Evaluation of standard procedures for inoculum transfer for homogeneous distribution during the start-up of a fungal solid state fermentation process

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A normal observation in solid state fermentation (SSF) is uneven growth on fermented substrate. This situation will usually result in unsatisfied outcome. Therefore, a proper technique for inoculum transfer prior to the start of the fermentation process was developed in this study to prepare inoculum transfers for the inoculation process into solid substrates. This was done to make sure the spore inoculum distributed homogenously into all solid particles before the fermentation was started. Three (3) techniques of inoculum transfer were tested in SSF using wheat bran as a model solid substrate while Aspergillus awamori was used as a model microorganism. Technique 1: the spore suspension was transferred into the center of the solid substrate over the solid surface; Technique 2: the spore suspension was distributed on the surface of solid substrate randomly and Technique 3: the spore suspension was distributed on the surface of solid substrate followed by homogenously mixed with spatula to form uniformly on the solid substrate. Fungal growth, spores and enzymes production (glucoamylase, protease, xylanase and cellulose) showed the highest values obtained with Technique 3 followed by Technique 2 and Technique 1. This study provides an idea of how important such parameter as inoculum transfer influence the growth performance of fungi during the SSF process. The choice of inoculum transfer technique had the greatest overall impact on the growth and most important targeted productivity of product. Such information enables the design of a better homogenous distribute of microorganisms into the whole solid substrate fermented bed.

Keywords: Inoculum; solid state fermentation; Aspergillus awamori; fungal growth; spore; enzyme.

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Introduction

Solid state fermentation (SSF) refers to the microbial fermentation, which takes place in the absence or near absence of free water, thus being close to the natural environment to which the selected microorganisms, especially filamentous fungi, since the process imitates their natural habitat [1]. SSF was developed for the manufacturing of traditional fermented foods; its application has been extended to the pharmaceutical and biochemical industries. Despite the fact that there is already much information available in the literature concerning SSF, it is always interesting to explore fresh ideas that may provide a better understanding. Literature studies clearly show that SSF offers a great and promising technology

Figure 1. Some of examples observed fermented substrate from the current study on uneven growth during SSF performed in petri dishes.

for future research and development in industry. Many benefits can be obtained through SSF technology and it can potentially be as advantageous as submerged fermentation (SmF) technology in bioprocessing [2]. Lacks and disadvantages seen with SSF need to be overcome in order to establish SSF technology as a possible solution to treat and process food and agroindustry waste for the generation of added-value products [3]. The growth of modern SSF as a tool in the development of biorefinery processes is necessary to realize more advances especially in bioprocess engineering and bioreactor system design in terms of both theoretical and practical features of this system [4].

The most important factor besides the choice of microorganisms and substrates to be considered during the development of an SSF is the transfer of inoculum or starter cultures to start-up the process. In contrast to liquid fermentation, distribution of starter culture homogeneously into every degree of solid particles could be a problem and difficult in SSF. The inoculum is generally used at high ration in most fermentation processes for the production of secondary metabolites, with the aim of producing the desired level of product in a short period [5]. The commonly applied inoculum preparation techniques for SSF include spore suspension, mycelia disc, mycelia suspension, and pre-inoculated substrates [6], while inoculation transfer/strategy is important aspect that needs attention in the development of successful SSF technology [7, 8]. Preparation of the solid substrate and subsequent inoculation are very important because the initial conditions strongly affect the entire SSF process [9]. Usually, it is very difficult to correct the inoculation transfer and there is always an uneven distribution of spores in the solid substrate bed [10]. In microbial SSF, from the time it is transferred from its preserved state until it is inoculated into the solid substrate where the microbial activity of interest is expressed is referred to as inoculum development [11]. According to Krishna and Nokes [12], inoculum quality and the influence of inoculum quality are important factors which need in-depth investigation before scaling-up of high-yielding fermentation process. Figure 1 shows some situation happened during SSF where it clearly showed the bad growth. Growth occurs unevenly. There is a certain part of fermented bed showing no growth either on the surface or other part of solid substrate. The penetrating process by fungal mycelium does not occur in general, especially at the bottom of fermented bed. This entire situation can be correlated with the inoculum transfer at the beginning of the fermentation process. It was identified as non-homogenized inoculum into solid substrate as the real factor for the cause. Among all, fungi are shown to be well adapted to SSF as their mycelium can grow on substrates

surfaces and penetrate into the inter-particles spaces, thereby colonizing solid substrate.

