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Overproduction of Laccase and Pectinase by Microbial Associations in Solid Substrate Fermentation

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Abstract The growth and the enzymatic production of two microbial fungal associations were studied: *Aspergillus niger* and *Fusarium moniliforme* and *Trametes versicolor* and *Aspergillus niger*. The synergistic interrelations between the species of the first mixed culture increased the biosynthesis of α-amylase and pectinase. *T. versicolor* and *A. niger* proved to be compatible partners in the overproduction of the enzyme laccase, whose synthesis surpassed 8.4 times the enzymatic level in the monoculture, with both of the mixed microbial populations cocultivation facilitating the amplified synthesis of enzymes rather than their growth acceleration. A further proof of the presence of synergism established by the cultures was the enzyme volumetric productivities in both of the mixed microbial cultures, which increased parallel to the rise in the combined biomass synthesis. The competent selection of compatible partners can adjust the desired enzymatic levels and compositions in mixed fungal systems aimed at a number of specified designations. Thus, a very high level of laccase production (97,600 IU/g dry weight) was achieved. The chosen fungal strains produce a variety of different enzymes, but first microbial association produces mainly amylase and pectinase, necessary for their growth, and second association produces mainly laccase and pectinase.

Keywords Solid substrate fermentation · Laccase · Pectinase · Mixed fungal culture

Introduction

Filamentous fungi are typically found in nature as symbiotic associations on solid substrates like wood, plant seeds, stalks, roots, and leaves. Similar to natural microbial processes, the mixed cultivation of fungi may bring about more effective assimilation of the substrate, increased productivity of desired metabolites, improved adaptation to the changing environmental conditions, and higher resistance to contamination by undesired micro-

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organisms [1]. The advantages of mixed cultures may become even more convincing in solid substrate cultivation, as the colonization, penetration, and degradation of the substrate has the chance to occur in a symbiotic association where each species is provided its own niche for growing and substrate degradation due to the availability of a personal set of enzymes that may act synergistically with the rest. In the case of lignocellulose waste, the complexity of the substrate calls for the incorporation of a number of enzymes and for interactions between various microorganisms to make it possible for the biodegradation processes to occur. In addition, fungal solid substrate fermentation is a low-cost technology fermentation process particularly suitable for the needs of developing countries.

The synthesis of enzymes by solid substrate fermentation (SSF) can be carried out by joint cultivation of two or more fungal strains, which are capable of producing an optimal combination of the respective enzymes. Synthesis of cellulase by mixed culture solid substrate fermentation has been reported by a number of authors [2–4]. Significant success has also been achieved by mixed culture solid substrate fermentation in the biosynthesis of xylanase [5], α-amylase [6], inulinase [7], and phytase [8]. The augmentative interest in solid substrate cultivation of mixed fungal cultures is attributed to both the high levels of enzymatic production, especially of cellulolytic enzymes, and to the enzyme-provoked digestibility of lignocellulose materials designed for animal feed products. Despite the enlarged interest in solid substrate fermentation of various enzymes, scientific literature still lacks research information on enzyme laccase.

Laccase production has been found to be highly dependent on the conditions for cultivation and nutritive media composition [9, 10]. Laccases were generally produced at low concentrations, but higher yields were achieved with addition of various supplements to media [11]. The addition of xenobiotic compounds such as xylidene, lignin, and veratryl alcohol is known to increase and induce laccase activity [10]. Some of these compounds affect the metabolism or growth rate, while others, such as ethanol, indirectly trigger laccase production [11]. Lu et al. [12] found that the addition of cellobiose can induce profuse branching in certain *Trametes* spp. and consequently increase laccase activity. The addition of low concentrations of copper to the cultivation media of laccase-producing fungi stimulates laccase production [13].

The main object of our study was to investigate the interrelations between the species in two microbial associations as well as to find the conditions ensuring maximum laccase and pectinase production without any special supplements to the solid media of cultivation.

Materials and Methods

Fungal Cultures

The fungal cultures used in this work were: *Trametes versicolor*, *Aspergillus niger*, and *Fusarium moniliforme*. The cultures were supplied from the collection of the Department of Biotechnology at the University of Food Technologies, Plovdiv, Bulgaria. *T. versicolor* was chosen with respect to its potential for laccase production, and *A. niger* for pectinase production. *F. moniliforme* was selected as a producer of α-amylase and glucoamylase. The combinations of the fungi were chosen to achieve maximum level for production of laccase and pectinase, taking into consideration the enzyme activities of the monocultures. Stock cultures were maintained on malt agar slants.

Mycelial inocula for SSF were prepared by inoculating 10^7 spores of each fungus from agar-slant culture to each of several 300-ml shake flasks containing 50 ml beer must, 7.5°B.

