Physiological and Enzymatic Activities of *Rhizopus oligosporus* in Swing Solid-State Reactor*

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Dedicated to the 80th birthday of Professor Elemír Kossaczký

Hydrated, acidified, dehulled, and autoclaved *Pisum sativum* seeds were fermented with *Rhizopus oligosporus* NRRL 2710. Solid-state fermentations were carried out in a SWiNG-Solid-State-Reactor S-10-F and in Petri dishes. Effects of loading rates (23 %, 46 %, 58 %, and 70 %) and aeration intensity (5.0 dm^3 min^{-1} kg^{-1}, 7.5 dm^3 min^{-1} kg^{-1}, and 10.0 dm^3 min^{-1} kg^{-1}) on morphological and physiological characteristics of *R. oligosporus* NRRL 2710 and activity of its selected extracellular hydrolases were determined. Loading rate of 58 % and aeration of 10 dm^3 min^{-1} kg^{-1} provided the highest physiological activity of the fungus and most intensive secretion of α-amylase, cellulase, lipase, and proteases.

Solid-state fermentation (SSF) is frequently applied for manufacturing of high value added, healthy food [1]. Tempe, the traditional Indonesian fermented dish is produced from soybean or other leguminous seeds, such as pea, bean, and mixtures of leguminous and corn seeds, inoculated with *Rhizopus oligosporus*, which is regarded as a safe microorganism (GRAS) [2]. The fungus covers the seeds and in a relatively short time completely penetrates and binds them into the cake-like product [3].

The most important mycelium function during tempe manufacturing is the synthesis of enzymes, which hydrolyze raw material thus providing the desired texture, taste, and flavour of the final product. *R. oligosporus* secretes hydrolases, which split lipids, polysaccharides, and proteins, and enzymes which digest these ingredients of raw materials that are harmful to humans [4].

Physiological activity of filamentous fungi depends on the morphological form and the growth phase [5]. Study of the effect of hyphal metamorphosis on the dynamics of enzymes and other products synthesis requires application of highly sensitive and accurate methods. Staining of fungal cells with methylene blue, which is reduced by active mitochondria, and the counterstain (carbol solution of basic fuchsine), which renders the cells bright orange, white, greyish orange or dark grey, dependently on their respiratory activity was found to be the fast and accurate method of determination of the physiological state of fungi [6]. It facilitates finding correlation between culture conditions (*e.g.* medium composition, loading rate, parameters of agitation and aeration), physiological activity of mycelium, and synthesis of enzymes. Relevant models have been established for submerged cultures but not for solid-state fermentations [7].

Traditionally, tempe is produced under static culture conditions, on trays with perforated bottom. In the present study, tempe was produced in the SWiNG-Solid-State-Reactor S-10-F (SSSR) under agitated and aerated culture conditions, using *Pisum sativum* seeds inoculated with *R. oligosporus* NRRL 2710. The experiments focused on effects of loading rate and aeration intensity on morphology, physiology, and biochemical activity of *R. oligosporus*.

EXPERIMENTAL

Pisum sativum var. Agra seeds were soaked in 0.85 % lactic acid solution (300 cm^3 per 100 g of seeds) overnight, manually dehulled, autoclaved, and inoculated with $w = 0.01$ % spore suspension (10^6 *R.*

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oligosporus NRRL 2710 spores per cm^3). Fermentation in a SSSR (Tec Bio, Germany) was carried out at 37 ℃ for 72 h. The bed was agitated for 1 min every 3 h. The loading rates were 23 %, 46 %, 58 %, and 70 % of the bioreactor working volume and the air volumetric flow rates of 5.0 dm^3 min^{-1}, 7.5 dm^3 min^{-1}, and 10.0 dm^3 min^{-1} per kg of the cotyledons were used.

Samples of tempe were collected after 24 h, 48 h, and 72 h of the culture and gently suspended (2 rotations per 15 s, to avoid fragmentation of hyphae) in 0.2 % aqueous Tween 20 solution (10 cm^3 per 1 g of tempe) using Omni Mixer Homogeniser. This suspension was 5-fold diluted in distilled water and filtered through 1.2 μm filter (47 mm in diameter). The hyphae retained on the filter were suspended in 5 cm^3 of 0.8 % Tween 20 (pH = 7.0) and stained with methylene blue [8]. Microscopic observations were conducted using an Olympus BX-40 light microscope under a magnification of 200 with phase contrast. The microscope was coupled with a camera and a computer equipped with Micro Image 4.0 software. Methylene blue staining (without counterstaining with basic fuchsine) enabled observation of four physiological areas designated as A, B, C, and D, within the *R. oligosporus* NRRL 2710 hyphae [8—10]. The colour of zones B (apical, growing cells), A and C (less active cells), and D (inactive cells) was white, violet, blue, and black, respectively. Approximately 25 images of hyphal entities were acquired during the whole process. These images were processed by median and high-pass Gauss filter. After their segmentation, the zone fractions (the arithmetical mean taken from 25 objects) corresponding to four different physiological states of *R. oligosporus* hyphae were determined. The zone fraction is the ratio of the zone area to total hyphal area.