Thus, standard procedures for inoculum transfer were developed to obtain satisfactorily homogenous transfer of the spores into the solid substrate particles prior the fermentation process. Here, we studied three (3) inoculum transfer techniques using spores suspension as inoculum. These simple preliminary studies also explore how development a proper technique can be exploited to improve process knowledge and definitely would have a great impact on the whole process. Hence, the objective of this study was to evaluate three inoculation techniques for better homogeneity of transfer to solid particles in order to establish the process of SSF for future bioprocess development.

Materials and methods

Microorganisms

Aspergillus awamori obtained from the School of Chemical Engineering and Analytical Science, Faculty of Engineering and Physical Science, University of Manchester are used throughout this study. A small amount of the spore suspension (0.5 mL) was spread on the surface of a 100 mL solid sporulation medium [5% (w/v) whole wheat flour and 2% (w/v) agar] in 500 mL Erlenmeyer flasks, and the inoculated flasks were incubated at 30°C for 7 days. Several glass beads (4-mm diameter) and 50 mL of sterile 0.1% (v/v) Tween 80 solution (Merck, Kenilworth, New Jersey, USA) were added to the flasks to form a stock spore suspension after gentle shaking. Fungal spores in universal bottle were stored at 4°C in agar slopes of solid sporulation medium as a stock culture and they were sub-cultured in the time interval of every two months. This strain produces compact colonies with huge spores and accumulates large quantities of black pigments during growth in the form of mycelium.

Solid state fermentation – The procedures

Wheat bran was obtained from Cargill Wheat Processing Plant, Manchester, UK. The substrate was kept in airtight container and stored in cold room for future use. Briefly, 12 g wheat bran was weighed and put into the 250 mL flask before being sterilized at 121°C for 15 min. The substrates were allowed to cool at room temperature before inoculating with *A. awamori* spores and being moistened with an amount of sterile distilled water to obtain the initial moisture content 65%. For technique 1 and 2, sterile uninoculated moistened wheat bran was distributed into petri dishes. By using a sterile pipette, a spore suspension (1.2×10^6 spores/g substrate) was transferred onto the solid surface according to strategies 1 and 2 described below. While for technique 3, inoculum transfer was performed inside the flask and mixed homogenously by stirring with a sterile spatula. After mixing well, the content was distributed onto petri dishes. All petri dishes were incubated at 30°C for 16 days. At defined time intervals, one petri dish for every technique was taken out for organic matter loss analysis.

Inoculum transfer

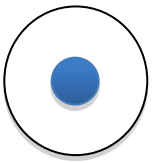
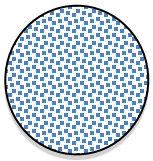
All experiments were carried out in commercial petri dishes, 1.5 cm high and 9 cm in diameter. This simple SSF system, which has similarities with tray bioreactors, was filled the inoculated solid substrate. In order to make sure the spores homogenously transfer into every solid substrate, three techniques for inoculum transfer were performed in this study (Table 1).

Sample analyses

1. Organic matter loss

Biomass was estimated on the basis of loss of organic matter or dry solid during the course of fermentation [13]. It was assumed that organic matter loss is due to carbon dioxide formation by fungal metabolic activities during the fermentation process. This technique involves a drying process at 80°C using an oven. Fermented substrate was dried in an oven overnight to a constant weight, which was recorded as the final dry weight of solid substrate after fermentation.

Table 1. Three strategies for inoculum transfer were performed in this study.

Technique	Procedures
Technique 1 	The spore suspension was transferred into the center of the solid substrate over the solid surface.
Technique 2 	The spore suspension was distributed on the surface of solid substrate randomly.
Technique 3 	Following the standard procedure described above with homogenous distribution of the spore suspension in the flask before being distributed into petri dishes.

