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PHYSIOLOGICAL PROPERTIES AND ENZYMATIC ACTIVITIES OF *RHIZOPUS OLIGOSPORUS* IN SOLID STATE FERMENTATIONS

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ABSTRACT

The physiology of the fungus *Rhizopus oligosporus* in the solid state fermentation of pea seeds was investigated by means of digital analysis of microscopic images. The correlations between the hyphal fractions within physiological zones, the release of glucose and soluble proteins, and enzymatic activities of the examined strain were also estimated.

Key words: digital image analysis, *Rhizopus oligosporus,* SSF

INTRODUCTION

Tempe is the traditional, fermented Indonesian food, produced from soybean by the filamentous fungus *R. oligosporus*. Apart from the soybean, also other leguminous plants, such as pea, bean, and mixtures of leguminous plants and corn, are applied for tempe production. *R. oligosporus* is a GRAS-organism (generally regarded as safe). This fungus grows rapidly at 34-45°C, and penetrates the bed, thus giving the uniform cake of bound beans. Like other filamentous fungi, it forms various morphological forms, and furthermore, not all hyphae are equally active during the culture. The studies of effects of various morphological forms of hyphae on biochemical properties of the fungus and product synthesis dynamics were facilitated by digital image analysis of microscopic images. Estimation of hyphal fractions, enable their correlation with other process and physiological parameters, such as culture medium composition and enzymatic activity of the fungus. However,

such analysis was done exclusively for the submerged cultures [1] and no data on solid state fermentation have been reported.

During tempe fermentation *R. oligosporus* synthesizes various enzymes, which hydrolyse the raw material and change its texture, taste and aroma. This process also reduces or even eliminates the anti-nutritive components in the fermented product. *R. oligosporus* synthesises the enzymes, that hydrolyse lipids, polysaccharides and proteins. It gives rise to partial liquefaction of the fermented material [4].

The presented work focused on changes in the physiology of *R. oligosporus* during the solid state fermentation, observed by digital image analysis, and finding the correlations between the alterations in concentration of selected bed components and the biochemical activity of the fungus.

MATERIALS AND METHODS

Production of tempe

The seeds of pea (Agra cultivar, harvested in 2001, UNGRET) were soaked in 0.85% lactic acid solution (1:3 w/v) all night at room temperature. Then they were manually dehulled, autoclaved and inoculated with spore suspension (10^6/ml, 1% v/w). Finally, the mixture was transferred to Petri dishes and incubated at 37°C for 72 hours.

Analytical techniques

The samples of *R. oligosporus* mycelium were obtained from the raw material of the bed, hyphae were suspended in 0.2% aqueous Tween 20 solution (10 ml per 1 g of tempe), homogenized (speed 2 for 15 sec, Omni Mixer Homogenizer), 5 fold diluted in distilled water, filtered through 1.2 µm filter (47 mm in diameter), suspended in 5 ml of 0.8% Tween 20 (pH 7.0), stained with methylene blue and observed under the light microscope OLYMPUS BX-40 with phase contrast (x200 magnification). The digital camera was mounted on the microscope and connected to the PC computer, equipped with digital image analysis software (MicroImage 4.0, Media Cybernetics for OLYMPUS). For each experimental point, at least 25 images of each hyphae sample were taken and subjected to analysis. The images were filtered by median and high-pass Gauss filter. Then they were segmented in the HSI colour model and finally the projected areas of the zones were calculated. The zone fraction was computed as the weighed mean of ratios of projected area of the given zone to the total projected area of mycelium. Different colours of filaments were obtained dependenting on the activity of mycelium. They ranged from white for apical and growing cells (zone “B”), through violet (zone “A”) and blue (zone “C”) for poorly active cells, to completely inactive black cells (zone “D”).

Spores were harvested from 1 g of fermented pea with 9 ml of 0.1% (w/v) Tween 80. A direct visual method was used for spore counting by using a counting chamber [8].

Enzymatic activities were assayed by using standard biochemical methods. They were expressed in activity units per gram of the product. One activity unit of the given enzyme refers to the number of released nanomoles of: fatty acids (from sunflower oil) for lipases, maltose (from soluble starch) for amylases, glucose (from salicine) for beta-glucosidase, glucose (from carboxymethylcelulose, CMC) for endoglucanase, xylose (from oat xylane) for xylanase, and tyrosine (from casein) for proteinases. The assays were done at optimum pH and temperature of each enzyme. Duplicate samples were prepared for each assay.

