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Lignin Peroxidase Production by *Streptomyces viridosporus* T7A

Use of Corn Oil as a Carbon Source

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Abstract

Lignin peroxidase (LiP) production cost should be reduced to justify its use in the control of environmental pollution. In this work, we studied the enzyme production by *Streptomyces viridosporus* T7A using glucose or corn oil as a carbon source having 0.65% yeast extract as a nitrogen source. Enzyme activity, observed using either 0.65% glucose or corn oil at 0.1, 0.5, and 1.0% concentration, was 300, 150, 300, and 200 U/L, respectively. Although higher enzyme activity was obtained in both media containing 0.65% glucose and 0.5% corn oil, the use of corn oil resulted in a better LiP stability. When combined carbon sources were used, higher values of enzyme activity (360, 350, and 225 U/L) were observed in media with 0.65% glucose and supplemented with 0.1, 0.5, and 1.0% corn oil, respectively. Although the presence of both glucose and 0.5% corn oil is favorable for LiP production, satisfactory results in terms of enzyme production and stability could be also observed using 0.5% corn oil as a sole carbon source, which may lead to reduced production costs of the LiP enzyme.

Index Entries: *Streptomyces viridosporus*; Lignin peroxidase; enzyme production; corn oil; medium optimization.

Introduction

Lignin is the second most abundant polymer in the biosphere, representing one of the largest reservoirs of aromatic structure in the world. The petroleum crisis in the 1970s encouraged many research groups to look for renewable sources of raw materials for chemical industries. Lignin was shown to be a very convenient alternative *(1)*. The biodegradation of this

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polymer is an oxidative process involving extracellular enzymes produced by certain microorganisms. Lignin peroxidase (LiP), a component of the ligninolytic complex, is responsible for lignin degradation in the presence of hydrogen peroxide *(2–5)*. Fungi, especially *Phanerochaete chrysosporium (6,7)*, and actinomycetes, particularly *Streptomyces* species *(8–12)*, have the capacity to produce this enzyme. An important characteristic of LiP is its ability to oxidize, besides lignin, a great variety of compounds such as chlororganics *(13,14)*, polycyclic aromatic hydrocarbons *(15)*, phenolic compounds *(16)*, azo dyes *(17)*, lignosulfonated compounds *(18)*, and pesticides *(19,20)*. This property makes LiP a potentially useful enzyme in industries that use lignocellulosic raw material, such as cellulose and pulp industries, in organopollutant's treatment systems and in lignin conversion for obtaining raw material for chemical industries *(5)*. Its use in treating effluents in pulp and dye industries is estimated to be $2 billion, thus, more detailed studies on the production and the characterization of the enzyme are needed.

Various carbon sources that aim to improve LiP production by *Streptomyces viridoporus* T7A have been studied by our research group. It was demonstrated that in fermentation with glucose as a carbon source, the addition of corn oil as an antifoaming agent improved enzyme production *(21)*. The use of oil revealed enzyme activity for longer fermentation periods, which is relevant considering the enzyme production on a large scale. In antibiotic production by streptomycetes, various vegetable oils have been used because they are low-cost raw materials and nonrepressive carbon sources *(22–24)*. Many studies have confirmed a lipolytic activity of several *Streptomyces* species, proving that oil can be used as an excellent carbon and energy source *(25)*. In this work, the use of corn oil as a carbon source for LiP production was investigated for reducing the cost of enzyme production.

Materials and Methods

Microorganism and Media

S. viridosporus T7A (ATCC 39115) stock spores were maintained at –20°C in a 20% (w/v) glycerol aqueous solution *(26)*. LiP enzyme was produced in duplicate agitated 100-mL submerged cultures using 500-mL shaker flasks. Medium YE consisted of 6.5 g/L yeast extract (Difco, Detroit, MI), mineral salts and trace metal stock solution *(27,28)*. Medium Glu consisted of medium YE supplemented with 6.5 g/L glucose (Reagen, Rio de Janeiro, RJ). Corn oil (Milleto, Gaspar, SC) was added to the YE medium in concentrations 0.1, 0.5, 1.0% to form media oil 0.1%, oil 0.5%, and oil 1.0%, respectively, and to the medium Glu in the same concentrations to form media Gluoil 0.1%, Gluoil 0.5% and Gluoil 1.0%, respectively. Cultures were incubated at 37°C in a shaker (Tecnal BTC 9090, Tecnal, Piracicaba, SP) for 105 h at 200 rpm.

Glucose Concentration

Glucose concentration was determined automatically by a Beckman Glucose Analyzer 2 (Beckman Instruments Inc., Fullerton, CA).

Biomass Accumulation

Microbial growth was monitored by determining cellular dry weight. Four mL of culture were filtered using Whatman filter paper #1. Collected cells were dried at 80°C overnight in an oven and then weighed. Dry weight was expressed as mg of cells/mL of culture.