Organic matter loss was then calculated as the weight difference and expressed as a percentage of the initial dry weight of the samples.

$$Organic\ matter\ loss\ (\%) = \frac{W_i - W_f}{W_i} \times 100$$

Where

W_i: The initial dry weight of solid substrate before fermentation

W_f: The final dry weight of solid substrate after fermentation

2. Spores

At the end of the incubation period, samples were harvested for spores' analysis. The whole fermented substrate was blended using a food-processing blender to get homogenous substrate. Approximately 5 g (on a wet basis) from all the fermented substrates was extracted with 100 mL distilled water and shaken for 30 h on a rotary shaker (Infors AG-CH4103, Switzerland) at 250 rpm and 30°C. Sample then filtered using muslin cloth to separate big particles and to obtain spores suspension. The concentration of the spore suspension was measured by hemocytometer. Spores suspension should be diluted enough so that the spores do not overlap with each other on the grid and should be uniformly distributed as it is assumed that the total volume in the chamber represents a random sample.

3. Enzymes

(1) Glucoamylase activity

Glucoamylase was assayed using the method as described by Ariff and Webb [14] using maltose as a substrate. Glucoamylase activity was determined by measuring the initial rates of glucose production and expresses as µmol of glucose liberated per minute per mL broth supernatant (µmol/min·mL) or unit per mL (U/mL). Glucoamylase activity is expressed throughout this study in units of U/g material measured on a dry basis.

(2) Protease activity

Protease activity was evaluated by the formation of free amino nitrogen (FAN). FAN concentration was measured using the ninhydrin colorimetric method as outlined by the European Brewery Convention [15] with modifications made by Wang [16]. The method based on the color reaction between ninhydrin and amino acids at pH 6.7, which gives an estimate of amino acids, ammonia and in addition the terminal alpha-amino nitrogen groups of peptides and proteins. The amounts of proteases for the production of one milligram FAN in one minute under controlled conditions was defined as one unit of activity (U/mL). The protease activity throughout this study was expressed in units of U/g material measured on a dry basis.

(3) Xylanase activity

Determination of xylanase activity was conducted according to the method developed by Bailey et al. [17]. The assay is based on the release of reducing sugars from 1% (w/v) xylan (Sigma-Aldrich, Saint Louis, MS, USA) solution prepared in 0.05 M citrate buffer pH 5.4 by 3,5-dinitrosalycylic acid method (DNS method) at 50°C by using xylose to generate a standard curve. The xylanase activity was expressed throughout this work in units of U/g material measured on dry basis. One unit of xylanase activity was defined as the amount of enzyme producing 1 µmol xylose equivalents per minute under assay conditions.

Cellulase activity

Filter paper cellulase activity was measured according to IUPAC recommendations [18] employing filter paper Whatman No. 1 as a substrate [19]. The procedures were designed to measure cellulase activity in terms of “filter paper units” (FPU) per milliliter (mL) of original (undiluted) enzyme solution. Quantitative results of the enzymes preparations must be compared on the basis of significant and equal conversion. The value 2 mg of total reducing sugars as glucose from 50 mg of filter paper in 60 min reaction time was designated as the intercept for calculating filter paper cellulase units (FPU) by IUPAC. The total reducing sugars was then determined by the DNS method [20]. The cellulase activity was expressed throughout this study in units of FPU/g material measured on a dry basis.

Results and discussion

The inoculation transfer is an important aspect in SSF especially when involves fungi. In large scale SSF, the inoculum transfer may become a major relevance, because of the difficulties in distribution of fungal spores. The rationale of the study:

- The first cultivation on a solid substrates solid state fermentation (SSF) system used an inoculum that serves as a starter microorganism to make the fermentation successful.

- Inoculum transfer process in SSF represents one of the important subjects that need to be focused on to make sure the process is successful. Compared to submerged fermentation (SmF) where inoculum preparation is very easy in terms of creating homogeneity within the substrate preparation. The homogenous conditions between inoculum and substrate in SSF sometimes become very difficult to achieve and absolutely will affect the process.