Activity of *R. oligosporus* NRRL 2710 enzymes (E.A.) was assayed in aqueous extracts of tempe by using the relevant standard methods and expressed in units per g of tempe. 10 cm^3 of distilled water was stirred in a mortar with 1 g of tempe. After incubation at 4 ℃ for 30 min the suspension was centrifuged (11 000 min^{-1}, 4 ℃, 30 min) and the supernatant was used for analysis. One activity unit (U) denoted 1 μmol of product liberated in 1 min from the substrate. The substrates for determination of activity of lipases, α-amylase, cellulase, and proteases were sunflower oil (40 % emulsion in 0.1 M-sodium phosphate buffer, pH = 7.0), soluble starch (1 % gel in 0.1 M-sodium acetate buffer, pH = 4.8), carboxymethylcellulose (0.5 % gel in 0.1 M-sodium acetate buffer, pH = 4.8), and casein (1 % colloidal solutions in 0.1 M-sodium acetate buffers, pH = 3.0 and 5.5), respectively. The assays of glycoside hydrolases were carried out at 50 ℃, and lipase and proteases were determined at 40 ℃. Fatty acids liberated by lipase were determined by titration with 5 mM-NaOH [4]. Reducing sugars released by α-amylase and cellulase were assayed with alkaline DNS [11]. The activity of proteases was estimated according to the method of *Anson* [12].

For comparison purposes, stationary cultures of *R. oligosporus* NRRL 2710 in Petri dishes (50 mm in diameter, 30 g of *P. sativum* cotyledons, which were inoculated with 0.3 cm^3 of spore suspension) were carried out. Samples of tempe were collected after 24 h, 48 h, and 72 h and analyzed as described above.

Fermentations in SSSR and on Petri dishes were carried out in triplicate. Triplicate samples of tempe were prepared for each determination listed above, and the Student *t*-test was applied for evaluating of the data distribution. Experimental results are presented as mean ± standard deviation.

RESULTS AND DISCUSSION

Enzymatic Activity of *R. oligosporus* NRRL 2710

The results of assays of lipase, α-amylase, cellulase, and proteases activity in samples of tempe obtained by fermentations in SSSR (for all examined loading rates and aeration conditions) are collected in Table 1. Table 2 presents results of determined enzymatic activity in samples of tempe derived by stationary cultures of *R. oligosporus* NRRL 2710 on Petri dishes.

Both the loading rate and the aeration intensity affected dynamics of synthesis of hydrolases by *R. oligosporus* grown in SSSR (Table 1, Fig. 1). The highest activity of amylases and proteases (with optimum pH for activity of 5.5) was observed on the third day of fermentation for the loading rate of 58 % and the air volumetric flow rate of 10.0 dm^3 min^{-1} kg^{-1} ((45.4 ± 0.9) U g^{-1} and (4.43 ± 0.13) U g^{-1}, respectively). The highest activity of lipases ((23.3 ± 1.0) U g^{-1}) was

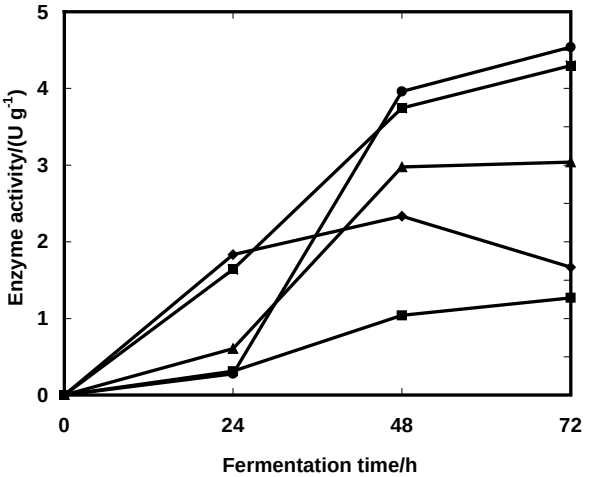

Fig. 1. E.A.(Biosynthesis of lipase) × 10/(U g^{-1}) (◆), E.A. (cellulase) × 10^{-2}/(U g^{-1}) (■), E.A.(α-amylase) (●), E.A.(protease at pH 3.0) (▲), E.A.(protease at pH 5.5) (■) during the fermentation in the SSSR.

