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Effect of Mixing on the Solid-State Fermentation of Coffee Pulp with *Aspergillus tamarii*

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Summary

Solid-state fermentation of coffee pulp with *Aspergillus tamarii* V12307 was carried out in laboratory scale reactors (bottle and column) to evaluate the effect of four different mixing frequencies (2.0, 2.7, 4.0 and 8.0 day^{-1}) on fungal growth, indirectly determined by carbon dioxide formation and the production of spores and pectin methylesterase. Coffee pulp was used as the sole source of nutrients. An increase in the fraction of bonded particles was observed in the bottle reactors after 12 h of cultivation when no mixing was applied. The use of any mixing frequency reduced the fraction of bonded particles. However, there was no significant difference in pectin methylesterase production between the mixing frequencies at the end of the fermentation. Similarly, there were no significant differences in CO_2 production, oxygen uptake or sporulation, demonstrating that the mycelium was not damaged by intermittent mixing. This strategy of mixing could be used in large scale reactors in order to reduce heat and mass limitations.

Key words: coffee pulp, solid-state fermentation, effect of mixing

Introduction

During solid-state fermentation (SSF) in pilot and industrial scale reactors, temperature and concentration gradients are observed along the substrate bed. Among cooling processes, evaporative cooling can be very efficient for reducing the temperature gradient. However, it dries up the solids and a reduction in microbial growth can be observed. The requirement for evaporative cooling can be reduced by mixing the solids of the bed, which increases heat transfer through the bioreactor wall. During the last two decades, qualitative observations have shown that the resulting sheer stress during the movement of the particles injures the cultured microorganism (*1*). In spite of that, little work has been done to quantify the effect of mixing on the growth of filamentous fungi, the group of microorganisms most often used in solid-state fermentation.

There are two possible methods of mixing solids: continuous and intermittent. Intermittent mixing gives a balance between the deleterious effect on the hyphae of the fungus and the positive effects on temperature control (*2*). The application of intermittent mixing requires evaluating how often the solids can be agitated without affecting the growth of the microorganism. Most reports have been done in pilot scale reactors where the temperature and concentration gradients are considerable (*3–5*). Therefore, it is important to evaluate the mixing of the solids in reactors without any limitation in heat and mass transfer phenomena.

Coffee pulp (CP) is an interesting SSF substrate due to its nutrient content (*6*) and the presence of bioactive compounds such as caffeine and antioxidants like hydroxycinnamic acids (*7*). The objective of this work is to evaluate the effect of intermittent mixing on SSF by *Aspergillus tamarii* V12307 on fungal growth, indirectly determined by carbon dioxide formation and the production of spores and pectin methylesterase (PME). CP was used as the sole source of nutrients.

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Materials and Methods

Microorganism

A. tamarii V12307 belonging to the collection of Metropolitan Autonomous University (Mexico) and Research Institute for Development (France), MAU-RID, was grown in potato dextrose agar at 30 °C for 6 days. A spore suspension was obtained with 0.1 % Tween 80 solution and scraping with a magnetic agitator. The spore concentration was determined with a Neubauer counting chamber.

Substrate

Sun-dried CP particles (free of beans, leaves, twigs and dust by passing through a number 6 mesh) were used as the sole source of nutrients for SSF. CP was composed principally of hollow oval particles (from (16.6±2.7) to (12.2±2.3) mm in diameter), resulting from the drying of coffee cherry. CP was humidified with distilled water until 50 % moisture content was reached. It was autoclaved at 121 °C for 10 min and subsequently inoculated. An inoculum size of $2.5 \cdot 10^6$ spores per g of dry matter (DM) was applied and the moisture was adjusted (69 %) with sterile distilled water.

Solid-state fermentation conditions

Laboratory scale SSF was carried out using two different bioreactors. Bottle reactors were used to measure the bonding of particles and PME production. Column reactors were used to measure carbon dioxide and spore production. A number of 35 bottles (4.5 cm in diameter×6.5 cm in height) were packed with 13 g of CP. Inoculated CP (20 g) was packed in glass columns (4.5 cm in diameter×20 cm in height). An air flow of 1 mL/(min·g of CP) was supplied. Both reactors were incubated at 30 °C for 48 h.

