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High-Yield *Bacillus subtilis* Protease Production by Solid-State Fermentation

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Abstract

A *Bacillus subtilis* isolate was shown to be able to produce extracellular protease in solid-state fermentations (SSF) using soy cake as culture medium. A significant effect of inoculum concentration and physiological age on protease production was observed. Maximum activities were obtained for inocula consisting of exponentially growing cells at inoculum concentrations in the range of 0.7–2.0 mg g^{-1}. A comparative study on the influence of cultivation temperature and initial medium pH on protease production in SSF and in submerged fermentation (SF) revealed that in SSF a broader pH range (5–10), but the same optimum temperature (37°C), is obtained when compared to SF. A kinetic study showed that enzyme production is associated with bacterial growth and that enzyme inactivation begins before biomass reaches a maximum level for both SF and SSF. Maximum protease activity and productivity were 960 U g^{-1} and 15.4 U g^{-1} h^{-1} for SSF, and 12 U mL^{-1} and 1.3 U mL^{-1} h^{-1} for SF. When SSF protease activity was expressed by volume of enzyme extract, the enzyme level was 10-fold higher and the enzyme productivity 45% higher than in SF. These results indicate that this bacterial strain shows a high biotechnological potential for protease production in solid-state fermentation.

Index Entries: *Bacillus subtilis;* protease; solid-state fermentation; submerged fermentation; soy cake.

Introduction

Proteases amount to 60% of the world sales of industrial enzymes, owing to their use in a wide range of different applications, such as food and beverage processing, detergent manufacture, and pharmaceuticals production. The production of proteolytic enzymes by different micror-

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ganisms has been widely reported in the literature, especially by the submerged fermentation (SF) processes *(1,2)*.

Solid-state fermentation (SSF) presents some advantages over SF, because it is a simple, low-investment process, which requires low energy consumption and allows easy control of contamination due to the low moisture levels employed. Additionally, low-cost, abundant materials of plant origin, such as agroindustrial wastes (soy cake, wheat bran, sugar cane bagasse, rice bran, etc.) can be employed as raw materials *(3)*. However, SSF processes present some limitations, such as the restricted range of microorganisms that are able to grow under reduced moisture levels. Most research found in the literature employing SSF use filamentous fungi, because these microrganisms are able to grow well under low-moisture conditions *(4)*. However, attempts to produce enzymes by SSF using bacteria have been performed in the recent years *(5–7)*.

The aim of this work was to investigate the production of an extracellular *Bacillus subtilis* protease by a SSF process and to compare it with a submerged fermentation process.

Materials and Methods

Microrganism and Conservation

The strain *Bacillus subtilis* 22_1 was isolated from excrements of larvae of *Bruchidade pachymerus nucleorum*, a parasite of the babassu coconut *(8)*, and identified in the Soil Microbiology Laboratory of IMPPG/UFRJ. This microrganism was cultivated for 7 d in agar medium [0.5% (w/v) yeast extract, 1.0% (w/v) peptone, 0.5% (w/v) NaCl, and 2.0% (w/v) agar], and was kept at 4°C.

Inoculum Development

B. subtilis cells conserved in agar medium were inoculated in 25 mL of a culture medium, previously optimized for protease production, containing organic nitrogen sources and no repressive carbon source [0.5% (w/v) yeast extract, 1.0% (w/v) peptone, and 0.1% (w/v) olive oil, pH 7.5] *(9)*. These cultures were incubated at 37°C and 200 rpm. For submerged fermentations, cultures propagated for 15 h were centrifuged and 1.6 mg of cells were inoculated into shaken flasks containing 120 mL of liquid medium. For solid-state fermentations, cultures propagated for either 7 h or 15 h were centrifuged and inoculated at concentrations of 0.3–2.5 mg cells per g of solid medium. Inoculation was carried out by resuspending appropriate amounts of centrifuged cells in 11.7 mL distilled water and distributing the resulting cell suspensions throughout the solid media. This volume of water was used in order to give an initial medium moisture content of 70% (w/w).

