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Lignin Peroxidase Production by *Streptomyces viridosporus* T7A

Nitrogen Nutrition Optimization Using Glucose as Carbon Source

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Abstract

The production of lignin peroxidase by *Streptomyces viridosporus* T7A was studied in submerged batch fermentations using growth media containing 6.5 g/L yeast extract and 2.5–10.0 g/L glucose, corresponding to carbon to nitrogen (C/N) ratios from 7.1–12.4. The kinetics for biomass and enzyme accumulation and glucose consumption were followed allowing definition of optimized conditions for enzyme production. Considering the physiological response of the microorganism in relation to enzyme production, a sharp increase on enzyme activity was consistently observed upon glucose depletion, indicating glucose regulation. In accordance to that the plot of maximal enzyme vs maximal enzyme per gram of glucose consumption showed a linear inversely proportional relationship, indicating that the characteristics of the metabolic pool at the studied C/N ratios affected enzyme biosynthesis even after glucose depletion.

Index Entries: *Streptomyces viridosporus*; lignin peroxidase; medium C/N ratio; nitrogen nutrition; glucose regulation.

Introduction

The interest in the production of the enzyme lignin peroxidase of *Streptomyces* is quite recent and is mainly due to its potential application in environmental pollution control. According to a number of publications, this enzyme has the ability to degrade recalcitrant xenobiotics, including dyes and pesticides *(1–4)*.

Considering the microorganism requirements for enzyme production as to nitrogen nutrition, organic nitrogen as yeast extract at 0.6% has been

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used as the sole nitrogen source *(5–7)* or at 0.25% *(8)* or 0.05% *(9)* in combination with 0.1% glutamic acid, 0.1% asparagine, and 0.1% proline. The enzyme activities under these conditions indicated that the individual use of yeast extract at 0.6% is the best option. In agreement with that, its addition at 0.05% to a medium containing only the above aminoacids increased enzyme activity threefold *(9)*.

The induction of lignin peroxidase by lignocellulose, xylans, or cellulose has been evaluated by their incorporation in culture media containing the aforementioned nitrogen sources. No conclusive data have thus been far obtained in terms of a real inductive effect owing to the heterogenity of the reported results *(5–10)*. Moreover, although in some cases a higher enzyme activity was observed in the presence of lignocellulose, this could be related to its role as carbon/energy source, through the provision of more favorable metabolic conditions for enzyme biosynthesis.

The use of glucose as carbon source has been also investigated. Thus the effect of the addition of 5.0 g/L glucose to an yeast extract/amino-acids medium or this medium supplemented with 0.05 g/L lignocellulose was compared. Only the glucose media resulted in high enzyme production, indicating the importance of the carbon/energy source for ligninperoxidase biosynthesis. The activity peak, however, was delayed until glucose depletion, indicating glucose regulation *(10)*. The effect of glucose concentration was studied in media containing 1.5% sucrose, 0.1% proline, 0.1% asparagine, 0.1% glutamic acid, and glucose concentrations 0.05, 0.1, and 0.5%. The results indicating glucose repression were described by a mathematical model *(9)*.

In despite of the fact that the foregoing studies indicate that yeast extract is an adequate nitrogen source, that glucose, although being repressive until depletion, is necessary for high enzyme accumulation and that induction by lignocellulose is debatable in *Streptomyces*, there is still not a clear qualitative and quantitative picture as to the carbon and nitrogen nutritional requirements for optimal ligninase production by this microorganism. In submerged fermentations, nitrogen and carbon nutrition and the carbon to nitrogen (C/N) ratio are important aspects for product-yield optimization, owing to its relation to the metabolic pool characteristics, which will affect the cell pathways flux and the regulation of gene expression.

In the present work, the production of lignin peroxidase by *Streptomyces viridosporus* was studied in submerged batch fermentations using 6.5 g/L yeast extract and 2.5–10.0 g/L glucose, showing C/N ratios within 7.1–12.4. The kinetics for biomass and enzyme accumulation and glucose consumption were followed, aiming at the definition of the optimum C/N ratio for enzyme accumulation. Moreover, aiming at a better understanding of the physiological response of the microorganism, in terms of enzyme production, in relation to glucose availability, maximal enzyme production per normalized glucose consumption, (U/L)/(g glucose/g biomass) was also determined.