LiP Enzyme Assay

LiP activity was assayed using 2,4-dichlorophenol (2,4-DCP, Sigma, St. Louis, MO) as a substrate *(29).* Reaction mixtures contained 50 m*M* sodium phosphate buffer, pH 7.0, 164 µ*M* 4-aminoantipyrine (Sigma), 3.0M 2,4-DCP, 4.0 m*M* hydrogen peroxide and 200 µL enzyme preparation in a final volume of 1.0 mL. Reactions were initiated by the addition of hydrogen peroxide, and the increase in absorbance at 510 nm was monitored for 1 min at room temperature (about 29°C). One unit (U) of enzyme activity was expressed as the amount of enzyme required for an increase of one absorbance U/min.

Results

Glucose and Yeast Extract in LiP Production

When glucose was used as a carbon source at the level of 0.65%, a sharp increase of LiP activity was observed after total glucose depletion (Fig. 1A). According to Pasti et al. *(30)* and Zerbini *(21)* glucose may have a regulator effect LiP production. The maximum LiP activity (300 U/L) was reached 24 h after the maximum biomass accumulation, which corresponded to 57 h of fermentation. Enzyme activity then decreased gradually throughout the fermentation process. The pH value decreased during glucose consumption and then increased steadily. When 0.65% yeast extract was used as a carbon and nitrogen source, a lower level of LiP activity was observed (200 U/L). The maximum activity occurred 24 h after the maximum biomass accumulation. The pH value increased slightly during the fermentation process (Fig. 1B).

Glucose and Corn Oil in LiP Production

LiP production by *S. viridosporus* T7A was first evaluated by experiments performed in fermenters using medium Glu with corn oil as an antifoam agent *(21)* to reduce foam formed during fermentation. The addition of corn oil caused cellular dry weight and LiP activity to increase throughout the fermentation process. The maximum LiP activity under these conditions was 190 U/L at 96 h. To evaluate the effect of corn oil on the LiP