For all conditions studied, the cultures remained static during the first 12 h and a one-minute mixing was applied. Then, mixing was applied intermittently using four different daily frequencies: 2.0, 2.7, 4.0 and 8.0 day^{-1}, corresponding to the mixing of solids every 12, 9, 6 and 3 h, respectively. The mixing was carried out with a fixed-speed vortexer (Model 16700 Barnstead/Thermolyne, Dubuque, IA, USA) in a similar way as the test tubes are mixed. Each SSF assay was carried out at least in duplicate.

Analytical techniques

The water content was determined by a gravimetric method using a moisture analyser MB45 (Ohaus Corporation, Pine Brook, NJ, USA) and the water activity was measured using Aqualab CX-2 equipment (Decagon Devices, Inc., Pullman, Washington, DC, USA). CP (5 g) was mixed with 45 mL of distilled water and magnetically stirred for 20 min. After settling, the supernatant was used to determine the pH and the spore concentration using a Neubauer chamber.

The bonding of CP particles due to the growth of the microorganism was quantified using a modification of the proposed technique for wheat grains (*5*). For each sampling time, the CP particles incubated into one bottle were carefully unpacked. The free CP particles were separated from the particles joined to one or more particles (bonded particles). The bonded particle fraction, *w*(bonded particles), was expressed as the percentage of the mass of the bonded particles in the total mass.

Carbon dioxide production was used as an indirect measurement of growth. The O_2 and CO_2 concentrations in the dry exhaust air of the column reactors were measured using a gas chromatograph GOW-MAC 580 (Gow-Mac Instrumentation Co, Bethlehem, PA, USA) equipped with a thermal conductivity detector and an automatic sampler. The respiratory activity parameters were calculated using a reported methodology (*8*).

PME was measured using a titrimetic technique (*9*). A crude extract was obtained by mixing 1 g of dry CP with 6 mL of distilled water. A volume of 19 mL of 0.5 % citric pectin solution, used as the substrate, and 1 mL of crude extract were incubated at 37 °C. The volume of 0.005 M NaOH added to maintain the pH constant (6.0) was registered continuously for 40 min using a Mettler DL21 automatic titrator (Mettler-Toledo AG, Greifensee, Switzerland). One unit of PME activity was defined as the amount of enzyme necessary to release 1 μmol of acid per minute at 37 °C and pH=6.0.

An analysis of variance was carried out ($p<0.05$) in order to determine the difference between the mixing frequencies.

Results and Discussion

Effect of mixing on the SSF in bottle reactors

Heat accumulation in large scale reactors is a well-known problem in SSF processes. Heat dissipation can be promoted by mixing; however, it has been suggested that mixing has a negative effect on mycelium activity and sporulation (*10*) using large scale reactors. Nevertheless, in these kinds of vessels, the effect of mixing on the reduction of heat, mass gradients and mycelium damage is difficult to evaluate. The intermittent mild mixing studied in this work was intended to promote the rupture of the bonded CP particles resulting from the growth without damaging the mycelium. The use of small scale laboratory reactors allowed for evaluation of the effect of mixing without problems of temperature control, since small reactors are isothermal.

Fig. 1 shows the bonded particle fraction during SSF by *A. tamarii* V12307 under static conditions using four different daily frequencies of intermittent mixing of cultures in bottle reactors. A bonded particle fraction higher than zero was found even at the beginning of the culture as a likely consequence of the stickiness of the moist coffee pulp particles. Under static conditions, this fraction increased from 18 % in 12 h of culture to a relatively constant value of 69 % after 33 h of incubation. In contrast, under intermittent mixing conditions, the bonded particle fraction was lower than 33 % at any time of culture. The use of the lowest frequency of mixing (2.0 day^{-1}) maintained the bonded particle fraction at a constant value (24.5 %) during the entire SSF procedure, while higher frequencies (2.7, 4.0 and 8.0 day^{-1}) reduced the bonded particle fraction gradually during the fermentation. The four frequencies of mixing reduced the amount of bonded CP particles, for instance, when the lowest