Table 1. Activity of *R. oligosporus* Enzymes during the Fermentation in the SSSR

t/h	Loading rate/% 23			46			58			70	
	Aeration rate/(dm^3 min^{-1} kg^{-1}) 5.0	7.5	10.0	5.0	7.5	10.0	5.0	7.5	10.0	5.0	7.5
	Lipase activity/(U g^{-1})										
24	11.7 ± 0.9	13.3 ± 0.8	13.3 ± 0.3	18.3 ± 0.9	15.8 ± 0.4	4.2 ± 0.1	16.7 ± 1.0	13.3 ± 0.8	13.3 ± 0.7	23.0 ± 1.2	20.0 ± 1.2
48	5.0 ± 0.3	18.3 ± 0.4	20.0 ± 0.9	23.3 ± 1.0	22.9 ± 0.9	6.7 ± 0.4	8.3 ± 0.1	5.0 ± 0.7	11.7 ± 0.7	13.3 ± 0.9	11.7 ± 0.9
72	3.3 ± 0.1	15.0 ± 0.5	15.0 ± 0.2	16.7 ± 0.6	17.4 ± 0.4	10.0 ± 0.7	3.3 ± 0.2	1.7 ± 0.2	5.0 ± 0.9	6.7 ± 0.3	5.0 ± 0.4
	Cellulase activity/(U g^{-1})										
24	0.09 ± 0.003	0.03 ± 0.005	0.03 ± 0.004	0.03 ± 0.001	0.02 ± 0.002	0.01 ± 0.002	0.18 ± 0.003	0.06 ± 0.001	0.17 ± 0.02	0.23 ± 0.08	0.21 ± 0.07
48	0.06 ± 0.004	0.04 ± 0.001	0.04 ± 0.002	0.10 ± 0.005	0.08 ± 0.007	0.04 ± 0.006	0.13 ± 0.009	0.05 ± 0.003	0.11 ± 0.01	0.16 ± 0.05	0.15 ± 0.05
72	0.07 ± 0.002	0.06 ± 0.002	0.07 ± 0.009	0.13 ± 0.008	0.09 ± 0.002	0.06 ± 0.007	0.13 ± 0.01	0.05 ± 0.005	0.11 ± 0.03	0.15 ± 0.04	0.13 ± 0.04
	Amylase activity/(U g^{-1})										
24	3.1 ± 0.2	1.6 ± 0.01	2.3 ± 0.3	2.8 ± 0.4	4.2 ± 0.4	2.2 ± 0.3	5.9 ± 0.3	2.9 ± 0.1	3.8 ± 0.2	7.5 ± 0.5	4.6 ± 0.4
48	5.2 ± 0.4	17.8 ± 0.21	31.1 ± 1.2	39.6 ± 1.0	35.3 ± 1.2	14.8 ± 0.9	8.2 ± 0.9	4.3 ± 0.3	15.2 ± 0.9	20.0 ± 1.1	16.7 ± 0.8
72	3.1 ± 0.1	39.6 ± 1.3	41.7 ± 1.3	45.4 ± 0.9	43.5 ± 1.3	21.0 ± 1.1	4.7 ± 0.3	2.7 ± 0.4	10.8 ± 0.7	16.6 ± 0.8	13.3 ± 0.6
	Protease activity at pH = 3.0/(U g^{-1})										
24	0.71 ± 0.07	0.44 ± 0.05	0.51 ± 0.08	0.61 ± 0.09	0.62 ± 0.10	0.67 ± 0.08	0.92 ± 0.07	0.58 ± 0.04	1.54 ± 0.09	2.35 ± 0.09	1.99 ± 0.17
48	1.32 ± 0.09	1.53 ± 0.11	1.56 ± 0.19	2.97 ± 0.11	1.76 ± 0.17	1.65 ± 0.19	1.62 ± 0.09	0.91 ± 0.12	2.20 ± 0.23	3.71 ± 0.34	2.83 ± 0.24
72	1.83 ± 0.11	2.39 ± 0.32	2.78 ± 0.21	3.46 ± 0.20	3.04 ± 0.23	2.76 ± 0.21	2.33 ± 0.31	1.06 ± 0.08	2.86 ± 0.21	4.40 ± 0.29	3.12 ± 0.28
	Protease activity at pH = 5.5/(U g^{-1})										
24	0.10 ± 0.01	1.03 ± 0.09	0.99 ± 0.08	1.64 ± 0.12	0.59 ± 0.07	0.54 ± 0.06	0.14 ± 0.02	0.08 ± 0.004	0.96 ± 0.01	1.08 ± 0.12	0.99 ± 0.02
48	0.36 ± 0.08	1.79 ± 0.18	3.2 ± 0.19	3.74 ± 0.26	2.55 ± 0.21	1.63 ± 0.22	0.41 ± 0.07	0.25 ± 0.01	1.38 ± 0.21	1.92 ± 0.22	1.61 ± 0.18
72	0.41 ± 0.04	3.37 ± 0.24	3.82 ± 0.30	4.43 ± 0.13	4.3 ± 0.28	2.65 ± 0.11	0.43 ± 0.05	0.32 ± 0.01	1.98 ± 0.27	2.82 ± 0.31	2.10 ± 0.21