Table 1
Growth Media Composition
Showing the Carbon and Nitrogen Source Concentrations,
Total Millimolar Concentrations, and the Respective C/N Ratios

	Medium/amounts				
Compounds	YG1	YG2	YG3	YG4	YG5
Yeast extract (g)	6.5	6.5	6.5	6.5	6.5
Glucose (g)	2.5	4.0	5.0	6.5	10.0
Distilled water (mL)	1000.0	1000.0	1000.0	1000.0	1000.0
C (m*M*)	327.1	377.0	410.4	460.4	577.1
N (m*M*)	46.4	46.0	46.4	46.4	46.4
C/N ratio	7.1	8.1	8.8	10.0	12.4

Materials and Methods

Microorganism

For its propagation and sporulation, stock cultures of *Streptomyces viridosporus* T7A (ATCC 39115) kept at 4°C, were inoculated on agar slopes (malt extract and yeast extract 0.3 g each, bacto peptone 0.5 g, glucose 1.0 g, agar 1.5 g plus water to a total volume of 100 mL) and incubated at 37°C for 1 wk when a dense sporulation was observed.

Fermentations

Batch fermentations were performed in 500 mL shake flasks containing 100 mL of growth medium and incubated at 37°C and 200 rpm for 5 d. The flasks were inoculated with 3.0 ml of a spore suspension with Abs_{570} of 0.36. Growth media compositions are presented on Table 1. All media contained: 5.3 g Na_2HPO_4, 1.98 g KH_2PO_4, 0.20 g $Mg_3(PO_4)_2 \cdot 7H_2O$, 0.20 g NaCl. 0.05 g $CaCl_2$/1000 mL and 1.0 mL of a trace elements solution with the following composition: 0.2 g $FeSO_4 \cdot 7H_2O$, 0.02 g $MnSO_4 \cdot 7H_2O$, 0.01 g $CuSO_4 \cdot 5H_2O$ and 0.18 g $ZnSO_4 \cdot 7H_2O$/1000 mL.

Analytical

Glucose concentration was determined using a Glucose Analyser Beckman II (Beckman Instruments, Inc., Fullerton, CA). *Biomass concentration* was evaluated as cell dry weight. *Lignin peroxidase activity* was determined using 2,4-dichlorophenol as substrate, according to Ishida et al. *(11)* and modified by Pasti et al. *(10)*. The reaction mixture contained in a total volume of 1.0 mL, 200 µL of 15 m*M* 2-4 dichlorophenol, 100 µL of 1.64 m*M* 4-aminoantipyrine, 250 µL of 200 m*M* potassium phosphate buffer, pH 7.0, 150 µL of distilled water, 200 µL of the sample. The reaction was started by the addition of 100 µL of 40 m*M* hydrogen peroxide and the increase in absorbance at 510 nm was monitored during 2 min at 37°C using a SHIMADZU spectrophotometer model UV-2022 (Shimadzu Scien-

