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Protoplast fusion of *Rhizopus oryzae* and *Rhizopus microsporus* for enhanced fumaric acid production from glycerol

MONIKA KORDOWSKA-WIATER*, MAGDALENA POLAK-BERECKA, ADAM WAŚKO, ZDZISŁAW TARGOŃSKI

Department of Biotechnology, Human Nutrition and Science of Food Commodities,
University of Life Sciences in Lublin, Lublin, Poland

* Corresponding author: monika.kordowska-wiater@up.lublin.pl

The first three authors have contributed equally to this work

Abstract

Rhizopus oryzae and *Rhizopus microsporus* strains were screened for their ability to produce fumaric acid on glycerol as the sole carbon source in the medium. After seven days of stationary culture, fumaric acid was assayed by HPLC analysis, and maximum concentrations of 0.3% (w/v) and 0.33% (w/v) were recorded. Protoplast fusion was used to improve fumaric acid production. A selective medium for the fusant culture was composed on the basis of biochemical differences between parental strains, as examined using the Biolog FF MicroPlate™ Fungi Identification Test. Double fusion rounds led to a 1.46-fold increase in fumaric acid productivity relative to the parental strains. Individual *Rhizopus* fusants demonstrated a various ability to produce fumaric acid from 2.0% (w/v) of glycerol, with the most effective ones producing from 0.2 to 0.27 $g \cdot g^{-1}$ of this acid. To date, no studies have been carried out to improve fumaric acid production by *Rhizopus* with the use of glycerol as the only carbon source in the medium.

Key words: *Rhizopus*, fumaric acid, protoplast fusion, glycerol

Introduction

Protoplast fusion technology is a type of genetic modification by which two protoplasts are fused to form a hybrid cell. This technique has a great potential for genetic manipulations, particularly for obtaining industrially useful microorganisms, and is also an important approach in the strain improvement program. It involves digestion of the cell wall by carbohydrase enzymes (glucanase and chitinase) and transfer of genetic material to the host strain. The various factors affecting protoplast formation include slant age, inoculum age, contact time, level of lytic enzymes, mycelium level, temperature, buffer type, and osmotic stabilizers (Anjani Kumari et al., 1994; Reddivari et al., 2003). The isolated protoplasts can be induced to fuse by mild electric stimulation (electrofusion) or chemicals such as sodium nitrate, polyethylene glycol (PEG), and calcium ions. Chemical fusogens cause the isolated protoplasts to adhere to one another and lead to tight agglutination followed by protoplast fusion (Jogdand, 2001; Pasha et al., 2007). Production and regeneration of protoplasts is a useful technique for fungal transformations. Sawicka-Żukowska et al. (2004), for example, performed a protoplast fusion between auxotrophic mutants of the filamentous fungus *Rhizopus cohnii* and obtained a fusant with approximately 3.5 times higher lipase activity than that of the original strain. Similarly, in another study protoplast fusion between different strains of *Trichoderma reesei*, resulted in more than a two-fold increase in the extracellular carboxymethyl cellulase activity for the resulting fusants (Prabavathy et al., 2006).

Rhizopus sp. is a filamentous fungus known for its ability to produce sustainable platform chemicals. One of the main metabolites produced by *Rhizopus* is fumaric acid. It is currently used in the food industry as a pH adjuster, preservative, and flavor enhancer. It can also be used for the production of polyester and alkyd resins. So far the main substrates used for biotechnological conversion have been glucose, molasses, cassava bagasse, wood hydrolysates and corn straw (Roa Engel et al., 2008; Xu et al., 2010; Ferreira et al., 2012). Fumaric acid production by *Rhizopus* can be improved by different metabolic engineering techniques such as random mutagenesis, transformation, gene knockout (Meussen et al., 2012) and protoplast fusion.

Crude glycerol is the main biodiesel waste stream. Yang et al. (2012) predict production of approximately 18.5 million tons of this byproduct by year 2016. Its biotechnological utilization has been substantially developed during the last few years and various microbial metabolites e.g. industrially important acids (citric, fumaric, malic, oxalic and succinic), 1,3-propanediol, erythritol, alcohols, fungal proteins and enzymes (lipase, phytase) have been produced (Nicol et al., 2012; Yang et al., 2012). Some strains of *Rhizopus* can produce fumaric acid from glycerol but with poor yield.