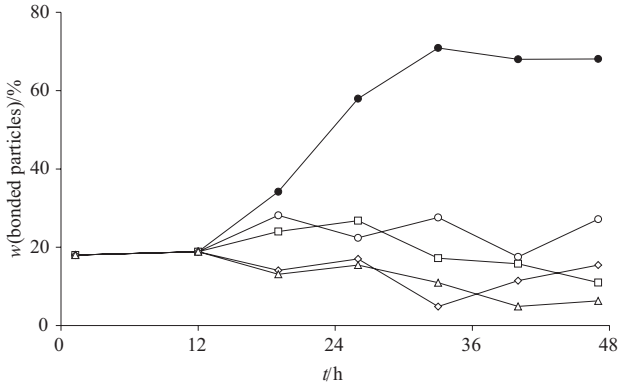

Fig. 1. Bonded particle fraction during the solid-state fermentation of coffee pulp by *A. tamarii* V12037 in bottle reactors. After a static period of 12 h, four different daily mixing frequencies were applied: (○) 2.0, (□) 2.7, (◇) 4.0 and (△) 8.0 day^{-1}, (●) without mixing

frequency (2.0 day^{-1}) was applied, the final fraction was (27.1±1.2) %, while the highest frequency (8.0 day^{-1}) produced a significantly ($p<0.05$) lower final fraction ((6.3± 0.2) %).

The substrate size and shape might play an important role in the global effect of mixing of solids on the growth of filamentous microorganisms. Photographic analysis of fermenting particles (not shown) indicated that CP particles are shell-like, with convex and concave surfaces. A random disposition of CP particles is found inside the fermenting beds. CP presents two surfaces for the growth of the mycelium. Under static conditions, it is expected that the apparition of agglomerates is due to mycelial growth between the surfaces of different CP particles. The outer and inner surfaces of the particles are covered completely by the fungus, forming bridges between particles, resulting in the agglomerates. The movement of the particles causes shear stress forces that restrict the growth of the fungus over the outer (convex) surface, decreasing the development of agglomerates during fermentation with intermittent mixing.

Reports in the literature in regard to the intermittently mixed SSF reactors (rotary drums) are scarce and qualitative. For instance, in the fermentation of soy beans by *Rhizopus oligosporus*, a cake-like mass is found under static conditions, while a granular product is observed under a mixed process (*11*). On the other hand, a decrease in the bonded particles when continuous mixing was applied to an SSF culture of *A. oryzae* in a rotary drum reactor has been reported (*12*). However, there were still agglomerates of 3–9 particles at the end of the fermentation. In a similar report, the application of a 5--minute mixing event at the end of the SSF process with *A. oryzae* led to around 25 % of the wheat particles remaining bonded (*5*).

Table 1 shows the final values of the measured variables of the SSF process with CP in a bottle reactor. The water content and the water activity remained constant during fermentation. In all the studied cases, the final water content was lower than the initial value. There was a tendency towards the decrease in CP moisture (Table 1) when the frequency of mixing was increased, in comparison with static conditions; this could be explained by the evaporation of water caused by the mixing of solids. Nevertheless, when an analysis of variance was applied, no significant differences were observed. Water activity values were kept almost constant, slightly above 0.98 during cultivation, that is, higher than the critical value reported for the culture of *A. oryzae* (*3*).

Table 1. Pectin methylesterase (PME) production, water content and water activity (a_w) after 48 h of coffee pulp fermentation by *A. tamarii* V12307 in bottle reactors using different mixing frequencies

Mixing frequency / day^{-1}	PME / mU/g	w(water) / %	a_w
0.0 (static)	47.0±15.7	67.2±0.4	0.988±0.004
2.0	81.0±18.6	62.2±1.8	0.985±0.001
2.7	63.4±6.9	64.5±4.0	0.987±0.003
4.0	73.8±16.2	62.2±2.0	0.988±0.003
8.0	88.2±31.4	61.7±1.1	0.989±0.001

The main polysaccharide reported in the mucilaginous constituents of CP is pectin (*13*). The utilization of this agricultural by-product first requires the breakdown of pectin. This can be achieved efficiently when different enzymes act together. Specifically, PME activity is involved in the breakdown of pectin. It releases methyl groups, which are covalently esterified to the polygalacturonic acid chains, thus promoting the action of depolymerising enzymes (*14*).