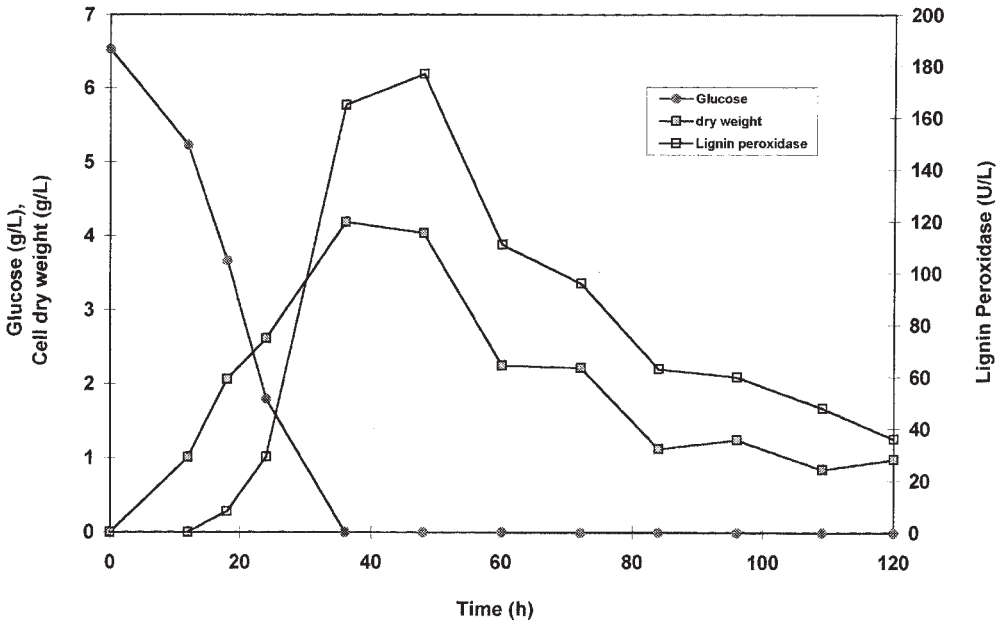

Fig. 1. Time course for lignin peroxidase and biomass accumulation and glucose consumption in *Streptomyces viridosporus* shake-flask fermentation in medium YG4 (glucose 6.5 g/L, yeast extract 6.5 g/L plus salts).

tific Instruments, Inc., Columbia, MD). One unit of enzyme activity corresponded to the increase of one unit of absorbance/min. Enzyme concentration was expressed as U/L of the culture supernatant.

Results and Discussion

Figure 1 shows the time course for medium YG4 fermentation which also represents the overall pattern for all the media studied. All experimental data represents the average of duplicate. As expected, a sharp increase of lignin peroxidase activity concomitant with glucose depletion and maximal biomass concentration was observed. After maximal growth, cell lysis was observed concomitant to the decrease in enzyme activity. Figure 2 compares the activity profiles in media YG1 to YG5. A gradual increase of enzyme accumulation was observed with the augmentation of glucose concentration within the range of 2.5–6.5 g/L, with minimal and maximal values of 103 and 177 U/L, respectively. The presence of glucose at 10.0 g/L resulted in a considerable delay on the activity peak as glucose depletion was prolonged to 48 h. This fermentation condition was not favorable for lignin peroxidase because its maximal concentration was 120 U/L.

The data for maximum biomass and enzyme concentrations, maximum units of enzyme produced per gram of cell dry weight ($Y_{P/X}$) and per gram of consumed glucose ($Y_{P/S}$) are compared in Table 2. According to this set of data biomass accumulation increased within the whole range of glucose

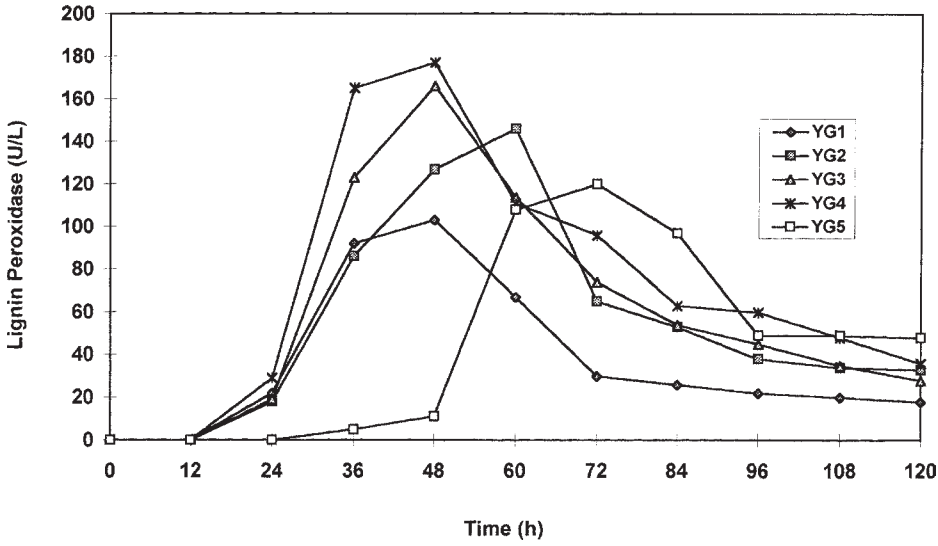

Fig. 2. Profiles of lignin peroxidase concentration in *Streptomyces viridosporus* shake-flask fermentations at 37°C and 200 rpm using growth media containing 6.5 g/L yeast extract and variable glucose concentrations (YG1 2.52 g/L, YG2 4.02 g/L, YG3 5.07 g/L, YG4 6.53 g/L, and YG5 10.04 g/L).

