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Lignin Peroxidase and Protease Production by *Streptomyces viridosporus* T7A in the Presence of Calcium Carbonate

Nutritional and Regulatory Carbon Sources

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Abstract

Streptomyces are good producers of enzymes of industrial interest, such as lignin peroxidase (LiP) and proteases. To optimize production of these enzymes by *Streptomyces viridosporus* T7A, two parameters were evaluated: carbon sources and calcium carbonate. Shake-flask fermentations were performed using culture media, with and without $CaCO_3$, contained yeast extract, mineral salts and either glucose, lactose, galactose, or corn oil. In the absence of calcium carbonate, the maximum values for LiP and protease activities occurred during the idiophase with LiP activity being favored by glucose, corn oil, and galactose, and protease activity being favored only by corn oil. Calcium carbonate affected the cell morphology by reducing the size of the pellets. Moreover, in the presence of the salt, LiP production was growth-associated in all media but the glucose medium. Higher enzyme levels were observed when galactose and glucose were used as carbon sources. Protease activity was repressed by both glucose and galactose, whereas corn oil was the best carbon source for the enzyme production. Calcium carbonate increased LiP production by up to 2.6-fold. Such improvement was not observed for protease production, suggesting a selective effect of $CaCO_3$ on LiP activity.

Index Entries: *Streptomyces viridosporus*; lignin peroxidase; protease; carbon sources; calcium carbonate.

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Introduction

Streptomycetes are important industrial microorganisms owing to their ability to produce a large number of secondary metabolites, such as antibiotics, and several classes of enzymes having different industrial applications *(1,2)*. Because of their capacity to produce extracellular enzymes involved in biodegradation of lignocellulosic materials *(3,4)*, increasing attention has been given to these microorganisms during the last two decades *(1)*.

Biodegradation of lignin is an oxidative process that involves extracellular enzymes—including lignin peroxidase (LiP)—responsible for lignin degradation in the presence of hydrogen peroxide *(5)*. Fungi, particularly *Phanerochaete chrysosporium*, are the most studied microorganisms with respect to LiP production. However a few *Streptomyces* species have also been reported to produce the enzyme *(4–7)*. LiP has been extensively studied because of its application in the conversion of low-values biomass, such as agricultural wastes, into products such as fuel, animal feeds, and industrial feedstocks *(1)*. Also, highly recalcitrant pollutants and waste compounds such as azo dyes and pesticides can be degraded by LiP *(8–12)*. These factors make this particular enzyme attractive for use in environmental pollution control.

According to the literature, the Gram-positive bacterium *S. viridosporus* T7A produces four extracellular LiP isoforms when grown on lignocellulose *(7)*. All the isoforms require hydrogen peroxide as an electron donor, but each of them has a different substrate range. The isoform ALiP-P3, which has the greatest known substrate range, is capable of catalyzing the oxidative cleavage of C_α-C_β and C_α-carbonyl bonds, and is the only isoform detectable with an enzyme assay using 2,4-dichlorophenol as substrate *(7,13)*. This method has been preferred over the veratryl alcohol-oxidation assay in the studies of LiP of actinomycetes because of its sensitivity *(7)*.

Culture media composed of different nitrogen and carbon sources have been used in order to investigate nutritional conditions favoring LiP production aiming at the improvement of enzyme yield and stability *(14–16)*. Yeast extract proved to be the best nitrogen source *(7,15,17)*. Among the carbon sources studied, including some that may act as inducers, such as lignocellulose, cellulose, and xylans, glucose resulted in the highest LiP activity in spite of showing a repressive effect *(14,15)*.

In previous studies developed by our group *(18)*, it was also observed that glucose favored LiP production by *S. viridosporus* T7A using a medium containing yeast extract as nitrogen source. The maximum LiP activity was detected during the stationary growth phase, just after glucose depletion (33 h), and was followed by a rapid decline, which could have been related to proteases released during the cell lysis.

To better investigate the roles of glucose and other carbon sources in the regulation of LiP and protease activities, this work reports the produc-

tion of these enzymes by *S. viridosporus* T7A, using culture media containing different carbon sources in the presence and absence of calcium carbonate. The effects of this salt on the enzymes activities in the culture supernatant are also discussed.