Data are presented as mean values ± SD; n (number of determination) = 3.

Table 2. Activities of *R. oligosporus* Enzymes during the Fermentation on Petri dishes

t/h	Enzyme activity/(U g^{-1})				
	Lipase	Cellulase	Amylase	Protease	
				pH = 3.0	pH = 5.5
24	18.3 ± 1.1	0.26 ± 0.10	6.10 ± 0.52	1.71 ± 0.45	0.72 ± 0.22
48	13.3 ± 0.9	0.18 ± 0.08	8.98 ± 0.11	2.54 ± 0.19	1.42 ± 0.21
72	6.7 ± 0.6	0.15 ± 0.05	7.45 ± 0.48	3.74 ± 0.34	1.97 ± 0.43

Data are presented as mean values ± SD; n (number of determination) = 3.

found on the second day under the same culture conditions. Most intensive biosynthesis of acidic proteases (with optimum pH for activity of 3.0) was observed on the third day for the loading rate of 46 % and aeration intensity of 7.5 dm^3 min^{-1} kg^{-1} ((4.40 ± 0.29) U g^{-1}), but only a slightly lower activity of these enzymes ((3.46 ± 0.20) U g^{-1}) was found for the loading rate of 58 % and aeration of 10 dm^3 min^{-1} kg^{-1}. The activity of cellulases in samples of tempe peaked ((0.23 ± 0.08) U g^{-1}) on the first day of fermentation in the SSSR for loading rate of 46 % and aeration intensity of 7.5 dm^3 min^{-1} kg^{-1}. At conditions providing the relatively high productivity of other hydrolases (72 h of fermentation, loading rate of 58 %, and aeration of 10 dm^3 min^{-1} kg^{-1}) also the most efficient biosynthesis of cellulases occurred ((0.13 ± 0.01) U g^{-1}). Production of examined enzymes *via* stationary cultures of *R. oligosporus* NRRL 2710 on Petri dishes was lower (Table 2, Fig. 2) compared to the results obtained for fermentation in the SSSR with loading rate of 58 % and aeration intensity of 10 dm^3 min^{-1} kg^{-1}. Activity of lipase and cellulase in samples of tempe derived by stationary cultures on Petri dishes peaked on the first day ((18.3 ± 1.1) U g^{-1} and (0.26 ± 0.1) U g^{-1}, respectively) whereas the highest activity of α-amylase ((8.98 ± 0.11) U g^{-1}) was found on the second day, and that of proteases on the third day ((3.74 ± 0.34) U g^{-1} and (1.97 ± 0.43) U g^{-1}). Higher productivity of *R. oligosporus* enzymes during 72 h fermentation of *P. sativum* cotyledons in the SSSR as compared to stationary cultures in Petri dishes implies that proper aeration of tempe stimulates biochemical activity of the fungus.