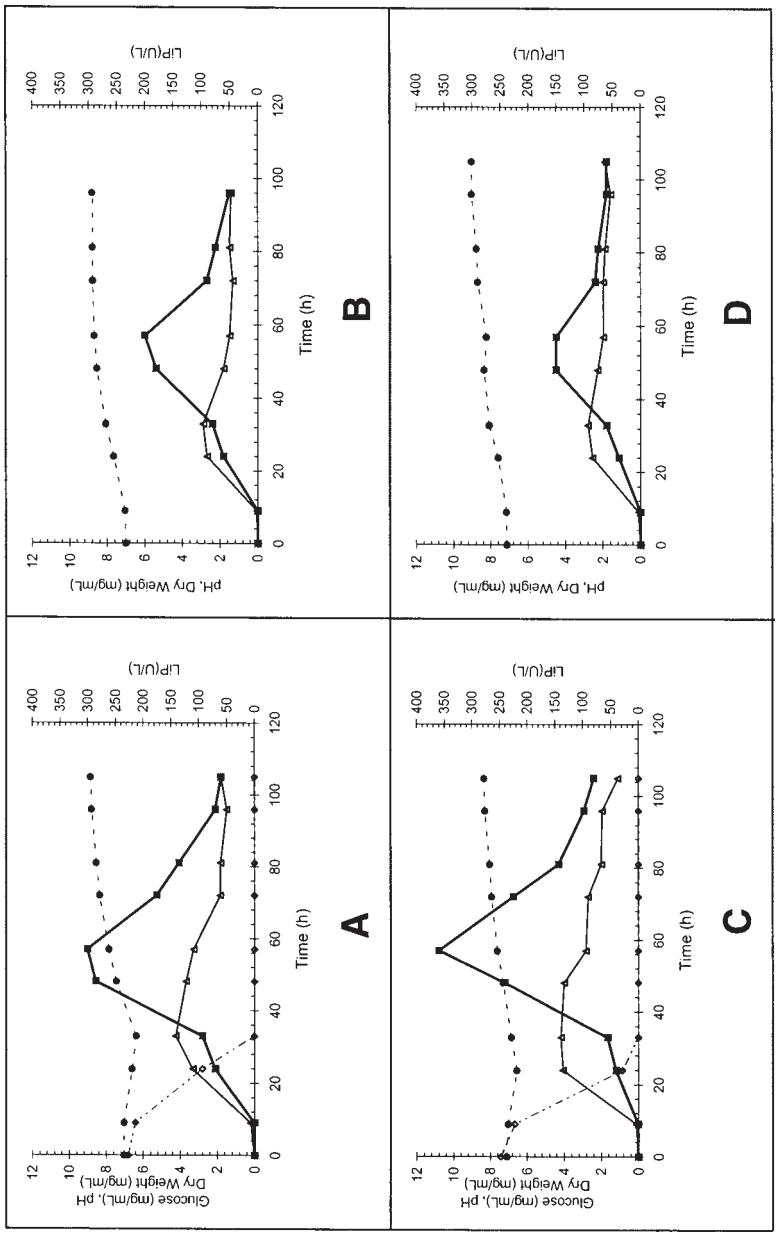

Fig. 1. Relationship between glucose consumption ◇, extracellular lignin peroxidase ■, biomass accumulation △ and medium pH ● during 5-d fermentations of *S. viridosporus* T7A in agitated submerged culture: **(A)** medium Glu and C/N ratio, 9.92. **(B)** medium YE and C/N ratio, 5.25. **(C)** medium Gluoil 0.1% and C/N ratio, 11.3. **(D)** medium Oil 0.1% and C/N ratio, 6.64.

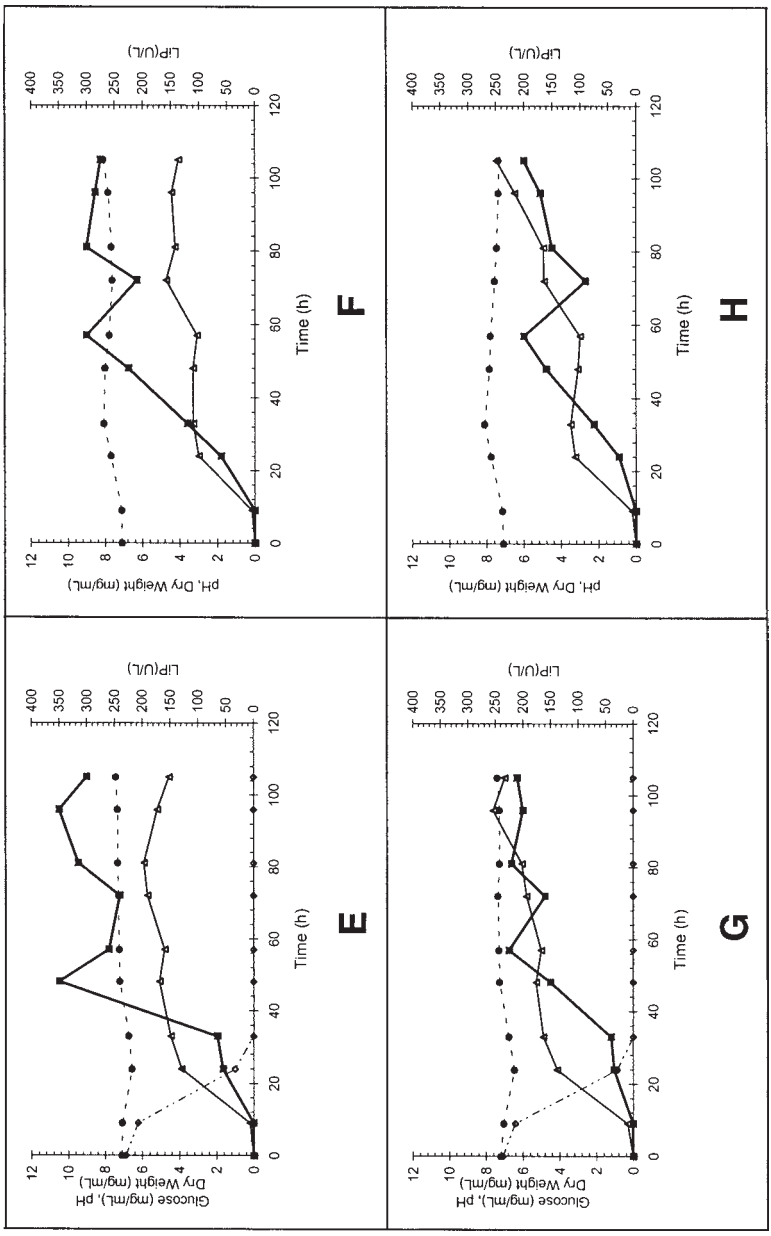

Fig. 1. *(continued)* Relationship between glucose consumption ◇, extracellular lignin peroxidase ■, biomass accumulation △ and medium pH ● during 5-d fermentations of *S. viridosporus* T7A in agitated submerged culture: **(E)** medium Gluoil 0.5% and C/N ratio, 16.9. **(F)** medium Oil 0.5% and C/N ratio, 12.2. **(G)** medium Gluoil 1.0% and C/N ratio, 23.8. **(H)** medium Oil 1.0% and C/N ratio, 19.2.

production, a series of experiments was carried out (Fig. 1C,E,G). Biomass accumulation was higher as the oil concentration increased in the medium, proving that the corn oil can be used as a carbon source by *S. viridosporus*. When the medium contained 0.65% glucose and was supplemented with 0.1% corn oil, enzyme activity reached 360 U/L, followed by a sharp decrease throughout the fermentation process (Fig. 1C). Interestingly, in the medium supplemented with corn oil at 0.5%, the maximum enzyme activity reached 350 U/L, but the enzyme did not drastically decrease; rather, it was relatively stable for another 48 h (Fig. 1E). This is probably owing to the relative stability of the medium pH. Moreover, higher oil concentrations, as in medium Gluoil 1.0%, resulted in a similar fermentation pattern compared to the medium Gluoil 0.5%; however, the level of maximum enzyme activity was lower (225 U/L) (Fig. 1G).