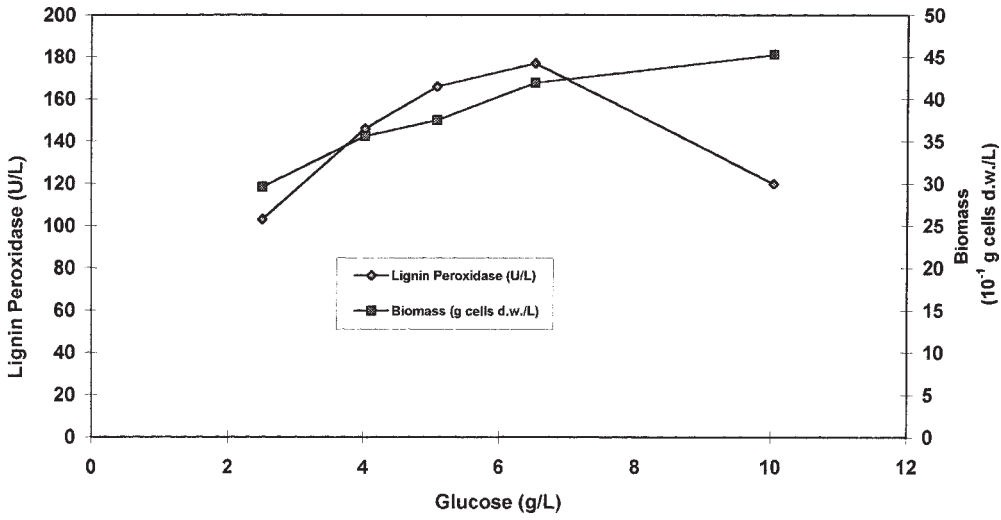

Fig. 3. Maximal values for lignin peroxidase and biomass concentrations in growth media containing YE 6.5 g/L and variable glucose concentrations in *Streptomyces viridosporus* shake-flask fermentations.

concentration, although enzyme accumulation increased only up to 6.5 g/L. According to the data, the use of yeast extract and glucose at 6.5 g/L (C/N ratio 10) provided the best nutritional condition for enzyme accumulation (Fig. 3).

Table 2
Maximal Values for Lignin Peroxidase and Biomass Concentration, and the Yield Coefficients $Y_{P/X}$, $Y_{P/S}$, $Y_{S/X}$, and $P/Y_{S/X}$ Also Showing the Glucose Concentrations of the Growth Media that Contained 6.5 g/L Yeast Extract

Medium	Glucose concentration (g/L)	Maximum activity (U/L)	Maximum biomass (g d.w./L)	$Y_{P/X}$ (U/g d.w.)	$Y_{P/S}$ (U/g glucose)	$Y_{S/X}$ (g glucose/ g biomass)	$P/Y_{S/X}$ (U/L/g glucose/ g biomass)
YG1	2.52	103	2.96	44.6	40.9	0.85	121.0
YG2	4.02	146	3.56	71.6	36.3	1.13	129.0
YG3	5.07	166	3.75	59.5	32.7	1.35	123.0
YG4	6.53	177	4.19	43.6	27.1	1.56	113.0
YG5	10.04	120	4.53	35.5	12.0	2.21	54.3