The goal of the present work was to isolate protoplasts from *Rhizopus* strains and carry out protoplast fusion to enhance fumaric acid production from glycerol in fusant progenies.

Materials and methods

Fungal strains and growth conditions

Rhizopus oryzae strain R15 (NBRC 4756) and *Rhizopus microsporus* strain R67 isolated from rye were maintained on potato-dextrose agar (PDA) slants at 4 °C. These strains were chosen from 90 screened isolates (data not shown), on the basis of their ability to produce relatively high yields of fumaric acid from glycerol.

The following media were used in the experiments. Medium for *Rhizopus* cultivation: KH_2PO_4 0.2% (w/v), $(NH_4)_2SO_4$ 0.14% (w/v), $MgSO_4 \times 7H_2O$ 0.05% (w/v), $CaCl_2$ 0.03% (w/v), yeast extract 0.1% (w/v) microelements solution ($FeSO_4 \times 7H_2O$, $MnSO_4 \times H_2O$, $ZnSO_4$) 0.05% (v/v), Tween 80 0.1% (v/v), glucose 1.0 or 2.0% (w/v), pH 6.0. Regenerative medium: PDA + 0.6 M sorbitol, pH 6.0. Selective medium: 0.2% (w/v) KH_2PO_4, 0.14% (w/v) $(NH_4)_2SO_4$, 0.05% (w/v) $MgSO_4 \times 7H_2O$, 0.03% (w/v) $CaCl_2$, 0.1% (w/v) yeast extract, 0.05% (v/v) microelements solution ($FeSO_4 \times 7H_2O$; $MnSO_4 \times H_2O$; $ZnSO_4$), 0.1% (v/v) Tween 80, 1.0% (w/v) sugar (sucrose, maltose, L-arabinose), 2.0% (v/v) agar, pH 5.5-5.7. Medium for fumaric acid production: 0.2 % (w/v) KH_2PO_4, 0.14% (w/v) $(NH_4)_2SO_4$, 0.05% (w/v) $MgSO_4 \times 7H_2O$, 0.03% (w/v) $CaCl_2$, 0.1% (w/v) yeast extract, 0.05% (v/v) microelements solution ($FeSO_4 \times 7H_2O$; $MnSO_4 \times H_2O$; $ZnSO_4$), 0.1% (v/v) Tween 80, 2.0% (w/v) glycerol, pH 6.0.

The lytic enzymes used are as follows: Lyticase from *Arthrobacter luteus* (2000 $U \cdot mg^{-1}$, Sigma), Lyzing Enzymes from *Trichoderma harzianum* (Sigma), and Chitinase from *Trichoderma viride* (600 $U \cdot g^{-1}$, Sigma).

Cultivation of Rhizopus to obtain young mycelium

The medium for *Rhizopus* cultivation (100 cm^3) was prepared in 200 cm^3 Erlenmeyer flasks. It was inoculated with a spore suspension and incubated at 32 °C and 180 rpm for 7 h. Every one hour, microscopic preparations were made to observe spore germination and define the time needed for the formation of young mycelium. Observations were made at 600 × magnification, and the hyphae were photographed.

Protoplast formation

The young mycelium of *Rhizopus* was obtained from the culture as described above, 50 cm^3 of hyphae of both strains was filtered through sterile cotton and cellulose membranes. Thereafter, the filter membranes with fungal cells were washed in Petri dishes with sterile 0.1 M phosphate buffer, pH 6, for 20 min on a laboratory shaker at room temperature. Mycelium cells were dispensed into Eppendorf tubes and centrifuged (8000 × g, 10 min). To lyse the cell walls, pellets were suspended in solutions of lytic enzymes (Lyticase 1200 $U \cdot dm^{-3}$, Lysing Enzymes from *T. harzianum* 6 $g \cdot dm^{-3}$, and Chitinase 180 $U \cdot dm^{-3}$) or in an enzyme cocktail in 0.1 M phosphate buffer containing 0.6 M mannitol as an osmotic stabilizer. The mixtures were incubated at room temperature in an orbital shaker at 75 rpm. The contents were examined at 1-h intervals under a light microscope for protoplast formation. After 1 h, the mixtures were centrifuged at 3000 × g for 10 min. The supernatant was discarded, and the protoplasts were suspended in phosphate buffer with the osmotic stabilizer.

