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Effect of Aeration on Lignin Peroxidase Production by *Streptomyces viridosporus* T7A

Leda M. F. Gottschalk,[1] Ronaldo Nobrega,[1] and Elba P. S. Bon*,[2]

[1]Programa de Engenharia Química, COPPE; and
[2]Departamento de Bioquímica, IQ Universidade Federal do Rio de Janeiro, RJ, Brazil, 21949-900, E-mail: elba1996@iq.ufrj.br

Abstract

The effect of aeration on lignin peroxidase production by *Streptomyces viridosporus* T7A was studied in a bench-scale bioreactor using a previously optimized growth medium (0.65% yeast extract and 0.1% corn oil, pH 7.0) at 37°C and natural pH. Airflow rates of 0.3, 1.0, and 1.5 vvm and a fixed agitation of 200 rpm were initially studied followed by 1.0 vvm and 200, 300, 400, and 500 rpm. The use of 1.0 vvm and 400 rpm increased enzyme concentration 1.8-fold (100–180 U/L) and process productivity 4.8-fold (1.4–6.7 U/[L·h]) in comparison with the use of 200 rpm and 0.3 vvm. The inexpensive corn oil, used as carbon source, besides its antifoam properties, proved to be nonrepressive for enzyme production.

Index Entries: *Streptomyces viridosporus*; lignin peroxidase production; aeration; productivity; corn oil.

Introduction

Since World War II, human activity has introduced a great variety of xenobiotic chemicals into the environment on a large scale. Every year some 1000 new chemicals are introduced on the market, many of them displaying a rather poor biodegradability *(1)*. Lignin peroxidase (LiP), which in nature is responsible for lignin degradation in the presence of hydrogen peroxide *(2)*, also presents the ability to oxidize, besides lignin, a larger number of aromatic substances, including highly polluting and recalcitrant compounds, such as azo dye and pesticides *(3,4)*. Although such properties make LiP potentially useful in environmental pollution control *(5)*, this application in an industrial scale requires its production at low cost.

*Author to whom all correspondence and reprint requests should be addressed.

Since the first characterization of LiP by Ramachandra et al. *(6)*, several aspects of LiP production by *Streptomyces viridosporus* T7A have been studied. Yeast extract proved to be the best nitrogen source *(6–8)*. Concerning carbon sources, glucose; lactose; galactose; corn oil; and some sources considered to be inducers, such as lignocellulose, cellulose, and xylans, have been tested to optimize LiP production. The use of glucose allowed the highest LiP activity in spite of showing a repressive effect during cell growth *(7–10)*. Corn oil, besides being a low-cost carbon source, favored enzyme production in higher yields, improved enzyme stability during fermentation, and prevented the formation of foam, a particularly interesting feature considering large-scale fermentations *(11)*. Calcium carbonate increased the enzyme levels and the industrially relevant parameter productivity *(12,13)*. The positive effect of this salt might be related to the enzyme molecule stabilization by the calcium ion and/or to a higher enzyme release from the *Streptomyces* cells that displays a more filamentous mycelium in comparison to the traditional pellet morphology *(12)*.

The study of airflow rates is important in submerged bioreactors because the microorganism grows completely immersed in the culture medium without direct contact with the gas phase, which induces oxygen mass transfer limitation both inside and outside the pellets (14). Thus, the production of enzymes by aerobic filamentous microorganisms, such as *S. viridosporus*, in submerged fermentations depends strongly on the bioreactor airflow rates and agitation conditions, since oxygen, which has a low solubility, is often a limiting nutrient *(15)*. A better mix in the culture medium, making oxygen and also nutrients more accessible for the cells, would improve enzyme synthesis *(16)*.

The present work studied the effect of airflow and agitation rates on LiP production by *S. viridosporus* in a bioreactor with a 3-L working volume.

MATERIALS AND METHODS

Microorganism

S. viridosporus T7A (ATCC 39115) sporulating cultures were obtained by growing the microorganism at 37°C for 6–8 d on an agar medium containing malt extract (3 g/L), yeast extract (3 g/L), peptone (5 g/L), glucose (10 g/L), and agar-agar (20 g/L). Spores were suspended in a 20% (w/v) glycerol aqueous solution and maintained at –20°C *(17)*.