Materials and Methods

Organism

S. viridosporus T7A (ATCC 39115) spores from slants containing the medium composed of 3 g/L malt extract, 3 g/L yeast extract, 5 g/L peptone, 10 g/L glucose, and 10 g/L agar-agar, which had been incubated at 37°C for 6–8 d, were used for preparing spore suspensions in 20% glycerol *(19)*. The working and the long-term spore suspensions were stored in small aliquots at –20 and –70°C *(20)*, respectively.

Culture Media

The basal medium, YS, was composed of 6.5 g/L yeast extract, and mineral salts and trace metal stock solution, in phosphate buffer (1.98 g/L KH_2PO_4 and 5.3 g/L Na_2HPO_4) *(18)*. The medium YS was supplemented with either 6.5 g/L glucose, 6.2 g/L lactose, 6.5 g/L galactose, or 0.5 g/L corn oil to give a C/N of about 10, constituting media, Glu, Lac, Gal and Oil, respectively. In a second set of experiments, the phosphate buffer in the media YS, Glu, Lac, Gal, and Oil was substituted by 5 g/L calcium carbonate. These media were designated YSC, GluC, LacC, GalC, and OilC, respectively.

Culture Conditions

All experiments were carried out in 500-mL Erlenmeyer flasks containing 100 mL culture medium inoculated with a spore suspension in order to give a final A_{570nm} of 0.01 with regards to cell density/mL. The cultures were incubated in a shaker (model Tecnal BTC 9090, Piracicaba, SP) at 200 rpm and 37°C for 105 h. Samples of 2 mL were taken at the relevant time intervals for determination of cellular growth, extracellular LiP, and protease activities. All experiments were performed in duplicate.

Cellular Protein

The cellular growth was evaluated by determination of the protein content of the cellular material appropriately treated with 1 *N* NaOH *(17)*. Protein was assayed using the modified Folin-phenol method and the microassay procedure proposed by Peterson *(21)*. A standard curve of bovine serum albumin (BSA) (2–40 µg) was used to estimate protein concentration in the samples, which was expressed as g of cellular protein/L of culture.

Lignin Peroxidase Activity

Extracellular peroxidase activity was assayed by a modified method based on enzymatic oxidation of 2,4-dichlorophenol (2,4-DCP) in the presence of hydrogen peroxide and 4-aminoantypirene *(13)*. The product formed during the reaction is a colored antipyrilquinonimine that absorbs at 510 nm. Many variations of the original assay *(13)* can be found in the literature *(7,12,14)*. In the present work a reaction mixture of 1.0 mL contained 50 m*M* of potassium phosphate buffer, pH 7.0, 3 m*M* 2,4-DCP, 0.164 m*M* aminoantipyrine (Sigma, St. Louis, MO), 4 m*M* hydrogen peroxide, and 200 µL of the enzyme preparation *(14)*. The reaction was initiated by the addition of hydrogen peroxide and the increase in A_{510} was monitored for 30 s at 25°C *(12)*. Color development was not observed in the reaction mixture without either hydrogen peroxide or enzyme. After the addition of H_2O_2, the absorbance values considered for determination of LiP activity represented initial rates of reaction. One unit of LiP activity corresponded to an increase of 1.0 U of absorbance/min. Extracellular LiP activity was expressed as units of enzyme/L.

Protease Activity

The extracellular protease activity was assayed by a modified azocasein method *(22)*. The compound sulfanilamide-azocasein when hydrolyzed produces a specific color, what allows the hydrolysis to be followed by the increase of absorbance at 428 nm. One unit of protease activity corresponded to an increase of 1.0 U of absorbance/min. Extracellular protease activity was expressed as units of enzyme/L.

Results and Discussion

Effect of Carbon Sources on LiP and Protease Activities

Figure 1 shows the cellular-growth profiles obtained with the different carbon sources tested. The initial growth rates were quite similar in media YS, Gal, and Oil, but slightly higher in media Glu and Lac, suggesting that glucose and lactose were more promptly uptaken and/or metabolized than the other carbon sources. Glucose depletion was observed within 33 h (data not shown). The overall cellular-growth profiles were quite similar in all media containing carbohydrates, whereas the peaks of cell concentrations were followed by lysis. In medium Oil, the initial cell-growth rate was probably solely related to the uptake of yeast extract. After 24 h, time needed for reaching the maximum cellular growth (0.7 g/L) in medium YS, the cells seemed to have adapted to oil metabolism, and the maximum biomass concentration was kept during more than 30 h. After this period, biomass rapidly increased, reaching a higher value (1.2 g/L) that was maintained for the subsequent 50 h. These results indicated that the carbon