Figure 2 shows an image of growth for comparison of three inoculum transfer techniques. Through visual observation, inoculum transfer upon the cultivation of SSF showed different patterns of fungal growth. For more details on the degree of fungal growth, data from organic matter loss are shown in figure 3. Fungal growth occurred where almost 75% substrates were consumed (measured at day 16) when inoculum transfer was homogenously transferred into solid substrate using technique

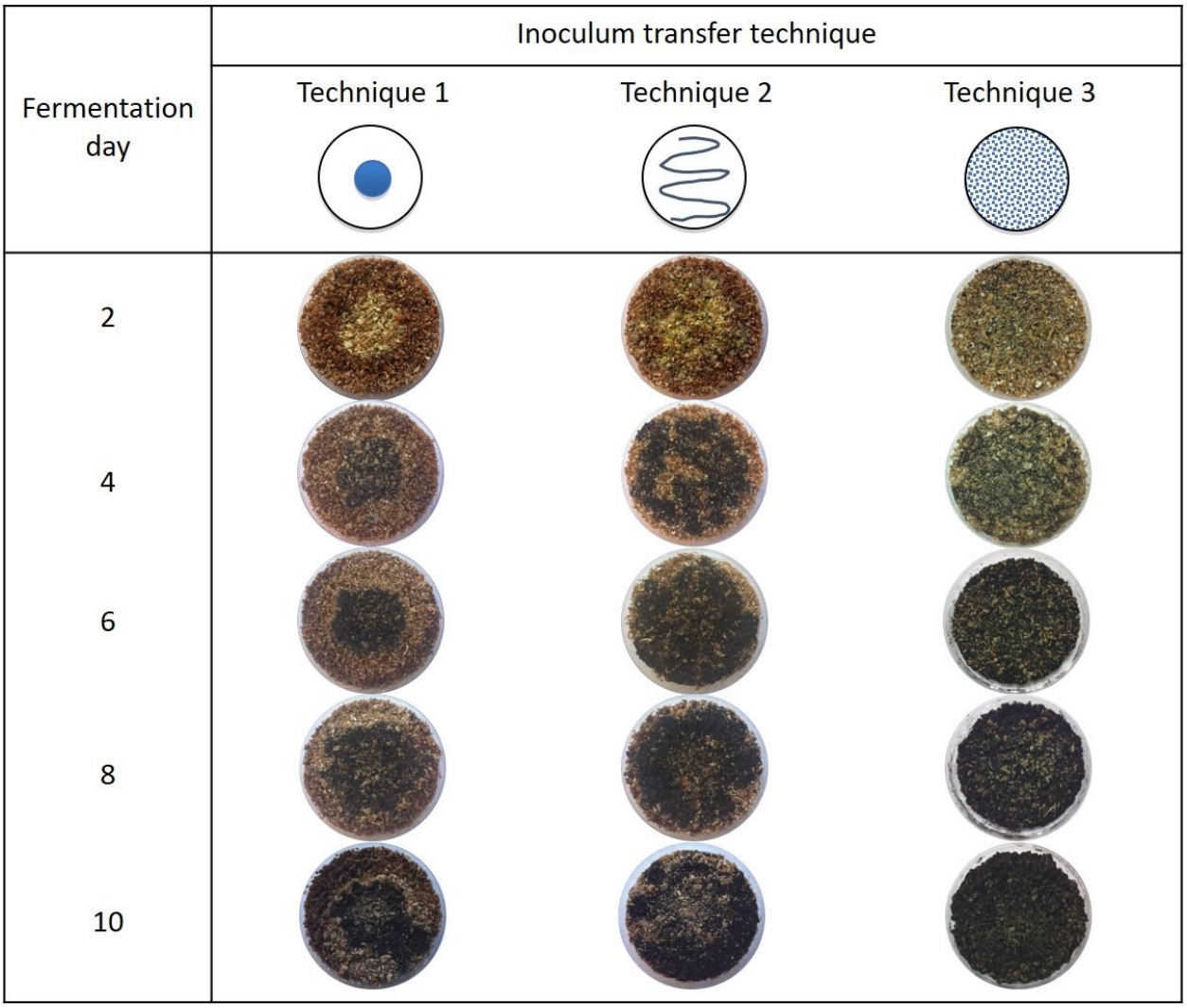


Figure 2. Image of the fermented substrate during fermentation process with three different inoculum transfer techniques.