Fermentations

LiP was produced in batch fermentations using an optimized growth medium (6.5 g/L of yeast extract, 1 g/L of corn oil, 5 g/L of calcium carbonate, 0.20 g/L of $MgSO_4 \cdot 7H_2O$; 0.20g/L of NaCl; 0.05 g/L of $CaCl_2$, and a trace metal stock solution, all at pH 7.0 *[18,19]*) in a bioreactor model Applikon, 3-L working volume, coupled to a biocontroller ADI 1030. Inoculum was prepared in 500-mL shake flasks containing 100 mL of the same medium that was inoculated with the spore suspension to a final

absorbance at 570 nm of 1.0. Flasks were incubated in a Shaker Tecnal BTC 9090 at 200 rpm and 37°C for 16 h. Three flasks (total volume of 300 mL) were used to inoculate the bioreactor (10% [v/v]). Fermentations were carried out at 37°C for approx 80 h. Airflow rates of 0.3, 1.0 and 1.5 vvm (1 L of air /[L of substrate·min]) and agitation of 200 rpm were initially studied. Subsequently, an airflow rate of 1.0 vvm and agitation rates of 200, 300, 400, and 500 rpm were also evaluated. Samples, taken at different time intervals, were centrifuged at 4000 rpm for 10 min. The cell cake was used for cell growth evaluation, and the culture supernatant was used for measurement of pH and LiP activity.

Cell Growth

Cell growth was estimated through the cell cake protein content after pretreatment with 1 *N* NaOH *(7)* and expressed as grams of cell protein per liter of culture. Protein concentration was estimated by the modified Folin-phenol method and microassay procedure using a standard curve for bovine serum albumin (2–40 μg) *(20)*.

LiP Activity

LiP was determined by the enzymatic oxidation of 2,4-dichlorophenol (2,4-DCP) in the presence of H_2O_2 and 4-aminoantipyrene (4-AAP) *(21)*. The 1.0-mL reaction mixture contained 164 μ*M* 4-AAP (Sigma, St. Louis, MO), 3 m*M* 2,4-DCP (Sigma), 4 m*M* hydrogen peroxide, and 200 μL of the culture supernatant in a 50 m*M* potassium phosphate solution (pH 7.0). The reaction was initiated by the addition of hydrogen peroxide and the increase in absorbance at 510 nm was monitored for 1 min at room temperature. One unit of LiP activity corresponded to the increase of one unit of absorbance per minute under initial reaction rates. Extracellular LiP concentration was expressed as units of enzyme per liter of culture. Normalized LiP activity and productivity were expressed as units of enzyme per gram of cell protein and units of enzyme per liter of culture per hour, respectively.

Results and Discussion

Effect of Airflow Rates

Figure 1 shows data for cell growth, as measured by protein content; pH variation; and LiP concentration, in fermentations carried out at 200 rpm and different airflow rates. Cell growth was not favored by the use of 0.3 vvm; the peak biomass cell concentration of 0.229 g/L was about twofold lower in comparison to the findings observed for the use of 1.0 and 1.5 vvm: 0.404 and 0.489 g/L, respectively (Fig. 1A). Therefore, this condition, which also presented the lower peak enzymes titers (100 U/L), resulted in oxygen and/or substrate transfer rate limitations.

Improved enzyme titers (peak values of 120 and 170 U/L) were observed within 48 h of fermentation when airflow rate increased to 1.0 and 1.5 vvm, respectively (Fig. 1B). Besides the positive response of cell growth