Table 1 shows the PME activity produced by *A. tamarii* V12307 on CP in the bottle system. PME production attained under static conditions was 47 mU per g of dry mass. There was an increase (1.5-fold) in the mean PME activity (70.7 mU per g of dry mass) with any of the frequencies applied in comparison with static conditions. There was no significant effect on PME production among the four mixing frequencies studied. This value was lower to that reported with related microorganisms (*14*,*15*). Although these higher values could mainly be attributed to the differences in the composition of the substrates, it could also be the result of selected conditions for PME production. However, these results point to the possibility of improvement of the growth conditions to attain higher production.

Enzyme production using mixed SSF reactors has already been reported, but there are no conclusions on the effect of mixing. In some cases, it is a consequence of the evaluation of variables not related to mixing, such as culture composition or carbon dioxide concentration (*16*, *17*). On the other hand, the effect of the mixing rate on the production of α-galactosidase and invertase has been studied (*1*). No difference was found in the production of α-galactosidase at a mixing rate of 0.15–15 rpm, so these data are in agreement with our results. However, an increase in invertase production was reported when the mixing rate was higher. A comparison of the polysaccharidase and glycosidase production of traditional static soybean fermentation using *R. oligosporus* with that obtained in a rotating drum reactor has also been

done (*11*). The total enzyme production in the rotary drum and the traditional process were similar after 48 h of incubation. During the third day of incubation, the total enzyme production increased in the rotary drum, whereas it decreased in the traditional process.

In this work, experimental evidence has been shown indicating that intermittent mild mixing possibly reduces the heat and mass transfer limitations by the separation of bonded CP particles without damaging the mycelium. Therefore, the lack of a significant difference in PME production suggests the presence of the same amount of active mycelium. However, biomass cannot be reliably measured in SSF. The lack of a direct evaluation of the growth in bottle reactors led to the use of column bioreactors.

Effect of mixing on SSF columns

The carbon dioxide production after 48 h of SSF of CP by *A. tamarii* V12307 using column reactors under static conditions and intermittent mixing is shown in Fig. 2. It can be interpreted as an indirect measurement of biomass. No significant differences between the studied conditions (static or mixed) were observed for the total carbon dioxide production, presenting a mean value of 144.5 mg per g of initial dry matter. Higher carbon dioxide production, 417.4 mg per g of initial dry matter, in SSF of coffee pulp by *Penicillium commune* has been reported (*18*). However, it is difficult to compare these values originating from different microorganisms; an effect of mixing was not found either in terms of total oxygen uptake, with a mean value of 86.0 mg per g of initial dry matter. In addition, there were no significant differences in the carbon dioxide production rate or oxygen uptake rate (Table 2). The analysis of overall respirometry data indicates that the application of intermittent mixing did not damage the mycelium.

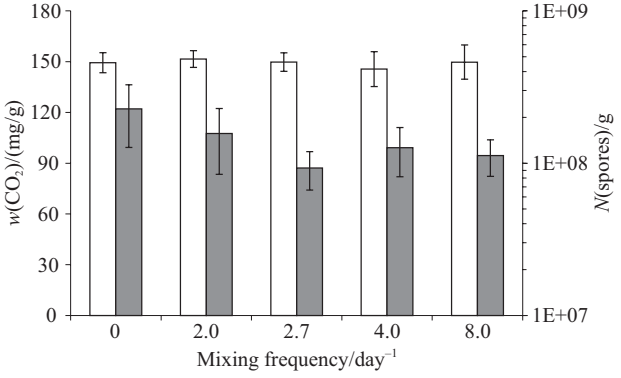

Fig. 2. Carbon dioxide (white bars) and spore (grey bars) production at the end of a 48-hour solid-state fermentation of CP by *A. tamarii* V12307 in column reactors using different mixing frequencies