3. With fermentation using technique 2, almost 50% of solid substrate was consumed and at the end of fermentation, there was solid substrate not utilized. Technique 1 promoted poor growth (33%) and growth was observed in the center and occurred a limited distance from the original inoculation. However, as eloquently stated by Soccol et al. [21], nutrients supplementation is needed in order to provide all necessary nutrient for optimum growth.

In SSF, fungi can sometimes grow well and produce larger amounts of targeted products. Complex fungal growth phenomena observed in SSF are associated with solid substrates that are typically heterogeneous and porous with void spaces [22]. The mycelium of fungal growth gives the filamentous fungi the ability to penetrate into solid substrates; however, this process can be influenced by many factors. These include the physical characteristics of the solid substrate, such as particle size and moisture content, which lead to the porosity of the fermented bed.

Production of spores

As expected, the production of spores was elevated in technique 3, followed by technique 2 and technique 1 (Figure 4). Spores count increased about twelve-fold when SSF was carried using technique 3 during the inoculum

transfer. Prior to starting the work, this study showed the importance of considering the inoculum transfer technique in assessing the productivity of any culture. This might determine the success of the SSF process.

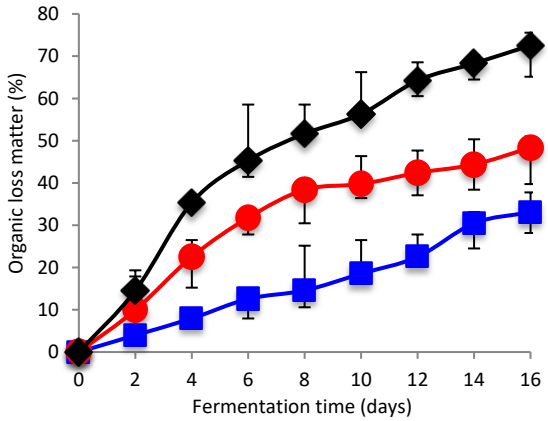


Figure 3. Comparison of growth profiles between inoculum transfer techniques into solid substrate based on organic matter loss. (■) : technique 1; (●): technique 2 and (◆): technique 3.

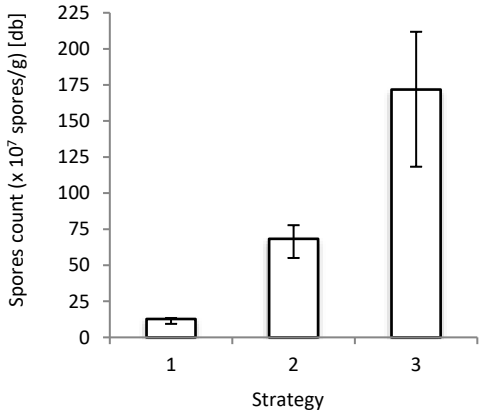


Figure 4. Spores count profile from fermented wheat bran with different techniques of inoculum transfer (at day 10).

Production of enzymes

This work also presents the SSF process of wheat bran using *A. awamori* for the production of an enzymatic consortium containing glucoamylase, protease, cellulase, and xylanase for each technique performed. Activity levels for glucoamylase that was produced for each technique are shown in figure 5A. Glucoamylase activity levels of 81.3, 148.29, and 301.23 U/g [db] were obtained after day 10 of fermentation time in technique 1, 2, and 3, respectively. Glucoamylase activity levels were higher when SSF was carried out with technique 3. However, almost a 4-fold glucoamylase activity decrease was recorded for with technique 1 compared to the technique 3. Protease activity levels measured in samples in figure 5B. Protease activity levels of 57.75, 104.71, and 170.32 U/g [db] were obtained after day 10 of fermentation time in technique 1, 2, and 3, respectively. Protease activity increased about three-fold when SSF was carried out with technique 3 compared to technique 1.