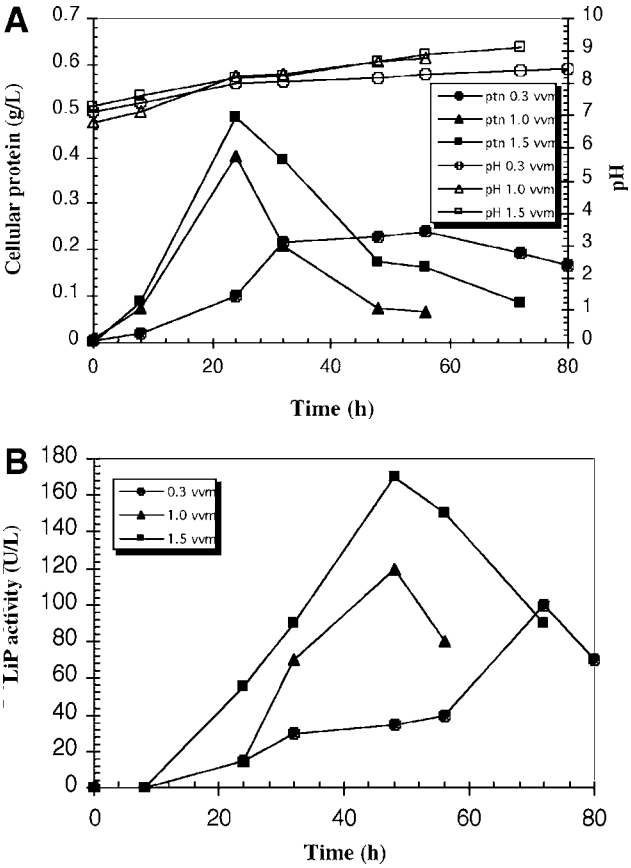

Fig. 1. Cellular protein (ptn), pH variation **(A)** and extracellular LiP activity **(B)** profiles of *S. viridosporus* T7A grown in agitated submerged culture at 37°C, 200 rpm using different airflow rates.

and enzyme production to the increase in aeration rate, maximum LiP activity was also anticipated in 24 h in both cases. These results corroborate those found in the literature; both lignin mineralization and LiP synthesis were increased in cultures grown under high oxygen tension *(14, 22)*.

The pH increased in all fermentations, reaching maximal values within the range of 8.5–9.5 (Fig. 1A). Enzyme titers decreased after 48 h of fermentation with the use of 1.0 and 1.5 vvm, probably owing to pH inactivation since previous work indicated pH 8.0 as a threshold value for enzyme stability *(8)*.

Effect of Agitation Rate

In filamentous microorganisms, such as *S. viridosporus*, pellet size and morphology are greatly affected by the agitation or shear stress. It is also reported that the average pellet size decreases strongly with the increase in agitation rate *(15)*.

Table 1
Comparison of Maximum LiP Activity, Maximum Biomass Accumulation, Maximum $Y_{LiP/X}$, and Productivity in All Fermentation Conditions Tested[a]

Fermentation conditions	Maximum LiP activity (U/L)	Maximum biomass accumulation (g cell protein/L)	Maximum $Y_{LiP/X}$ (U/g cell protein)	Productivity (U/[L·h])
0.3 vvm, 200 rpm	100	0.229	518	1.4
1.0 vvm, 200 rpm	120	0.404	1600	2.5
1.0 vvm, 300 rpm	120	0.236	1071	3.3
1.0 vvm, 400 rpm	**180**	0.162	**3750**	**6.7**
1.0 vvm, 500 rpm	140	0.129	2333	5.4
1.5 vvm, 200 rpm	170	**0.489**	1034	3.5

[a]Bold numbers indicate best parameter for each condition.

A previous work from our laboratory also showed that microorganism pellet size was affected by the presence of calcium carbonate in the growth medium since the predominance of a more filamentous morphology was observed *(12)*. Because the reduction in pellet size might be relevant for the mechanisms of mass transfer, facilitating nutrient uptake and enzyme release into the culture broth, calcium carbonate was added to our culture medium. Note also that calcium ions play important structural roles in peroxidases of different organisms *(23)*.

Although in the first set of experiments the airflow rate of 1.5 vvm resulted in the highest LiP production (170 U/L), the normalized production was higher for the aeration of 1.0 vvm, the condition selected to study the effect of agitation rate (Table 1).

Figure 2A shows that the increase in agitation rate from 200 to 500 rpm using a fixed 1 vvm airflow rate resulted in a consistent decrease in cell protein content that represented cell growth. This result was unexpected because a better mix allowing oxygen and nutrients more accessible for the cells should be beneficial for *S. viridosporus* growth. Therefore, it is possible that our mixing conditions resulted in high shearing effects that were detrimental to mycelium integrity. Enzyme production, however, was not likewise affected since the same peak concentration of 120 U/L was observed for 200 and 300 rpm within 48 h of fermentation and a peak enzyme concentration of 180 U/L was observed within 32 h when 400 rpm was used, resulting in gain in productivity. Although LiP also peaked within the same time interval for 500 rpm, enzyme levels were lower (140 U/L). The increase in agitation rate of 200–400 rpm, besides increasing LiP production, also anticipated maximum LiP activity in 16 h (Fig. 2B). Even though we did not measure corn oil consumption during the fermentation, it is conceivable that, since corn oil is an insoluble carbon source, better agitation conditions favored this carbon substrate mass transfer (24–26). As previously observed for the airflow rate studies, pH increased in all fermentations, reaching maximal values of about 9.0 (Fig. 2A).