Protoplast inactivation

Two methods of protoplast inactivation were studied: chemical and thermal. In the chemical method (ChI) (Zhao et al., 2009), protoplasts were inactivated by a sodium nitrite solution (0.1 M). One cm^3 of this solution and 1 cm^3 acetate buffer (0.1 M, pH 4.6) were mixed and preheated at 31 °C for 5 min. Next, protoplast suspensions were added to that mixture and gently vortexed at 31 °C for 9 min. Na_2HPO_4 solution (0.07 M, pH 8.6) was added to terminate the reaction. One cm^3 of the solution was spread on a Petri dish with the regeneration medium to check protoplast inactivation. The thermal method (TI) involved heating the protoplast suspensions in a stabilizing buffer in a water bath at 60 °C for 5 min. Thereafter, 1 cm^3 of the solution was spread

on a Petri dish with the regeneration medium to check protoplast inactivation.

Protoplast fusion

Inactivated protoplasts and intact protoplasts were mixed in the following combinations: R15 ChI + R67; R15 + R67 ChI; R15 ChI + R67 ChI; R15 TI + R67; R15 + R67 TI; R15 TI + R67 TI. Protoplast suspensions were mixed with a 40% PEG solution and incubated at room temperature for 30 min. Next, an equal volume of STC (1M D-sorbitol, 10 mM Tris-HCl, 10 mM $CaCl_2$,pH7.5) buffer was added, and the mixtures were centrifuged at 3000 × g for 10 min. The protoplasts were suspended in 0.5 cm^3 STC buffer, plated on regeneration agar and incubated for 48 h at 32°C.

Selection

The Biolog FF MicroPlate™ Fungi Identification Test was used to examine the ability of the parental strains to assimilate 95 different carbon sources. The fungi were grown on PDA at 32°C, and the spore suspension in FF Inoculating Fluid was prepared by swabbing the spores from the surface of the agar plate. Then, the optical density was measured (OD_{590}) and adjusted to approximately 0.5. Next, 100 mm^3 of the suspension was pipetted into each well of the FF MicroPlate. Plates were incubated at 30°C for 24-96 h and were then read using a Biolog MicroStation™ Reader. On the basis of the mycelium growth, an appropriate carbon source was chosen for the selective medium for the cultivation of parental strains.

Colonies which grew on the regeneration medium after the fusion were transferred onto the selective medium and incubated for 48 h at 32°C. Their ability to grow was observed, and colonies of isolates were transferred onto PDA slants.

Production of fumaric acid by Rhizopus strains and fusants from glycerol

The production medium (50 cm^3) prepared in 100 cm^3 Erlenmeyer flasks was inoculated with mycelium. Cultures of fusants were incubated at 32°C on an Infors Minitron rotary shaker at 200 rpm for 7 days. After 24 h, the pH of the cultures was measured, and 10% (w/v) solution of $CaCO_3$ was added to obtain pH 5.4. After 7 days, the pH of the cultures was analyzed, and all cultures were treated with a 7.8% solution of H_2SO_4 and sterilized for 15 min at 121°C to restore fumaric acid from calcium fumarate. Then, samples were obtained for the HPLC analysis of the fumaric acid, byproducts and glycerol. Before the analysis, the samples were treated with acetonitrile 1:1 and centrifuged (13 200 × g, 10 min) in order to remove proteins The mycelia after cultivation were rinsed in distilled water and dried at 104°C to constant mass in order to evaluate the production of the fungal biomass from glycerol.