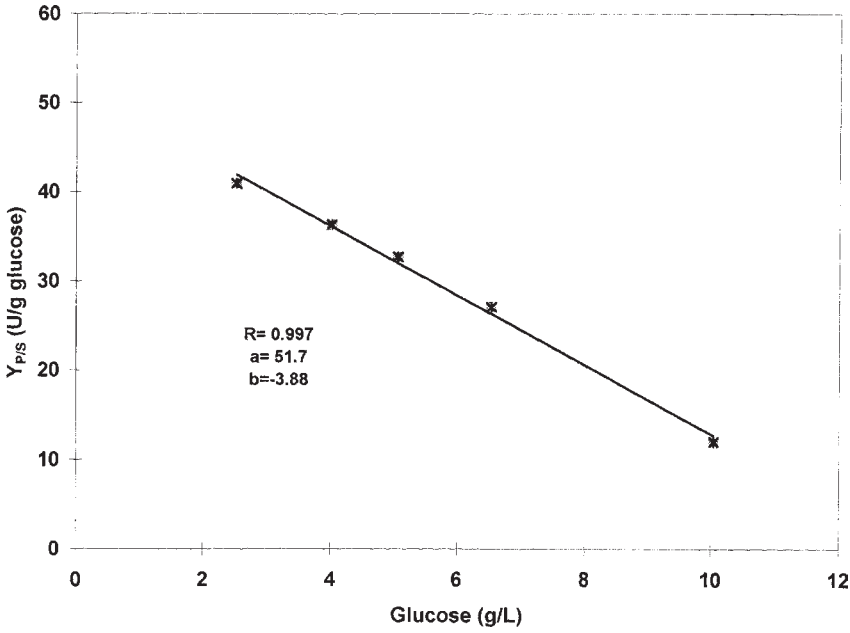

Fig. 4. Data for maximal enzyme produced per normalized glucose consumption, $Y_{P/S}$, in growth media containing YE 6.5 g/L and variable glucose concentrations in *Streptomyces viridosporus* shake-flask fermentations.

The plot of the yield coefficient $Y_{P/S}$ vs glucose concentration (Fig. 4) shows an inverse linear relationship, which is represented by the equation

$$Y_{P/S} = 51.7\ (U/g) - 3.88\ (U.L/g^2) \cdot [glucose]_o\ (g/L); \quad (1)$$

where $[glucose]_o$ is the initial glucose concentration.

The resolution of this equation as shown by:

$$LiP_{máx}\ (U/L) = 51{,}7\ (U/g) \cdot [glucose]_o\ (g/L) - 3{,}88\ U \cdot L/g^2\ .\ [glucose]_o^{\,2}\ (g/L)^2; \quad (2)$$

allowed the determination, for these working conditions, of the optimum glucose concentration for lignin peroxidase accumulation as 6.65 g/L. Considering now the data for normalized enzyme production per gram of biomass ($Y_{P/X}$) (Table 2) and the enzyme production per normalized glucose consumption ($P/Y_{S/X}$) the glucose concentration of 4.0 g/L (C/N 8.1) provided the less repressive condition for enzyme production (Table 2 and Fig. 5).

In conclusion, our results indicated that under our working conditions the C/N ratio 10 (YE 6.5 g/L and glucose 6.5 g/L) is the more favorable for enzyme accumulation, although the less repressive glucose condition for enzyme biosynthesis corresponded to the C/N 8.1 (0.65 g/L YE and 4.0 g/L glucose). The higher cell concentration provided by C/N 10 in comparison to C/N 8.1 allowed the higher enzyme accumulation.

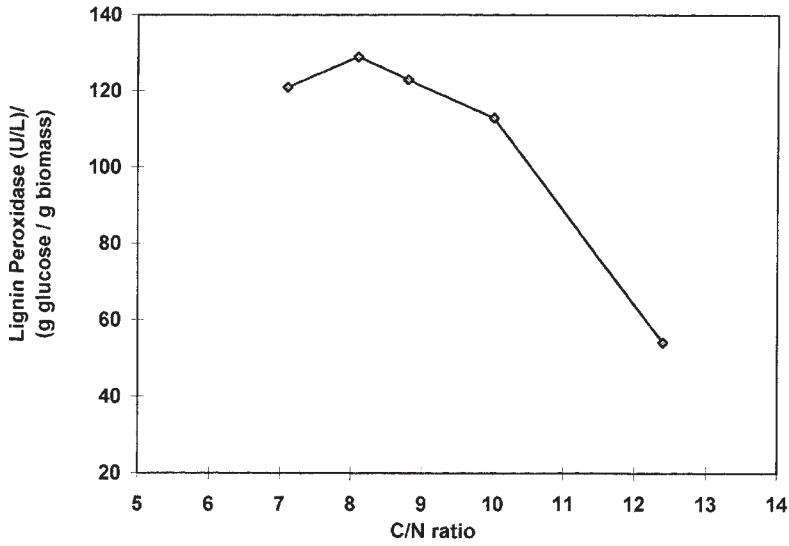

Fig. 5. Data for maximal lignin peroxidase production per normalized glucose consumption ($P/Y_{S/X}$) at the studied C/N ratios.

Acknowledgments

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