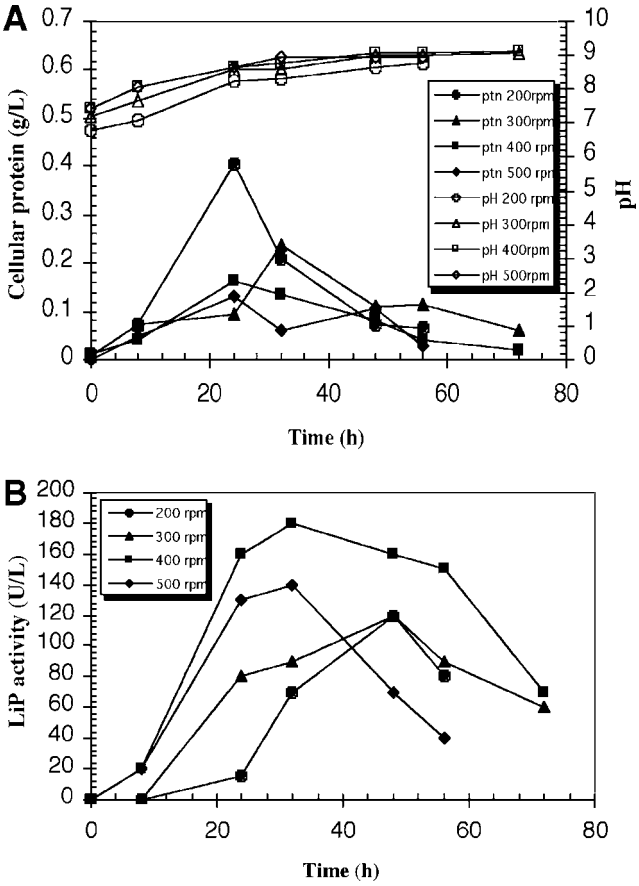

Fig. 2. Cellular protein (ptn), pH variation **(A)** and extracellular LiP activity **(B)** profiles of *S. viridosporus* T7A grown in agitated submerged culture at 37°C, 1.0 vvm using different agitation rates.

Effect of Different Aeration/Agitation Rates on Normalized LiP Production and Productivity

In submerged bioreactors, biomass grows with oxygen mass transfer limitation, suggesting the necessity of designing a suitable bioreactor configuration that provides good oxygen transfer in a low-shear environment *(14)*. The literature provides descriptions of different ways to increase oxygen availability, such as immobilization of microorganisms in airlift bioreactors, addition of a water-immiscible organic solvent in the medium, and/or exposure of the culture medium to hyperbaric oxygen *(27–29)*.

In the present work, different aeration and agitation rates in a submerged bioreactor were evaluated in order to improve LiP production and productivity. Figure 3 compares normalized LiP production (U/g of cell protein). and Fig. 4 compares LiP productivity (U/[L·h]) in all conditions tested. The maximum value for normalized LiP activity (3750 U/g of cell

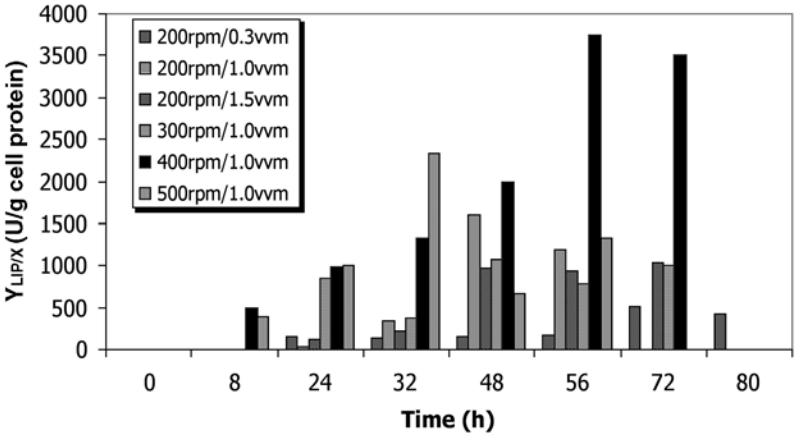

Fig. 3. Comparison of normalized LiP production of batch fermentations of *S. viridosporus* T7A grown in agitated submerged culture at 37°C, using different airflow and agitation rates.