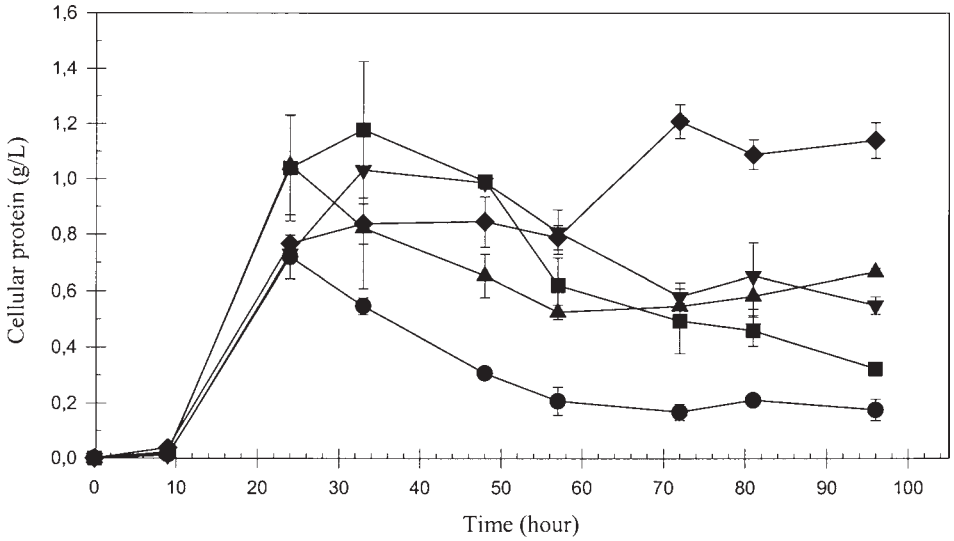

Fig. 1. Cellular growth profiles of *S. viridosporus* T7A in culture media containing phosphate buffer and different carbon sources. Data represent the average of duplicate 100 mL cultures of the media YS (●), Glu (■), Lac (▲), Gal (▼), and Oil (◆). Error bars indicate the maximum and minimum values of cellular protein (g cellular protein/L culture).

source corn oil, although being slowly metabolized, was effective in preventing cell lysis and allowed a second growth phase. The visual observation of the cultures showed a slow disappearance of oil. Low values of extracellular lipase activity were detected during the secondary growth phase (data not shown).

In all media, the maximum LiP activities were obtained only during stationary growth phase (Fig. 2), with the highest value being observed in the medium Glu (350 U/L). LiP activity peak in medium Lac (250 U/L) was maintained for longer periods of time, which represents an advantage in terms of enzyme production in a larger scale. However corn oil was the most favorable carbon source for lignin peroxidase production and stability, because high value of enzyme activity (300 U/L) was reached in 57 h, and after a small decline, the maximum value of LiP activity was recovered and kept up to the end of fermentation. In comparison with the carbohydrates, the use of corn oil enabled a less variation of the pH values (data not shown), probably by preventing cell lysis. This fact seems to be associated to the higher enzyme stability observed in medium Oil.

Extracellular protease activities were only detected after 33 h of fermentation in all media (Fig. 3); they were delayed in comparison with LiP activity. The use of corn oil resulted in the highest protease activity (38 U/L), followed by glucose (23 U/L). The other carbon sources resulted in maximum values of enzyme activities lower than 14 U/L.