The pH of the media was adjusted with 1 M NaOH to 4.5. The inoculated flasks were incubated at 30 °C and 220 rpm for 48 h.

Solid Substrate Fermentation

The solid substrate fermentation (SSF) was carried out in 300-ml Erlenmeyer flasks containing a medium consisted of 4.0 g wheat bran, 2.5 g oats straw, 2.5 g beetroot press, and 40 ml of basal salt solution of following composition (% *w*/*v*): $(NH_4)_2SO_4$, 0.14; KH_2PO_4, 0.2; $MgSO_4 \cdot 7H_2O$, 0.03; $CaCl_2$, 0.03; $FeSO_4 \cdot 7H_2O$, $ZnSO_4 \cdot 7H_2O$, $MnSO_4 \cdot 7H_2O$ and $CoCl_2$, 0.002. After sterilization (121 °C for 30 min) and cooling, each flask was inoculated with the respective mycelial inocula prepared as mentioned above.

We focused on the investigation of two different types of inoculation:

- Inoculation with *A. niger* as a first step and a subsequent addition of *F. moniliforme* after 3, 4 and 5 days.
- Inoculation with *T. versicolor* as a first step and a subsequent addition of *A. niger* after 3, 4 and 5 days.

SSF fermentation with monocultures was used as reference. All flasks were cultivated at 30 °C for 7 days. Triplicate flasks were set up for each experimental variation. After cultivation, the content of each flask was extracted with 30 ml distilled water for 30 min at a temperature of 22 °C using a shaker (220 rpm). The mixture was then filtered through double Watman 1 filter paper, and the supernatant was assayed for laccase, cellulase, α-amylase, glucoamylase, xylanase, and β-glucosidase activity. Triplicate flasks were set up for each experimental variation.

Biomass Determination

Fungal biomass was estimated indirectly by determining the mycelial glucosamine content according to Sakurai et al. [14]. The glucosamine content was related to mycelial protein by the following equation:

$$Y = 0.13x - 2.38,$$

where Y is the mycelial protein content and x is the glucosamine content (mg/g dry weight). Finally, fungal biomass amount was estimated by multiplying mycelial protein content by 2.5 [3]. The control sample was a flask containing only nutrient medium (non-inoculated).

Enzyme Assays

The total cellulase activity was measured as filter paper activity (FPA) using Whatman 1 filter paper according to Mandels et al. [15]. β-glucosidase activity was assayed according to Riou et al. [16] using *p*-nitrophenyl-β-D-glucopyranoside as a substrate. Xylanase activity was measured according to Ilieva et al. [17], α-amylase activity according to Pantschev et al. [18], and glucoamylase activity according to Dahlqvist, modified by Kurzina et al. [19]. Xylose, produced after hydrolysis, was measured with 3, 5-dinitrosalicilic acid according to Rutrlof et al. [20]. One international unit (IU) of enzyme activity was defined as the amount of enzyme that released 1 μmol product per minute (glucose equivalents for FPA and glucoamylase; xylose for xylanase; *p*-nitrophenol for β-glucosidase) at the assayed pH and temperatures. For α-amylase activity, one international

unit was defined as the amount of enzyme required to degrade 1 μmol starch to dextrins per minute. Laccase activity was assayed according to Marbah et al. [21] using syringaldazine as a substrate. One unit of laccase activity was defined as 0.001 ΔA_{530} for 1 min, pH 6.0 and 30 °C. Pectinase activity was assayed according to Roboz et al. [22] by following the decrease in viscosity of 1% pectin solution in phosphate buffer, pH 5, with a capillary viscometer at 30°C. One unit of pectinase activity was defined as the amount of enzyme that decreased the viscosity of 1% pectin by 30% for 10 min. The remaining analytical procedures were carried out under the following conditions: cellulase activity—pH 4.8, 50 °C; β-glucosidase activity—pH 4.8, 50 °C; xylanase activity—pH 4.2, 40 °C; α-amylase activity—pH 4.7, 30 °C; glucoamylase activity—pH 3.5, 62 °C.

Results and Discussion

The maximum biomass and enzyme production by single and mixed cultures are shown in Tables 1 and 2. The profile of enzymatic biosynthesis and the peak of biomass production in pure and mixed cultures from *A. niger* and *F. moniliforme* are presented in Table 1. The synergistic relations established by the two cultures can be witnessed by the increased combined growth and by the amplified simultaneous production of four enzymes: pectinase, β-glucosidase, α-amylase and xylanase, in the case where *F. moniliforme* was inoculated on the third day after *A. niger*. In the presence of the less productive *F. moniliforme*, the synthesis of pectinase by *A. niger* increased 1.7 times (when inoculation with *F. moniliforme* was done on the fourth day after *A. niger*), the synthesis of β-glucosidase increase 1.3 times and that of α-amylase 1.6 times. Sugar consumption by the *A. niger* probably suppresses feedback effect and stimulates enzymatic synthesis. The most significant synergistic effect arising from the combined cultivation of the fungi, with respect to both cultural growth and pectinase and α-amylase production, was observed when inoculation with *F. moniliforme* was done, on the fourth and fifth day after *A. niger*.