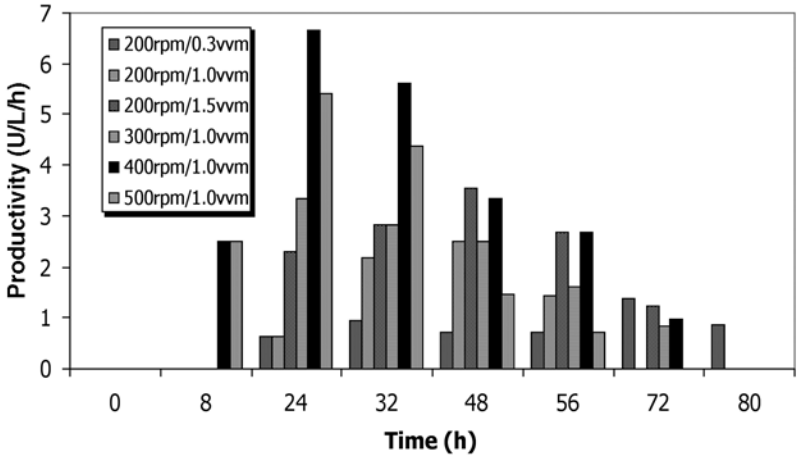

Fig. 4. Comparison of productivity of batch fermentations of *S. viridosporus* T7A grown in agitated submerged culture at 37°C, using different airflow and agitation rates.

protein) was obtained for the aeration/agitation resulting from the use of 400 rpm and 1.0 vvm within 56 h of fermentation (Fig. 3). The maximum value of LiP productivity (6.7 U/[L·h]) was also obtained in the same condition within 24 h of fermentation (Fig. 4).

Table 1 summarizes the maximum LiP activity (U/L), maximum biomass accumulation (g cell protein/L of culture), productivity (U/[L·h]) and maximum normalized LiP production (U/g of cell protein) in the six conditions studied. When the airflow rate increased from 0.3 vvm/200 rpm to 1.0 vvm/400 rpm, a 4.8-fold increase in productivity (from 1.4 to

6.7 U/[L·h]) and a 7.2-fold increase in normalized LiP activity (518–3750 U/g) were observed, suggesting an improvement in cell physiology toward enzyme biosynthesis. Although an airflow rate of 1.5 vvm/200 rpm also favored LiP production (170 U/L), the normalized production was lower (1034 U/g of cell protein). Thus, the higher cell growth, resulting from the better culture medium mix, allowing oxygen and nutrients more accessible for the microorganism, had detrimental effects on enzyme production, and by extension, on the normalized LiP production data *(16,29)*.

The increase in agitation rate from 400 to 500 rpm resulted in a severe fall in normalized enzyme production (3750 to 2333 U/g of cell protein), most probably owing to the negative effect of the shearing effects on the mycelium's integrity. Productivity increased 2.7-fold when the agitation rate increased from 200 to 400 rpm and decreased (6.7 to 5.4 U/[L·h]) when it increased from 400 to 500 rpm, confirming that the best condition for LiP production was 1.0 vvm and 400 rpm.

Conclusion

We studied in a bench-scale bioreactor the effect of aeration and agitation on *S. viridosporus* LiP production using the nonrepressive, although immiscible, corn oil as carbon source and $CaCO_3$ to induce a more filamentous cell morphology and, by extension, to improve nutrients and oxygen mass transfer. Cell growth, enzyme production, and productivity responded positively to the increase in aeration rate from 0.3 to 1.0 and 1.5 vvm. The increase in agitation rate from 200 to 500 rpm using a fixed 1 vvm airflow rate resulted in a consistent decrease in biomass accumulation; however, enzyme production was positively affected. It was possible to increase enzyme levels 1.8-fold (100–180 U/L), productivity 4.8-fold (1.4–6.7 U/[L·h]), and normalized enzyme production 7.2-fold (518–3750 U/g of cell protein) using 1.0 vvm and 400 rpm in comparison with 0.3 vvm and 200 rpm. The inexpensive corn oil, used as carbon source, besides its antifoam properties also proved to be nonrepressive for enzyme production.

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