and 6.0. Xylanase activity decreased at alkaline pH for the two types of wastewater, corroborating data in the literature (De Alencar Guimarães et al., 2013). Temperature was also an influential factor in enzymatic production and it may be related to thermophile or mesophile characteristics of the fungus used. The best temperatures for fungal xylanases lie between 30 and 60°C. Higher temperatures are usually associated with a short period production, unlike conditions in current study, where high temperatures were associated with a long period of production (7 days) with negative influence of the 40-60°C temperature range, indicating the mesophilic characteristic of *A. niger* used (Rizzatti, Sandrim, Jorge, Terenzi, & Polizeli, 2004; Chandra & Sankhwar, 2011; De Alencar Guimarães et al., 2013; Pérez-Rodríguez et al., 2014).

Acid and alkaline types of wastewater, albeit with different results, are capable of inducing xylanase production when employed in concentrations less than or equal to 50% of the culture medium, highlighting alkaline wastewater as the most efficient for production. Results represent a 50% reduction in water usage, which, taking into consideration enzymatic production on a large scale, becomes a sustainable alternative. In fact, it avoids the use of potable water, a scarce natural resource, and contributes towards the conservation and preservation of the environment (Moldes, González, Rodrigues, & Converti, 2013; Milinos, Viana, Brennan, & Donohuel, 2015).

Most studies using wastewater from the pulp industry are focused on physical, chemical and biological treatment processes that reduce its pollutant load (Kamali & Khodaparast, 2015). However, nutrients of wastewaters may be reused for better purposes, for example, production of enzymes of biotechnological interest, as reported in current study (Hay, Wu, Ng, Juan, & Jahim, 2016).

Residues reuse processes are very important since they reduce deposition in the environment. Another positive point in reuse is the possible reduction of wastewater toxicity, since biotechnological processes employ microorganisms as fungi, which degrade environmental pollutants such as dyes, heavy metals and organic pollutants, due to the large amount of oxidative enzymes produced. Fungal biomass from biotechnological processes, as wastewater treatment, may also be reused. However, the activity is very demanding because it is a treatment process that requires cell viability (Espinosa Ortiz, Rene, Pakshirajanb, van

Hullebusch, & Lens, 2016). Velásquez-Riaño, Lombana-Sánchez, Villa-Restrepo, & Fernández-Calle (2013) employed vinasse to obtain cellulose from *Gluconacetobacter kakiaceti* GM5 by fermentation, and reported the reduction (about 20%) of its toxic characteristics. Although post-production characterization tests were not performed in current study, it is also believed that, at the end of the fermentation process, *A. niger* changes wastewater characteristics and makes it less polluting. Therefore, future studies should be undertaken to elucidate these issues.

Current study showed that the fungus *A. niger* is a good xylanase producer even as a byproduct. Microorganisms, similar to the filamentous fungi, are preferred in industries due to their better performance in obtaining enzymes at low costs, as in the purifying processes. Moreover, the fungus is more efficient in the secretion of xylanolytic enzymes (Polizeli et al., 2005; Izidoro & Knob, 2014). The utilization of xylanase may be employed for pulp bleaching. It may also be obtained by fermentation processes using wastewater generated from the same industrial sector, as proposed in current study, resulting in a sustainable industrial process.

Conclusion

The two types of wastewaters induce xylanase production by *A. niger*. Alkaline wastewater provides better results under optimized conditions: 50% in relation to culture medium, 7-day production, 30°C, pH 6.0 and agitation at 160 rpm.

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