Corn Oil as a Sole Carbon Source in LiP Production

The profile of both LiP activity and biomass accumulation in media with corn oil at concentrations of 0.1, 0.5, and 1.0% as a sole carbon source was similar to those with 0.65% glucose supplemented with corn oil at the same concentrations. However, lower values of LiP activities and biomass accumulation were obtained. The maximum LiP activities reached 150, 300 and 200 U/L when corn oil was used at 0.1, 0.5 and 1.0%, respectively (Figs. 1D,F,H). The microorganism probably used yeast extract to grow, reaching a maximum biomass accumulation, followed by the first peak of LiP activities, then corn oil was used to resume growth and energy production. Accordingly, LiP activity, during the second growth phase, decreased then increased again after the second maximum cell growth. Maximum biomass accumulation was higher as the oil concentration increased in the medium, proving that corn oil can be successfully metabolized by *S. viridosporus*.

Discussion

Previous experiments using glucose as a carbon source and corn oil as an antifoam showed a continuous increase in cellular dry weight and LiP activity throughout the fermentation process *(21)*. Using glucose and corn oil at 0.65% and 0.5 or 1.0%, respectively, as carbon sources showed higher maximum enzyme activities and with certain stability. Consequently, corn oil can be used as a sole carbon source for LiP production to reduce the cost of enzyme production. The enzyme stability was previously studied by our group, and results showed that LiP activity decreased dramatically at pH values higher than 8.2 *(21)*. The maximum LiP activities (U/L), the maximum biomass accumulation (mg dry weight/mL of the culture), the productivity (U/L.h) and the C/N ratio obtained in all media tested are shown in Table 1.

Corn oil is an adequate carbon source for LiP production. It is also known to be a nonrepressive carbon source unlike glucose. Maximum

Table 1
Comparison of C/N ratio, Maximum LiP Activity (U/L), Maximum Biomass Accumulation (mg/mL), and Productivity (U/L.h) in all Media Tested

Medium	C/N ratio	Maximum LiP activity (U/L)	Maximum biomass accumulation (mg/mL)	Productivity (U/L.h)
YE	5.25	200	2.89	3.5
Glu	9.92	**300**	4.23	**5.3**
Gluoil 0.1%	11.3	**360**	4.17	**6.3**
Gluoil 0.5%	16.9	**350**	5.90	**7.3**
Gluoil 1.0%	**23.8**	225	**7.60**	3.9
Oil 0.1%	6.64	150	2.79	3.1
Oil 0.5%	12.2	**300**	4.73	**5.3**
Oil 1.0%	19.2	200	7.47	3.5

biomass accumulation was higher as the oil concentration was increased in the medium, proving that corn oil can be used as a carbon source by *S. viridosporus*. The maximum biomass accumulation, 7.60 mg/mL, was obtained in the medium containing 0.65% glucose and 1.0% oil, which have the highest C/N ratio (23.8). The best productivity, which is an important parameter in industrial fermentations, was obtained with the medium Gluoil 0.5%. However, the medium Glu showed the same productivity as oil 0.5%, proving that the 0.5% oil could replace the 0.65% glucose. Both media have a closer C/N ratio (10), which is the best C/N ratio for LiP production *(21)*. Results revealed that the use of 0.5% corn oil containing medium resulted in higher levels of LiP activity and relatively stable either in the presence or absence of glucose. The presence of 0.5 and 1.0% of corn oil in the media resulted in a second growth phase, which probably prevented cell lyses.

Results agree with previously reported data, which showed that the semicontinuous supplements of soy bean oil in streptomycetes fermentations for oxytetracycline production prevented premature autolysis of mycelia and consequently extended the biosynthetic process that resulted in maintaining the antibiotic production rate *(25)*. In this second growth phase, the pH values were maintained stably, which increased enzyme stability. In an attempt to relate LiP stability to the presence of extracellular proteases, the activity profile was determined in all media (data not shown). Our results indicated that there was no relationship between LiP inactivation and protease production, as higher proteolytic activities were observed in media containing corn oil. Our results support the use of corn oil as a carbon source for LiP production on a large scale, because corn oil is a low-cost raw material compared with other commonly used carbon sources.

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