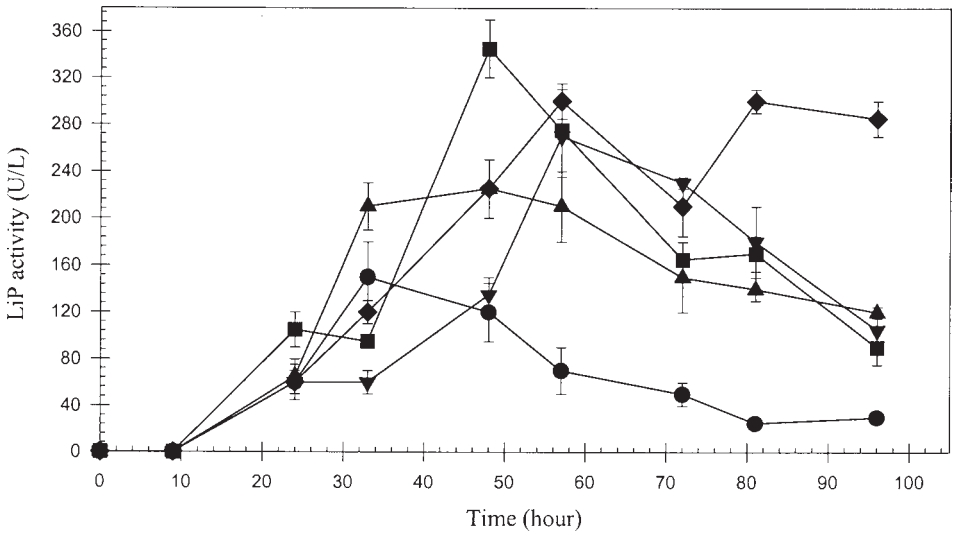

Fig. 2. LiP activity profiles of *S. viridosporus* T7A in culture media containing phosphate buffer and different carbon sources. Data represent the average of duplicate 100 mL cultures of the media YS (●), Glu (■), Lac (▲), Gal (▼), and Oil (◆). Error bars indicate the maximum and minimum values of LiP activity (U/L culture).

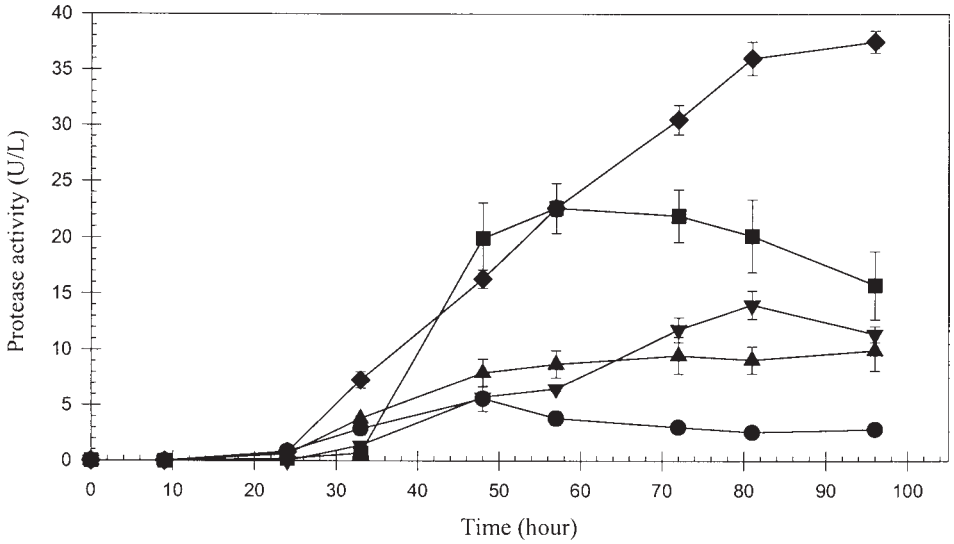

Fig. 3. Protease activity profiles of *S. viridosporus* T7A in culture media containing phosphate buffer and different carbon sources. Data represent the average of duplicate 100 mL cultures of the media YS (●), Glu (■), Lac (▲), Gal (▼), and Oil (◆). Error bars indicate the maximum and minimum values of protease activity (U/L culture).

Effect of Calcium Carbonate on LiP and Protease Activities

According to the literature, the presence of calcium carbonate in the culture medium favors LiP production by *S. viridosporus* T7A *(16)*. There-