Fermentations

Solid-State Fermentations

Soy cake (7.0% N) and babassu cake (3.2% N), kindly donated by Olfar Ltda. and Tobasa S.A., respectively, were tested as culture media. Five grams of cake were placed in 125-mL Erlenmeyer flasks and inoculated with cells at different concentrations and different physiological ages. The fermentations were carried out for up to 140 h at 30°C and initial pH 7.5 in incubators with humidified air injection, in order to maintain the moisture content of the media, which was initially adjusted at 70% (w/w). For soy cake, the effects of different initial pH values (5–10) and different temperatures (30–50°C) were investigated.

Submerged Fermentations

Fermentations were carried out for up to 19 h at 37°C in shaken flasks containing 120 mL (V_{flask}/V_{medium} = 4.2) of the aforementioned medium *(9)*. The effects of different initial pH values (5–10) and different temperatures (30–50°C) on enzyme production were investigated.

Crude Enzyme Preparations

Solid-State Fermentations

Enzymes were extracted from the fermented solids using 8 mL of distilled water (pH 7.4) per gram of solid medium, under agitation at 30°C and 200 rpm for 1 h. Subsequently, solids were removed by centrifugation at 3400*g* for 10 min, and the supernatant was used for pH and enzyme activity determination.

Submerged Fermentations

The culture broth was centrifuged for 10 min at 3400*g*. The supernatant was used for pH and enzyme activity determination, whereas the bacterial pellet was employed for biomass determination.

Protease Activity

Enzyme activity was determined according to the method proposed by Charney and Tomarelli *(10)*, which is based on the formation of azocasein derivatives in alkaline medium due to proteolytic cleavage. One unit of proteolytic activity is defined as the enzyme amount that produces a 0.01 difference in absorbance at 428 nm between the sample and its blank assay, per minute, under the assay conditions.

Biomass Determination

Biomass determination during inoculum development and submerged fermentations was carried out by determination of optical density

Table 1
Effect of Different Inoculum Physiological Ages on the Production of Protease by *Bacillus subtilis* in SSF[a]

Inoculum age (h)	Fermentation time for protease peak (h)	Activity (U g^{-1})	Productivity (U g^{-1} h^{-1})
7	62.5	960	15.4
15	72.5	290	4.0

[a]Inoculum Concentration: 2.0 mg cells per g of soy cake.

at 530 nm. For solid-state fermentations, the contents of whole flasks were agitated with 20 mL distilled water for 30 min and the resulting suspension filtered in filter paper for separating the solids from the bacterial suspension. After a further solids wash out with 10 mL of distilled water, the filtrate containing the cells was centrifuged at 3400*g* for 20 min at 4°C and the bacterial pellet suspended and washed with 10 mL of water four times. The pellet dry weight was determined at 70°C.

Results and Discussion

Protease Production by Solid-State Fermentation

Initially, experiments were carried out using babassu and soy cake as solid medium. However, the bacterial strain was not able to metabolize the babassu cake and, consequently, did not grow or produce protease in this medium. This result may be related to the fact that this agroindustrial residue presents a low nitrogen content (3.2% w/w), which seems to be crucial for the microbial protease metabolism *(11)*. On the other hand, when soy cake was used as culture medium, *B. subtilis* was able to grow and to produce large amounts of the enzyme. Probably, the higher nitrogen content (7.0% w/w) of this material makes it more adequate for the production of this enzyme. Other authors, who investigated the production of proteases by *Bacillus* sp. in SSF, observed that the type and composition of the medium are critical for biomass growth and enzyme production *(7)*.

Inoculum concentration and age are other critical factors concerning the production of enzymes, because the physiological state of the microorganism and its concentration are parameters that can significantly affect fermentation performance. Therefore, in the present work the effect of these parameters on protease production and productivity were investigated, as shown in Tables 1 and 2.