HPLC analysis

A high-performance liquid chromatograph (Gilson) with a UV-Vis detector, a refractive index detector, an automatic injector, and an integrator were used to analyze fumaric acid, byproducts and glycerol concentrations. A Bio-Rad Aminex HPX-87 H column was used at 65°C with the mobile phase of 0.05 M H_2SO_4 and a flow rate of 0.5 $cm^3 \cdot min^{-1}$. The analysis of concentrations of fumaric and lactic acids was performed using UniPoint software (Gilson, France). Concentrations of glycerol were determined using Chromax 2007 software (Pol-lab, Poland).

Results and discussion

In the first stage of the study, two strains of *Rhizopus*, R-15 and R-67, were cultivated to assess the time needed for spores to germinate and form a young mycelium susceptible to the lytic enzymes. Microscopic observations of strains R-15 and R-67 showed that spores started to germinate after 4 h and 5 h, respectively, and long germ tubes were observed in the medium after 6 h and 7 h, respectively (Figs 1 and 2). Consequently, these incubation periods were selected and considered sufficient for lytic enzymes to damage the cell walls of those fungi. After 7- and 8-h incubation, long and branched cells forming the mycelium were observed. Older mycelium was shown to be very difficult to separate single cells using the standard filtration techniques. It has been previously reported that protoplasts of *Rhizopus niveus* were most efficiently obtained from 4-6-hour-old germlings Younger germlings were resistant to lytic enzymes, and the efficiency of protoplast formation from older ones was lower (Yanai et al., 1990).

In the next stage of the study, the periods of action of three lytic preparations on *Rhizopus* R-15 and R-67 were compared to determine the optimum time for protoplast formation. It was observed that chitinase was the most effective lysing agent, with protoplasts of both fun-

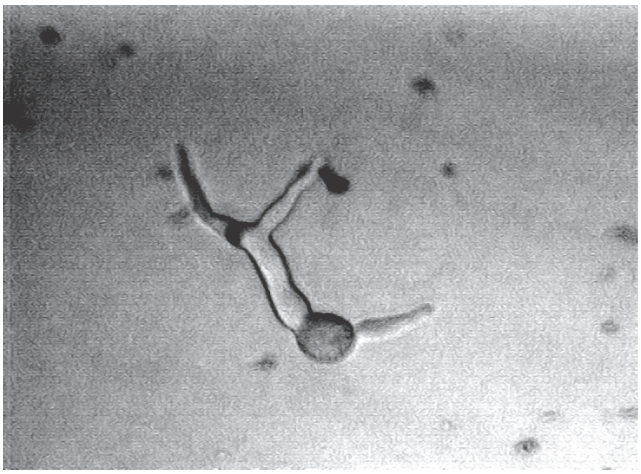

Fig. 1. Formation of young mycelium of *Rhizopus microsporus* R-67 after seven hours of incubation. Magnification 600 ×

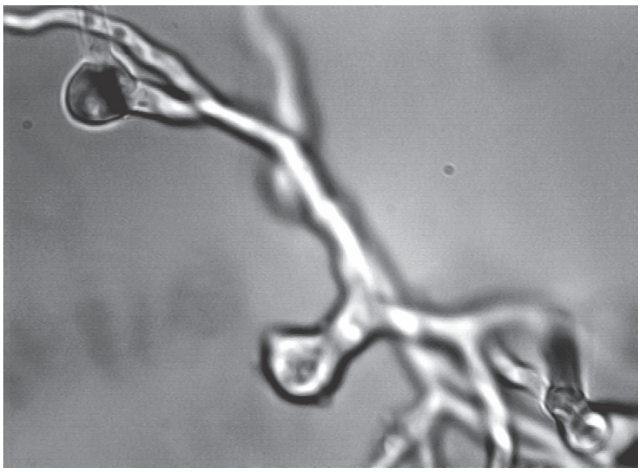

Fig. 2. Formation of young mycelium of *Rhizopus oryzae* R-15 after six hours of incubation. Magnification 600 ×

gal strains appearing in microscopic preparations after 1 h of incubation. In contrast, lyticase and lysing enzymes from *T. harzianum* needed 2 h for protoplast formation. This suggests that *Rhizopus* cells are not susceptible to the action of individual lytic enzymes, and so a mixture of these enzymes was prepared to intensify and shorten the protoplast formation time. Similar findings were reported by Eyini et al. (2006), who observed a maximum release of protoplasts in *Pleurotus florida* when using a mixture of enzymes containing cellulose, pectinase, and chitinase. A study by Reddivari et al. (2003) indicates that lytic enzymes from *T. harzianum* are not sufficiently effective in the formation of protoplasts of *Penicilium griseofulvum*, probably because of the low chitinase, β-1,3-glucanase, and cellulase activities.