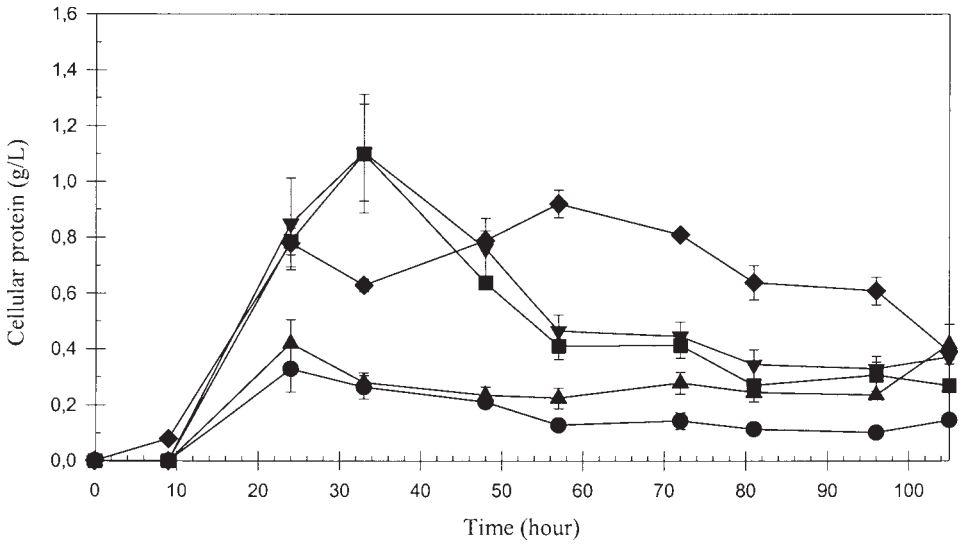

Fig. 4. Cellular growth profiles of *S. viridosporus* T7A in culture media containing calcium carbonate and different carbon sources. Data represent the average of duplicate 100 mL cultures of the media YSC (●), GluC (■), LacC (▲), GalC (▼), and OilC (◆). Error bars indicate the maximum and minimum values of cellular protein (g cellular protein/L culture).

fore in the second set of experiments, the phosphate buffer was replaced by calcium carbonate.

In comparison with media without calcium carbonate, lower levels of maximum biomass concentration were only observed in media YSC and LacC (Figs. 1 and 4). The general pattern for LiP and protease production, however, was greatly affected by the presence of this salt in the culture media (Figs. 5 and 6). The maximum values of LiP activity in media YSC (390 U/L), GluC (595 U/L), LacC (360 U/L), and GalC (660 U/L) were increased by 2.6-, 1.7-, 1.6-, and 2.4-fold, respectively, with regard to media without calcium carbonate. Concerning protease activity, medium YSC had a positive effect on the maximum value, whereas the other media, particularly GluC, were not beneficial for it.

Interestingly, the maximum values for both enzyme activities were detected earlier in some of $CaCO_3$-containing media, with the most significant effect observed in media GalC and OilC (Figs. 2, 3, 5, and 6). These may be explained by the reduction of the size of the pellets occurred in the presence of calcium carbonate, which could have favored the mass transfer resulting in faster uptake of substrates and release of enzymes. In the medium GalC, LiP activity was growth-associated with maximum values for cellular growth (1,1 g/L) and LiP activity (660 U/L) being attained at the same fermentation time (33 h) (Figs. 4 and 5). In this medium, the LiP activity peak was followed by a drastic decline. In contrast, the combination of glucose and calcium carbonate resulted in a more stable enzyme,

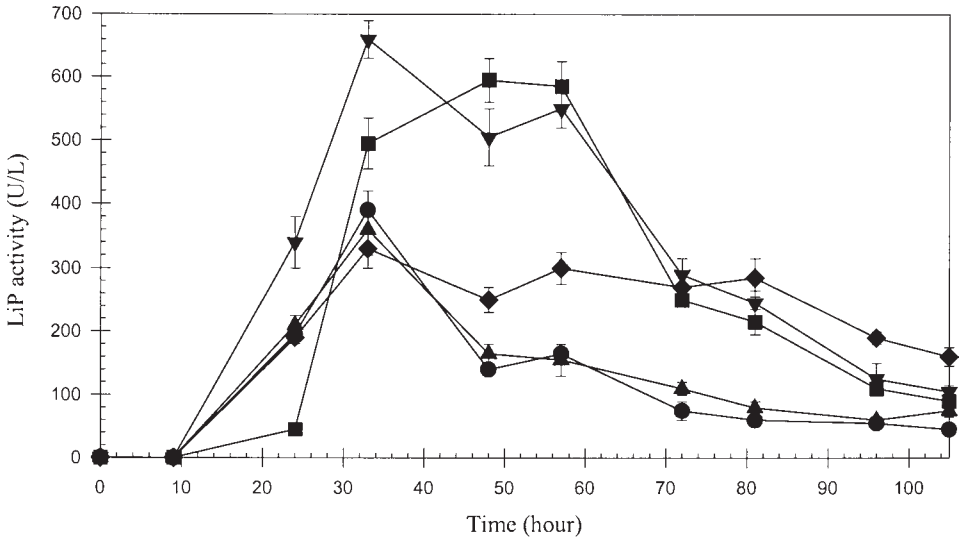

Fig. 5. LiP activity profiles of *S. viridosporus* T7A in culture media containing calcium carbonate and different carbon sources. Data represent the average of duplicate 100 mL cultures of the media YSC (●), GluC (■), LacC (▲), GalC (▼), and OilC (◆). Error bars indicate the maximum and minimum values of LiP activity (U/L culture).