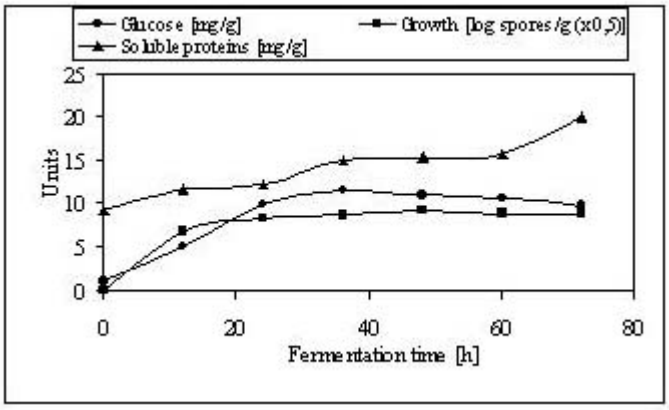
RESULTS

The solid state fermentation of *R. oligosporus* was carried out in the bed composed exclusively of pea seeds. This raw material contains approximately 21.6% proteins, 2.4% soluble saccharides, 47.6% starch, 16.7% cellulose, and 1.3% lipids.

Changes in the selected parameters during tempe fermentation

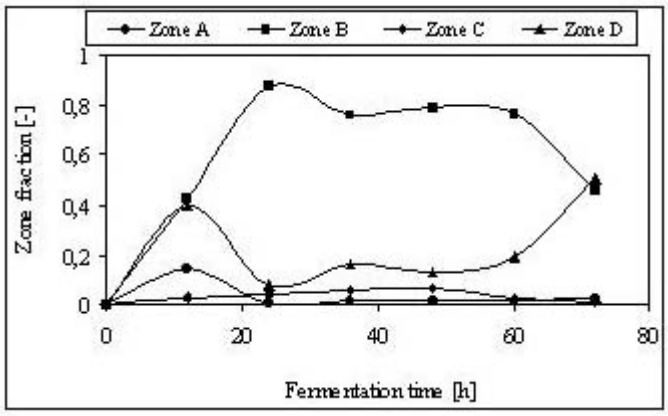
The fermentation process can be divided into three phases (Fig. 1). Glucose concentration continues to increase in the first and second phases of fungal growth. In the third phase, the autolysis of hyphae occurs. It is assisted by a decrease in glucose concentration (from 11 to 9.7 mg/g). Simultaneously, a significant increase in soluble proteins is observed (twice as high as in the beginning of the process).

Fig. 1. Changes in the selected process parameters during *R. oligosporus* solid state fermentation



In Fig. 2 the changes in hyphal fractions within the physiological zones are shown. Within the initial 24 h of the process, the linear growth of zone "B" (most-active apical cells) is observed. This growth is strictly correlated with the maximum rate of glucose release. Later in the process (till 48 h) the high but constant contribution of zone "B" is present in the system, and at the same time, the contribution of the zone "D" (inactive cells) is low. It proves that the growth of the fungus is balanced. At the end of the process, zone "D" forms more than half of all hyphae, and it is the proof of the biomass senescence. The result of hyphae autolysis was the secretion of proteinases to the bed, and the increase in soluble proteins concentration. The participation of zones "A" and "C" was practically negligible during the whole process.

Fig. 2. Changes in physiological zones fractions



Biosynthesis of enzymes during tempe fermentation

The qualitative analysis of *R. oligosporus* enzymes was done using the APIzym test. The activity of three enzymes, alkaline and acid phosphatase, and naphtol-AS-BI phosphohydrolase, was the highest (> 40 nmoles) during the whole process. After 24 hours of fermentation, the activities of esterase C4 and leucine arylamidase, lipase-esterase and valine arylamidase (activities of 30 and 20 nmoles, respectively), and a little lipase C14 (activity of 10 nmoles), were detected. In the second phase of the prolcess, the activities of the enzymes were as follows: beta-glucosidase - 30 nmoles, N-acetyl-beta-glucosaminidase - 20 nmoles, alfa-mannosidase – 10 nmoles, alfa-galactosidase – 3 nmoles.

Also the dynamics of biosynthesis of enzymes degrading polysaccharides, lipids and proteins, were investigated (Fig. 3 and 4). In the first phase of growth, the fungus synthesises lipase, the yield of which peaks at t=12 hours. Alfa-amylase activity reaches at maximum after 36 h and xylanase, endoglucanase and beta-glucosidase are synthesised with maximum yield after 48 h. The maximum glucose release rate correlates with the activity of these enzymes (apart from xylanase). In the last phase of the process, a fast increase in two acid proteases was found (optimum pH of these enzymes is 3.0 and 5.5, respectively). It correlates with soluble proteins release and increase in the fraction inactive zone "D". The secretion of acidic proteinases is crucial for the production process of high-quality tempe.