Table 1 Enzyme activities and growth of the fungal mixed culture of *A. niger* and *F. moniliforme* in SSF on the seventh day of their cultivation.

Cultures	Biomass (g/g dry weight)	Laccase	FPA	Enzyme activities (IU/g dry weight)				
				β-glucosidase	Pectinase	Xylanase	α-Amylase	Glucoamylase
A. niger (control)	0.09±0.002	10.0±0.1	1.0±0.02	93.4±2.0	961.5±15.2	1.0±0.01	138.3±1.5	79.0±2.02
F. moniliforme (control)	0.07±0.001	0	0.8±0.01	36.0±0.5	0	5.0±0.02	65.6±0.95	31.2±0.55
A. niger and *F. moniliforme*[a]	0.17±0.0045	0	1.6±0.03	134.7±3.0	1,041.6±25.3	6.0±0.025	227.0±5.9	74.85±1.95
A. niger and *F. moniliforme*[b]	0.19±0.004	0	1.3±0.02	118.6±2.0	1,666.6±28.5	6.8±0.03	227.5±6.1	74.85±2.05
A. niger and *F. moniliforme*[c]	0.22±0.005	0	0.6±0.01	118.6±2.2	961.5±16.8	10.0±0.032	250.0±6.3	68.7±1.8

[a] *F. moniliforme* inoculated 3 days after *A. niger*

[b] *F. moniliforme* inoculated 4 days after *A. niger*

[c] *F. moniliforme* inoculated 5 days after *A. niger*

Maximum biomass amounts of 0.19 and 0.22 g/g dry weight, respectively, as well as 1.7 times higher pectinase and 1.8 times higher α-amylase activity were achieved in the mixed culture compared to *A. niger* monoculture (1,666.6 and 250 IU/g dry weight). Of specific interest was that no synergistic interrelations were established in the case of the second amylolitic enzyme studied, glucoamylase, a situation which was probably more beneficial from an energetic point of view for the two microbial populations. The synergism between the cultures provides a possibility not only for the increasing of enzyme production but for the complete utilization of solid substrates by mixed cultures in comparison to the monocultures.

A second microbial association, between *T. versicolor* and *A. niger*, was made to help us study the impact of cultivation conditions on the codevelopment of two fungal species. *T. versicolor* is known to be an effective laccase producer. The strain also synthesizes pectinase, while its production of the amylolitic enzyme glucoamylase is considerably weak. Fungal growth and enzymatic production in the pure and the mixed cultures are presented in Table 2. The initiation of the second culture (*A. niger*) to *T. versicolor* on the third, fourth, and fifth day resulted in an acceleration of the co-growth of the strains and in a significant increase in laccase synthesis, respectively, 2.1, 7.5, and 8.4 times higher in the mixed cultures, reaching levels of 97,600 IU/g dry weight. The synergism established by the cultures was attributed to the higher pectinase productivity of *A. niger*, which destructed pectin, thus, facilitating the approach and destruction of lignin by *T. versicolor*. Cell walls of plant cells are extremely complex structures where the role of the pectin grid (covering cellulose, hemicellulose, and lignin) is to control cell wall pores dimensions, and, consequently, the access of enzymes to their substrates [23, 24]. *T. versicolor*, on its turn, contributed to the elevated enzymatic levels of cellulases and pectinase in the mixed culture (with *A. niger* inoculated on the fifth day) when compared to *A. niger* monoculture. Probably, the presence of sufficient substrate amounts in the medium eliminated the necessity of α-amylase synthesis by *A. niger* in the mixed culture as well. The interactions between the two microbial cultures favored, to a greater extent, the first of them and regardless of the increased biomass accumulation in the mixed cultures that could be attributed to the domination of *T. versicolor* over the second microbial strain.

Table 2 Enzyme activities and growth of the fungal mixed culture of *T. versicolor* and *A. niger* in SSF on the seventh day of their cultivation.