According to Table 1, when an inoculum concentration of 2 mg g^{-1} is employed, the highest protease activities are obtained when the inoculum is propagated for 7 h in liquid medium (exponential growth phase). In this physiological state the cells seem to be more productive, as a 70% reduction in enzyme activity and productivity is observed when a stationary-phase inoculum is employed (15 h of propagation).

Table 2
Effect of Different Inoculum Concentrations on Protease Production by *B. subtilis* in SSF[a]

Inoculum concentration (mg g^{-1})	Fermentation time for protease peak (h)	Activity (U g^{-1})	Productivity (U g^{-1} h^{-1})
0.3	109.0	323	3.0
0.7	114.0	947	8.3
2.0	62.5	960	15.4
2.5	41.0	437	10.6

[a]Inoculum age: 7 h.

Table 2 shows the effect of inoculum concentration on protease production. The highest protease levels (960 U g^{-1}) were obtained for inoculum concentrations in the range of 0.7–2.0 mg g^{-1}. However, when inoculum concentration was 2.0 mg g^{-1}, the protease activity peak was advanced from 114 to 62.5 h, resulting in a 1.8-fold increase in productivity. These results indicate that probably the best balance between substrate availability and initial cell concentration for this bacterial strain and soy cake was obtained at an inoculum concentration of 2.0 mg g^{-1}.

The present results confirm how significant the influence of inoculum concentration and physiological age on enzyme production by SSF can be. Similar results showing considerable increases in protease production in SSF due to increases in inoculum concentration were observed for *Bacillus* sp. *(7)* and *Penicillum* sp. *(12)*.

Effect of pH and Temperature on the Production of Protease by SF and SSF

Figure 1 presents the effect of medium pH on the production of proteases by *B. subtilis* growing in SF and SSF. For SF, maximum protease activities were observed in the pH range of 6–8. An increase in medium pH to 9 resulted in a 10% drop in activity, and a further increase to pH 10 caused a steep decrease in protease activity, although it was observed that the bacterium was able to grow in this rather alkaline medium. This fact could be related to protease self-digestion at higher pH values. For SSF, the activity levels remained approximately the same irrespective of the initial pH in the whole range tested (5–10), indicating that either the proteases that are preferentially expressed under SSF conditions have a higher stability in alkaline environments, or that there could be micro-pH differences in the solid medium.

Good cell growth and protease production at such high pH values were observed by other authors for alkalophilic strains producing alkaline proteases *(7)*. Thus, the present results indicate that the proteases produced by SSF are potential candidates for use in specific biotechnological applications that require enzymes to be active at such pH ranges (9–10).

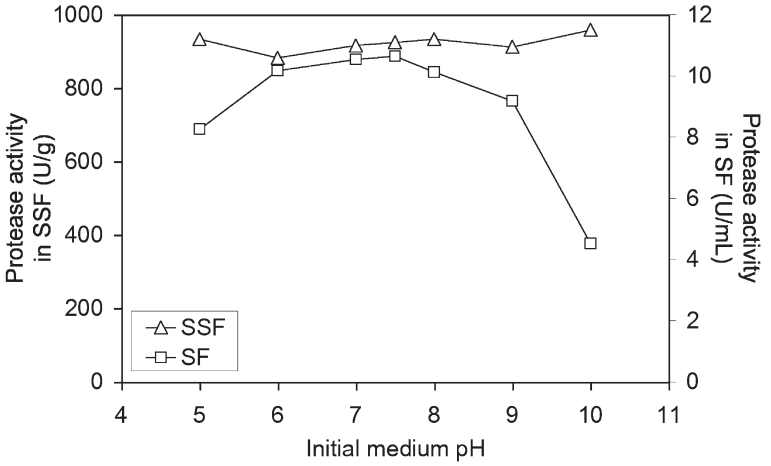

Fig. 1. Effect of initial medium pH on protease production by *Bacillus subtilis* in SF and SSF (fermentation time: 9 h for SF and 62 h for SSF; incubation temperature: 30°C).