Activity levels for xylanase extracted in every studied technique with *A. awamori* is shown in figure 5C. Maximum xylanase activity levels of 119.72, 389.65, and 686.17 U/g [db] were obtained after 10 days of fermentation time in technique 1, 2, and 3, respectively. Xylanase production was 6 times higher with technique 3 compared to technique 1 and 2. Activity levels of cellulase involved in every technique are shown in figure 5D. Maximum filter paper activity levels of 4.33, 10.19, and 16.99 FPU/g [db] were obtained after 10 days of fermentation time in technique 1, 2, and 3, respectively. Cellulase production was 4 times higher with technique 3 compared to technique 1.

These results proved that technique 3 is an excellent fungus for enzyme production. This was attributed to the efficient distribution of inoculum prior to start the process. Spores was homogenously distributed among the solid particles substrate and assumed that it was covered in every single part in petri dishes. Same observation in spore's production, technique 3 proved to be more favourable for enzymes production. Improvement of spores' distribution in the system might be a reason explaining this behaviour. Production of all enzymes seemed very poor with technique 1. We conclude that with proper distribution inoculum to start SSF given an advantage to shorten the fermentation process. The longer fermentation time needed for the technique 1 and 2 for fungus to convert all the substrate to produce spores or enzymes

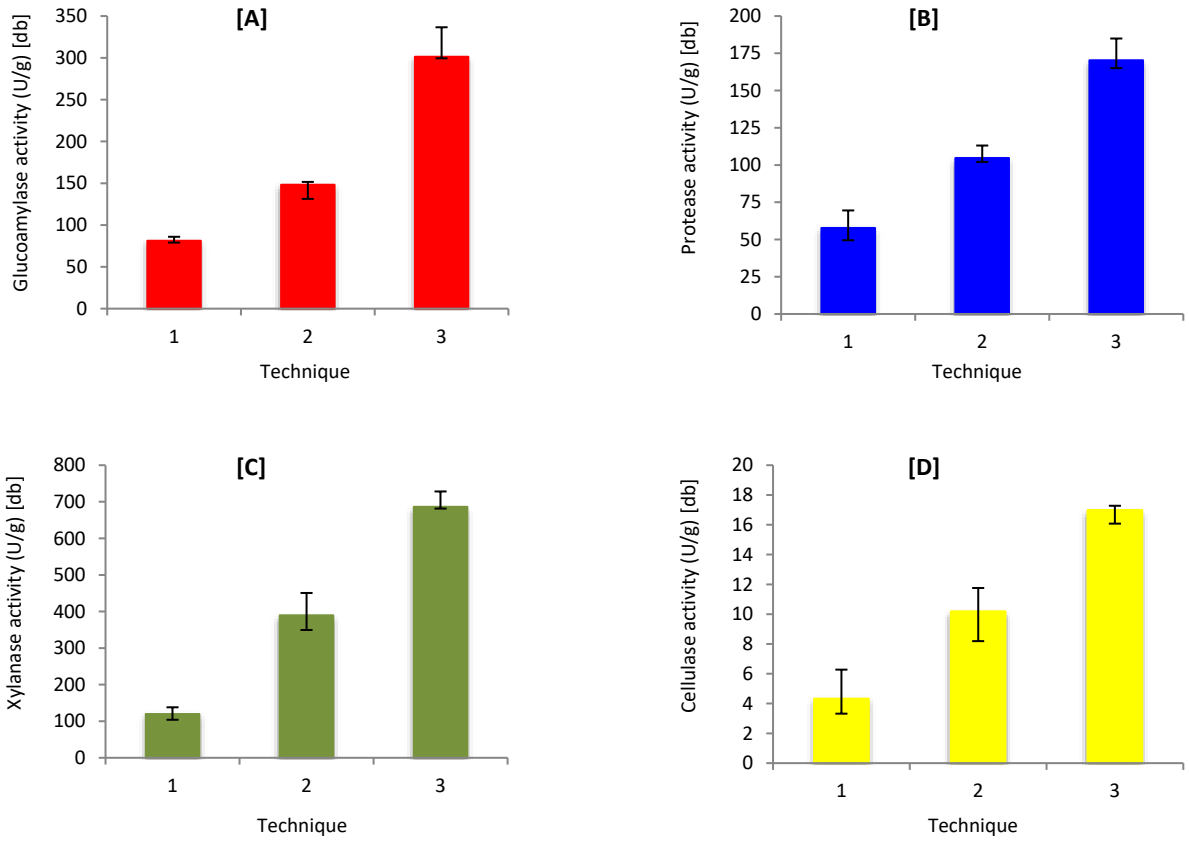


Figure 5. Activity of enzymes produced by the fungal culture of *A. awamori* with different techniques of inoculum transfer at day 10. [A]: glucoamylase activity; [B]: protease activity; [C]: xylanase activity and [D]: cellulose activity.

(or other targeted products). As studied by Ang et al. [23], the preparation of fungal inoculum used Cellophane Film Culture (CFC) technique provides an accurate estimation of fungal growth and the presence of laccase enzyme activity in SSF of rice husk.

In SSF, the inoculum process definitely will involve labor intensive due to the necessity to make homogenous conditions especially in large scale. Indeed, this will give a major advantage for the colonization of the substrate and the utilization of the available nutrients as shown in figure 6. The mycelium penetrates deeply into the inner solid substrate particles of wheat bran. The deep penetrating clean white mycelium and binding together the solid substrate, covered almost every part of the fermented bed and produced a compact fermented cake. Gutara et al. [24] developed strategies by using fermented cake of babassu cake as inoculum whereby increased production 1.5-fold with 10 times fewer spores than in conventional inoculation, indicating that fermented solids are an interesting alternative for inoculum development in SSF, mainly for large-scale processes. This also was supported by Kragh et al. [25] how important the inoculation method that could give huge impact the outcome of microbiological experiments in liquid cultures.