Fig. 3. Biosynthesis of polysaccharides-degrading enzymes

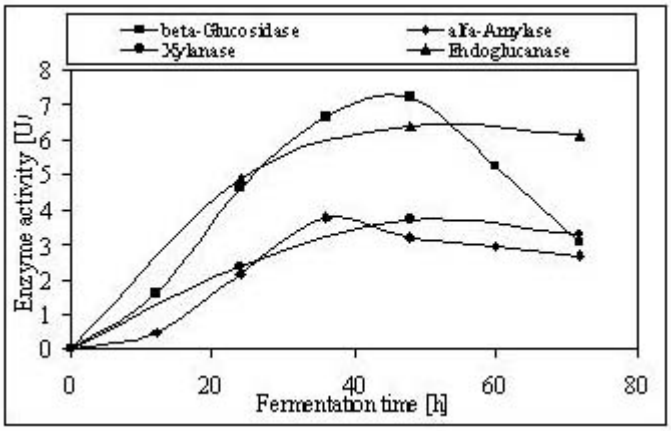
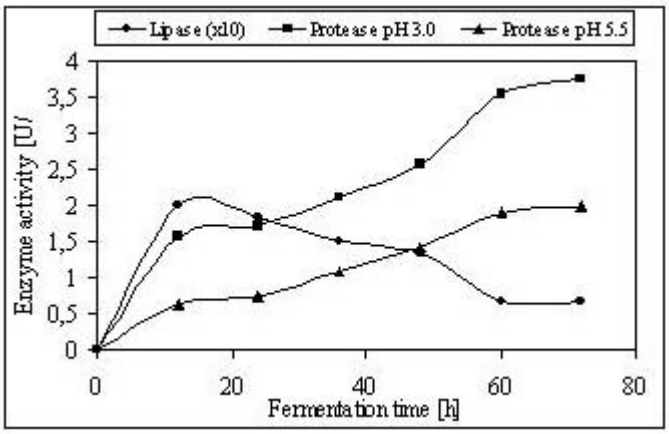


Fig. 4. Biosynthesis of enzymes degrading proteins and lipids



DISCUSSION

Due to a specific character of growth of filamentous fungi that form diverse morphological forms during cultivation, studies on their growth dynamics and the relationship between the morphological form and its biochemical properties are difficult. The most suitable method appeared to be the digital analysis of microscopic images, providing information on morphology and physiology of viable microorganisms based on microscopic observations [2, 7]. Investigations of the physiological state of microorganisms that can be done exclusively by using the appropriate methods of simple and differentiating staining, followed by the digital analysis of color images [2, 7, 9] yield numerous significant data on behavior of cells during fermentation. The percentage of zones of growth, and vacuole-containing and degenerated areas in the hyphae can be determined by using this method. Monitoring of changes in these fractions that are noticeable only by using this method, enables construction of models presenting variations in morphology and structure of the hyphae [1, 3, 6], and optimalization of fermentation processes conducted by filamentous fungi [2].

According to earlier reports, these techniques have been tapped exclusively for monitoring of submerged cultures of filamentous fungi [2, 6]. Therefore our studies on application of this method for characterization of the hyphae harvested from solid state cultures are unique. Preparration of the sample of hyphae in this case is more difficult because the fungus penetrates the substrate, and the residues of solid medium should be carefully removed from hyphae by washing prior to microscopic studies, to provide the satisfactory quality of the images. Methylene blue staining has been chosen because it is widely applied in studies on morphology and physiology of microorganisms [2, 5]. This method enabled observation of four physiological areas, designated as A, B, C, and D, within the *Rhizopus oligosporus* hyphae [1, 9]. The percentage of active (B) zones reached 80% in the initial period of fermentation. A gradual increase in the content of zone D demonstrated that the end of the process was achieved and the hyphae continued to degenerate. The percentage of zones A and C being intermediate stages between phases B and D was relatively low. Thus the control of hyphal physiology by using the digital analysis of microscopic images throughout the fermentation provides the accurate characterization of the process, and enables correlation between the physiological state of *Rhizopus oligosporus* and the dynamics of enzyme biosynthesis.

CONCLUSIONS

The digital image analysis of *R. oligosporus* hyphae from solid state fermentation, facilitates fast and accurate investigation of their physiological state. The changes in hyphal fractions within physiological zones correlate well with the trend of glucose release, and the increase in soluble proteins concentration at the end of the process. The multiphase character of these changes is strictly dependent on the enzymatic activities of the examined strain.

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