The mean value of maximum oxygen uptake rate under mixing conditions in comparison with static conditions was kept almost constant. Nagel *et al*. (*3*) reported a decrease of 15 % in the maximum oxygen uptake rate in SSF by *A. oryzae* in a paddle reactor, in comparison with laboratory conditions, indicating that continuous mixing does not affect negatively the respiration rate. Our results are in agreement with these findings. However, a significant decrease (37 %) in oxygen uptake rate was observed when static conditions were used (0.16–0.22 mol per kg of substrate per h) in comparison with continuous mixing (0.27–0.33 mol per kg of substrate per h) (*12*), indicating that mixing enhanced the respiration rate. In addition, the respiratory quotient found in this work was constant ((1.3±0.09) mol of CO_2 per mol of O_2) under the studied conditions.

The application of mixing did not disturb spore production as no significant difference was found between the distinct frequencies in comparison with static conditions (Fig. 2) during SSF in column reactors. In the present case, the mean production was $1.4 \cdot 10^8$ spores per g of dry matter. However, reduced *Penicillium glabrum* sporulation has been reported on polyurethane foam when mixing was applied (*10*). Our results are lower than those reported in the literature, which can probably be explained by the lack of the addition of nutrients to the CP, which was used as the sole nutrient source. This work was focused on analysing the effect of mixing.

A production of $2.3 \cdot 10^8$ spores per g of dry matter after 48 h in static culture was obtained. CP was used as substrate for *P. commune* spores with a production of $2.1 \cdot 10^9$ spores per g of dry matter (*18*). The *Aspergillus* genus spore production has been reported by SSF using other substrates and supports. For instance, Bapat *et al*. (*19*) produced *A. niger* spores with three substrates. The highest production ($3.1 \cdot 10^{10}$ spores per g of dry matter) was obtained using peas. On the other hand, a production of $2.5 \cdot 10^7$ spores per g of lupine or soy and $1.3 \cdot 10^8$ spores per g of lupine or soy were reported for *A. oryzae* and *A. sojae*, respectively (*20*).

Table 2. Maximum carbon dioxide production and oxygen uptake rates per initial dry matter during CP fermentation by *A. tamarii* V12307 in column reactors using different mixing frequencies

Mixing frequency	CDPR	OUR	OU	RQ
day^{-1}	mg/(g·h)	mg/(g·h)	mg/g	mol CO_2/mol O_2
0	6.7±1.06	3.9±0.01	85.0±7.4	1.29±0.15
2.0	6.3±0.15	3.9±0.45	89.5±3.3	1.23±0.06
2.7	6.2±0.18	3.9±0.19	84.6±3.5	1.29±0.05
4.0	5.9±0.28	3.8±0.28	87.8±9.0	1.21±0.06
8.0	6.0±0.61	4.1±0.53	83.1±12.7	1.32±0.12

CDPR=carbon dioxide production rate, OUR=oxygen uptake rate, OU=oxygen uptake, RQ=respiratory quotient

Singhania *et al*. (*21*) noted that one of the major challenges for the successful implementation of SSF is adequate mixing of the bed without damaging the microorganisms as well as the substrate particles. Alberton *et al*. (*22*) selected a mixture of substrates that does not suffer compaction and recommended its use for large scale bioreactors.

From the results shown above, it can be stated that the physical properties of the substrate (size, form, porosity) and the variables related to mixing such as rate, duration or frequency could protect the microorganism from shear stress during SSF processes with mixing.

Conclusions

A decrease of the bonding of CP particles was observed as a result of the application of intermittent mixing in laboratory SSF reactors using *A. tamarii* V12307, but it was not inhibitory in terms of CO_2 production, oxygen uptake, sporulation or PME production, demonstrating that the mycelium was not damaged by intermittent mixing. These results suggest that this mixing strategy could reduce heat and mass limitations in large scale reactors.

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