The production of protease in buffered media (results not shown) presented similar behavior as in the non-buffered media in the whole pH range tested, with slight activity variations being observed, probably due to the ions contained in the buffers. Other authors have already reported the influence of different buffers on activity and stability of microbial proteases *(4)*.

Figure 2 shows the effect of incubation temperature on protease production by *B. subtilis* in SF and SSF. It can be observed that the profile of protease production as a function of temperature is quite similar for SF and SSF. The highest enzyme levels were obtained at 37°C for both SF and SSF. An increase in temperature to 45°C resulted in a significant decrease in protease production for both fermentation types, probably due to heat inactivation of the enzyme.

Kinetics of Protease Production in SF and SSF

The kinetic profiles of protease production, biomass growth, and pH for SF and SSF are shown in Figs. 3 and 4, respectively. Based on the data presented in Fig. 3, a specific growth rate (μ) of 0.22 h^{-1} was found for SF. Protease production profile seemed to be associated with growth, but enzyme denaturation began to occur before biomass reached its maximum level. The enzyme production peak occurred at the mid exponential phase (9 h), when an enzyme activity of 12 U mL^{-1} and a productivity of 1.3 U mL^{-1} h^{-1} were obtained. After this fermentation time, a rapid enzyme inactivation process is observed, probably due to self-digestion, to the presence of other proteases not detected by the azocasein-based assay, or to medium alkalinization *(13)*. A similar behavior was observed by Yang and Wang *(5)*, when carrying out SF with *Streptomyces rimosus*. However,

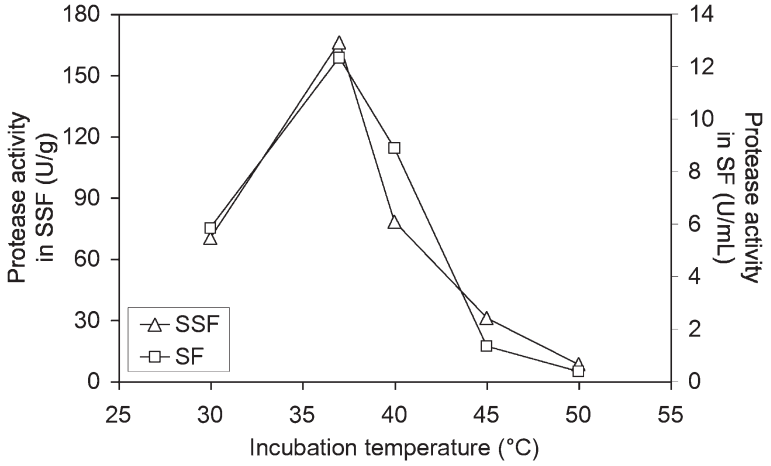

Fig. 2. Effect of cultivation temperature on protease production by *Bacillus subtilis* in SF and SSF (fermentation time: 9 h for SF and 24 h for SSF; pH: 7.5).

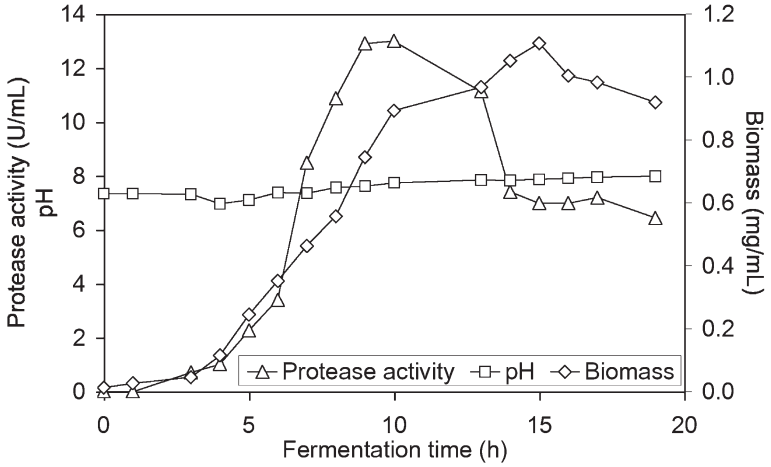

Fig. 3. Kinetic profile of protease production by *Bacillus subtilis* in submerged fermentation.

the protease peak for their biological system was obtained just after 166 h due to the slow growth of this microrganism.