Preparation of the solid substrate and subsequent inoculation are very important because the initial conditions strongly affect the entire SSF process [10]. Processing steps in inoculum transfer must be taken into account to obtain satisfying of fungal growth. Generally, this standard procedure applies in small-scale

Figure 6. Images of fermented wheat bran for technique 3 of inoculum transfer. (a) image taken from the top of the surface of fermented solid; (b) image taken from the bottom of the petri dish and (C) cross section pieces of solid fermented cake.

laboratory work. In large scale, small reactor with mixing device can be built upon the inoculation process under sterile conditions. Roussos et al. [26] have proved through Zymotis, dedicated solid state bioreactor for inoculum development which can produced five times higher production of spores as compared to the agar medium in flask, constitutes a success in the development of large scale inoculum. Another strategy reported by Cunha et al. [27] by proposed an unconventional pre-culture with an initial fungal growth phase under SSF, and then followed by transition to submerged fermentation by adding the liquid culture medium to the mycelium grown on solid substrate. The technique proposed improved an approximately 3-fold improvement in endoglucanase productivity compared to conventional submerged fermentation. Here we show that the technique of inoculation of fungus cultures has profound effect on the fungal growth observed, including spores and enzymes production.

Conclusions

Our results stress the importance of consistency in development and establishment of standard procedures for inoculum transfer into solid substrate for homogenous distribution to start the SSF process. Kragh et al. [25] stated, “the importance of inoculation consistency throughout experiments and the substantial impact aggregate development in liquid batch cultures may have on the outcomes of microbiological experiments”. This idea reflects to the solid state batch culture. In conclusion, we have discovered a connection between the technique of inoculation of fungus spores and the degree of distribution and homogeneity within those solid particles, thereby can affect the outcomes of experiments. Prior to starting the work, this study showed the importance of considering the inoculum transfer technique in assessing the productivity of any culture. A proper transfer technique was developed to achieve favorable distribution and homogeneity of inoculum within the fermented solid substrate. Technique 3 will be standard procedures for inoculum transfers for the future study. For scaling up, small reactors equipped with mixing devices or machinery to produce efficient homogenization. This factor might determine the success of the SSF process.

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