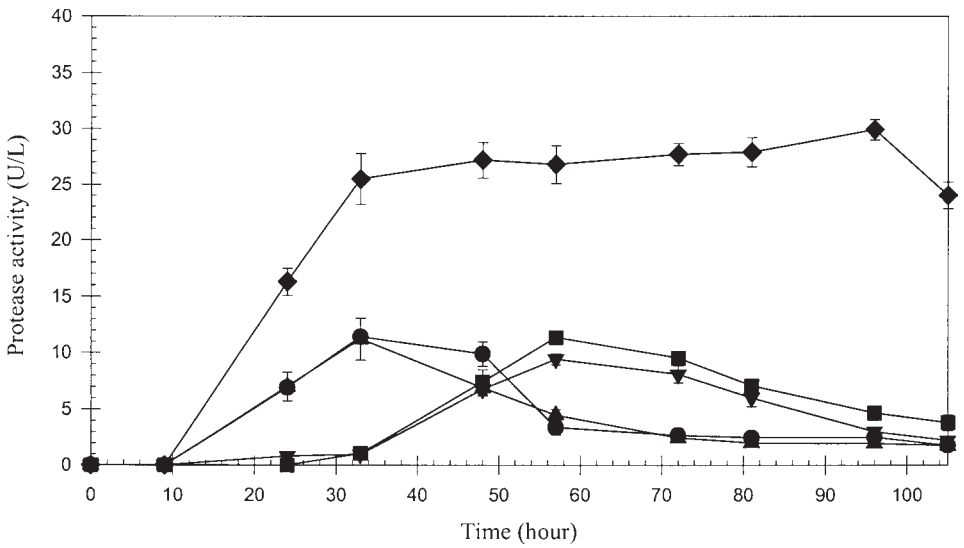

Fig. 6. Protease activity profiles of *S. viridosporus* T7A in culture media containing calcium carbonate and different carbon sources. Data represent the average of duplicate 100 mL cultures of the media YSC (●), GluC (■), LacC (▲), GalC (▼), and OilC (◆). Error bars indicate the maximum and minimum values of protease activity (U/L culture).

because high values of LiP activity was detected for a longer period of time (Fig. 5). According to the literature, this divalent cation plays a structural role in peroxidases of both *Phanerochaete chrysosporium* and *Arthromyces*

ramosus (23–25). In both cases, two calcium ions are required per peroxidase molecule, and they are important for the integrity of the active site, as confirmed by thermal inactivation experiments *(26,27)*. Furthermore, there is evidence that phosphates—the buffer system initially used in this work—repress antibiotic production by *Streptomyces (28)*, whereas calcium ions favor both extracellular protease and antibiotic production *(29)*. It also seemed that the higher enzyme stability observed in medium GluC was not related to the pH values during fermentation. The alteration of pH values owing to the presence of calcium carbonate was observed in all media containing carbohydrate as carbon source (data not shown), but the enzyme stability was not a characteristic of these fermentations.

Conclusions

Different carbon sources could be used for LiP and protease production. In the presence of phosphate buffer the extracellular enzyme activities was related to the stationary growth phase. The use of corn oil resulted in the highest level of extracellular protease activity and the most stable LiP activity in the culture supernatant. The presence of calcium carbonate resulted in an earlier detection of both enzyme activities, especially when corn oil and galactose were used as carbon sources. A more beneficial effect, however, was observed in galactose-containing medium where LiP production was growth-associated. In contrast, enzyme production in glucose-containing medium even in the presence of $CaCO_3$ remained as a product of secondary metabolism. The results presented in this work show a selective positive effect of calcium carbonate on LiP activity that could be related to a stabilization effect of calcium ion on the enzyme molecule and/or to its effect on cell morphology whereby mass transfer was favored.

Acknowledgments

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