For SSF, although the optimum temperature for protease production, as determined previously for a fermentation time of 24 h, was 37°C, the kinetic study was carried out at 30°C in order to avoid any large effect of thermal enzyme inactivation at longer fermentation times.

Figure 4 shows the kinetics of protease production, for an inoculum age of 7 h and an inoculum concentration of 2 mg g^{-1}. While in SF the microorganism attained stationary growth phase at a fermentation time of 12 h (Fig. 3), in SSF this occurred after 80 h, indicating that cell growth

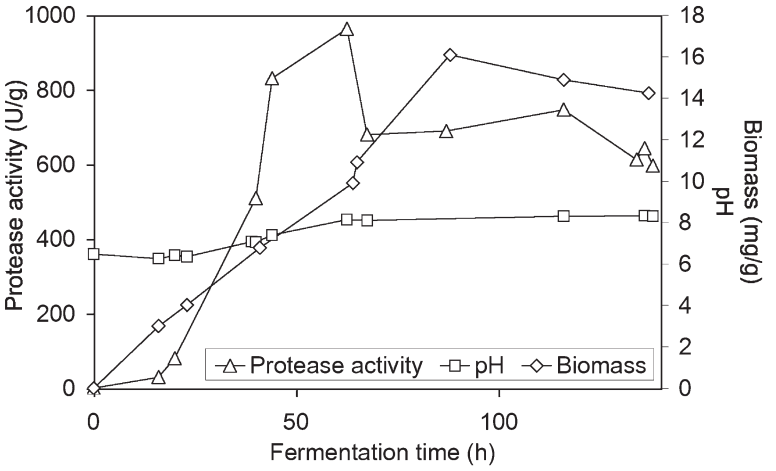

Fig. 4. Kinetic profile of protease production by *Bacillus subtilis* in solid-state fermentation.

and enzyme production in SSF occur much more slowly than in SF. However, the kinetic profile of protease production for both fermentation types presented a quite similar behavior, characterized by association with cell growth and beginning of enzyme inactivation prior to the biomass peak. Maximum protease activity (960 U g^{-1}) in SSF occurred after 62.5 h, when medium pH was near 8. Similarly as for SF, the enzyme inactivation that followed may be either due to self-digestion or cleavage by another protease, or to the increase in medium pH. Similar behavior was observed by different authors working with *Penicillium* sp. *(12)* and *Bacillus* sp. *(7)*.

The highest protease activity (960 U g^{-1}) obtained in SSF corresponds to an enzyme activity of 120 U mL^{-1} in the crude liquid extract, which is 10-fold higher than the highest protease level obtained for SF (12 U mL^{-1}). Considering the fermentation times required to achieve these maximum enzyme levels, the productivity obtained for SSF (15.4 U g^{-1} h^{-1} or 1.9 U mL^{-1} h^{-1}) was approx 45% higher than that obtained by SF (1.3 U $mL^{-1}h^{-1}$).

Conclusions

The *B. subtilis* strain isolated from excrements of *Bruchidade pachymerus nucleorum* larvae, a parasite of the babassu coconut, was able to produce protease by both SF and SSF. However, enzyme activity was 10-fold higher and productivity was 45% superior in the SSF process. Thus, the high levels of protease production presented by this bacterial strain growing in an agroindustrial by-product demonstrate the biotechnological potential of solid-state fermentation for the production of bacterial enzymes.

Acknowledgment

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