Cultures	Biomass (g/g dry weight)	Enzyme activities (IU/g dry weight)						
		Laccase	FPA	β-glucosidase	Pectinase	Xylanase	α-Amylase	Glucoamylase
T. versicolor (control)	0.08 ±0.002	11,600± 12.5	1.9± 0.05	50.0±0.75	367.6± 4.4	0.5± 0.0075	0	28.9± 0.4
A. niger (control)	0.09±0.002	10.0± 0.25	1.0± 0.04	93.4±1.4	1,003.8± 9.8	1.0± 0.008	138.3± 1.15	79.0± 0.6
T. versicolor and *A. niger*[a]	0.16±0.005	24,400± 23.9	2.4± 0.07	35.0±0.5	4,76.1± 4.9	0.9± 0.009	0	31.6± 0.4
T. versicolor and *A. niger*[b]	0.17±0.004	86,800± 36.5	2.8± 0.07	57.3±0.65	526.3± 5.1	0.8± 0.0085	0	33.3± 0.3
T. versicolor and *A. niger*[c]	0.20±0.004	97,600± 39.5	2.9± 0.06	71.9±0.7	1,163.8± 6.8	0.7± 0.0069	0	33.5± 0.4

[a] *A. niger* inoculated 3 days after *T. versicolor*

[b] *A. niger* inoculated 4 days after *T. versicolor*

[c] *A. niger* inoculated 5 days after *T. versicolor*

Table 3 Volumetric productivities in fungal mixed culture of *A. niger* and *F. moniliforme* in SSF.

Cultures	Biomass ($g\ l^{-1}\ h^{-1}$)	β-Glucosidase ($IU\ l^{-1}\ h^{-1}$)	Pectinase ($IU\ l^{-1}\ h^{-1}$)	α-Amylase ($IU\ l^{-1}\ h^{-1}$)
A. niger (control)	0.11±0.0016	111.2±1.2	1,144.2±12.5	164.5±2.5
F. moniliforme (control)	0.08±0.001	42.8±0.6	0	78.1±0.6
A. niger and *F. moniliforme*[a]	0.20±0.002	160.3±1.3	1,239.5±12.0	270.1±2.9
A. niger and *F. moniliforme*[b]	0.23±0.002	141.1±1.3	1,983.2±13.2	270.7±2.8
A. niger and *F. moniliforme*[c]	0.26±0.003	141.1±1.2	1,144.2±11.5	297.5±2.9

[a] *F. moniliforme* inoculated 3 days after *A. niger*

[b] *F. moniliforme* inoculated 4 days after *A. niger*

[c] *F. moniliforme* inoculated 5 days after *A. niger*

The results achieved with both of the mixed microbial cultures under study demonstrated clearly that the synthesis of all enzymes was highly responsive to the choice of moment at which the second microbial culture was inoculated. Another finding worth noting was the fact that the second microbial association provided its substrates mostly by taking advantage of laccase and pectinase, while the first microbial association, by utilizing pectinases, amylases and cellulases.

With both of the mixed microbial populations cocultivation facilitated the amplified synthesis of enzymes rather than their growth acceleration. According to Gutierrez-Correa and Tengerdy [25], the synergistic interrelations responsible for the increase of enzymatic synthesis were not directly related to growth. A further proof of the presence of synergism established by the cultures was the enzyme volumetric productivities in both of the mixed microbial cultures. Both the enzyme activities and the volumetric productivities increased proportionally with the combined biomass production (Tables 3 and 4).

Conclusion

Current studies undoubtedly prove that mixed cultivation is beneficial in the search for profitable synthesis of lignocellulases and pectinases on various agricultural wastes or for the enzymatic silage of these substrates. The competent selection of compatible partners can adjust the desired enzymatic levels and compositions in mixed fungal systems aimed at a number of specified designations. Thus, we achieved a very high level of laccase production

Table 4 Volumetric productivities in fungal mixed culture of *T. versicolor* and *A. niger* in SSF.

Cultures	Biomass ($g\ l^{-1}\ h^{-1}$)	Laccase ($IU\ l^{-1}\ h^{-1}$)	Pectinase ($IU\ l^{-1}\ h^{-1}$)
T. versicolor (control)	0.09±0.001	13,804±22.2	437.4±6.5
A. niger (control)	0.11±0.002	11.9±0.12	1,194.5±11.2
T. versicolor and *A. niger*[a]	0.19±0.002	29,036±23.5	566.5±5.3
T. versicolor and *A. niger*[b]	0.20±0.002	103,292±48.5	626.3±6.4
T. versicolor and *A. niger*[c]	0.24±0.003	116,144±49.2	1,384.9±12.2

[a] *A. niger* inoculated 3 days after *T. versicolor*

[b] *A. niger* inoculated 4 days after *T. versicolor*

[c] *A. niger* inoculated 5 days after *T. versicolor*

(97,600 IU/g dry weight) without any analog in the literature. The overproduction of laccase and pectinase achieved by mixed fungal culture of *T. versicolor* and *A. niger* suggests the possibility to produce this enzyme by solid substrate fermentation using a cheaper substrate for its industrial usage.

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