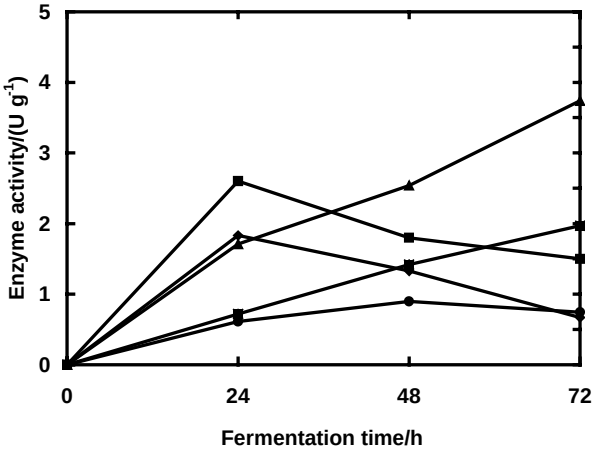

Fig. 2. E.A.(Biosynthesis of lipase) ×10/(U g^{-1}) (◆), E.A. (cellulase) × 10^{-2}/(U g^{-1}) (■), E.A.(α-amylase) (●), E.A.(protease at pH 3.0) (▲), E.A.(protease at pH 5.5) (■) during the fermentation on Petri dishes.

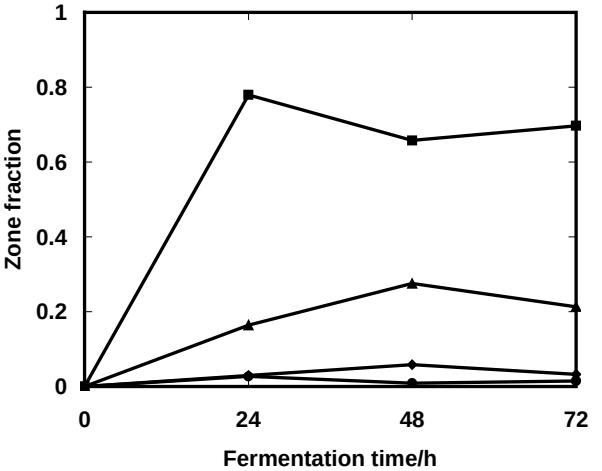

Fig. 3. Changes in zone fractions from "A" to "D" during the fermentation in the SSSR. Weighed mean fraction of the cells: A (●), B (■), C (◆), and D (▲).

Physiological Activity of *R. oligosporus*

Microscopic observations of *R. oligosporus* NRRL 2710 hyphae stained with methylene blue revealed that during the fermentation in the SSSR at optimum conditions (loading rate of 58 % and aeration intensity of 10 dm^3 min^{-1} kg^{-1}), the largest zone B (white cells with the highest physiological activity) fraction was observed on the first day (0.78, Fig. 3). On the second and the third day, this fraction was slightly smaller (0.66 and 0.7, respectively, Fig. 3). The zone D (black cells, inactive or with very weak physiological activity) fraction was concomitantly increased (from 0.16 on the first day to 0.28 and 0.21 on the second and the third day, respectively). Under stationary culture

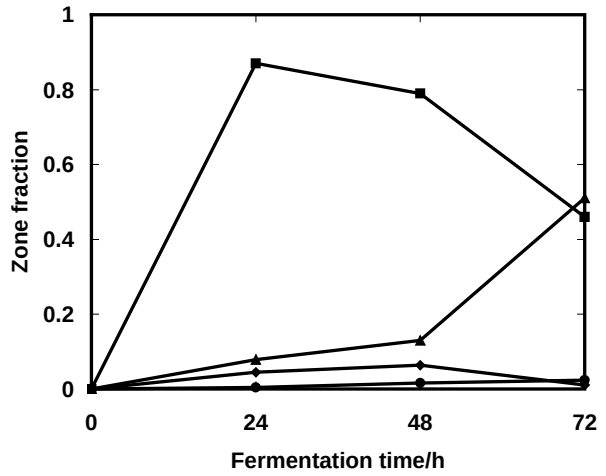

Fig. 4. Changes in zone fractions from "A" to "D" during the fermentation on Petri dishes. Weighed mean fraction of the cells: A (●), B (■), C (◆), and D (▲).

conditions (on Petri dishes) the largest zone B fraction (0.87, Fig. 4) was also found on the first day. On the second and the third day it dropped to 0.79 and 0.46, respectively (Fig. 4). The zone D fraction gradually increased from 0.08 on the first day to 0.13 and 0.51 (Fig. 4) on the second and the third day, respectively. Hence, the physiological activity of *R. oligosporus* NRRL 2710 cells on the third day of fermentation was significantly higher in aerated conditions.

Alterations in zone A (violet cells) fraction were negligible for both the examined culture conditions.

Presented studies revealed that the relatively high content of zone B fraction in *R. oligosporus* NRRL 2710 hyphae derived by fermentation in the SSSR on the second and the third day was correlated with higher yields of synthesis of hydrolases by this fungus as compared to cultures on Petri dishes.

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