In the present study, to simplify the selection, two protoplast inactivation methods were used before the fusion. Chemically and thermally inactivated protoplasts of R-15 were fused with non-inactivated protoplasts of R-67 (combinations A1 and D1) and *vice versa* (combinations B1 and E1) using a PEG solution. Additionally, a fusion between inactivated protoplasts of both *Rhizopus* R-15 and R-67 was performed (combinations C1 and F1). Followed by the fusions, the protoplasts were plated on regeneration agar, and different numbers of colonies were obtained from the particular combinations. It can be assumed that thermal inactivation was more effective because a total of 13 isolates were obtained after selection using this method. The results are listed in Table 1. It is believed that thermal inactivation damages the ribosome or ribosomal RNA in the cytoplasm (Ferenczy, 1984; Zhao et al., 2009). Whereas, the chemical methods used for protoplast inactivation cause lethal damage to cell structures (because of the oxidizing effects of free radicals), which results in the protoplasts losing their ability to regenerate.

Biolog FF MicroPlate substrate utilization and growth profiles of *Rhizopus* cultures were used to develop appropriate selection media (Buyer et al., 2001; Rice and Currah, 2005; Singh, 2009). The selection of fusants was based on media containing a specific sugar that was assimilated by one parental strain (inactivated before fusion) but not by the other (non-inactivated protoplasts). It was observed that sucrose and maltose were assimilated by strain R-15, but R-67 was unable to assimilate the former and assimilated the latter only very slowly. On the other hand, L-arabinose was metabolized by R-67 but not by R-15 (Table 1). A total of 14 isolates (fusants) obtained in this experiment were tested for their ability to produce fumaric acid from glycerol. All the fusants were able to produce this acid from glycerol in different concentrations during 7 days of incubation, as shown in Table 2. Singh (2009) reported that Biolog FF MicroPlate was suitable for substrate utilization studies of closely related fungi. Results of his study show that taxonomically distant cultures within the same genus group differ significantly in their substrate utilization profiles.

The present study is the first report on fumaric acid production by *Rhizopus* fusants on a growth medium containing glycerol as a carbon source. *Rhizopus* fusants displayed different abilities to produce fumaric acid from

Table 1. Number of fusants of the first round of fusion on selective media
(N – non inactivated strains, ChI – chemically inactivated strains, TI – thermally inactivated strains)

Combination	Fungal strains	Number of isolates	Selective sugars	Number of isolates after selection
A1	R-67 N + R-15 ChI	No growth	–	–
B1	R-67 ChI + R-15 N	1	L-arabinose	1
C1	R-67 ChI + R-15 ChI	No growth (C)	–	–
D1	R-67 N + R-15 TI	8	Sucrose, maltose	7
E1	R-67 TI + R-15 N	13	L-arabinose	6
F1	R-67 TI + R-15 TI	No growth (C)	–	–

Table 2. Production of fumaric acid and biomass from glycerol by the most effective *Rhizopus* fusants of double round of fusion after 7 days of incubation

Name of fusant	Fumaric acid concentration (g)[1]	Fumaric acid yield ($g \cdot g^{-1}$)	Biomass concentration (g)[1]
A2-1	3.8 ± 0.12[2]	0.23	7.81 ± 0.38
A2-2	3.6 ± 0.2	0.23	10.46 ± 0.31
A2-3	3.4 ± 0.41	0.21	8.54 ± 1.69
A2-5	3.9 ± 0.32	0.24	9.32 ± 0.85
A2-6	3.6 ± 0.15	0.22	10.2 ± 0.93
A2-7	4.1 ± 0.53	0.27	9.51 ± 1.48
B2-3	3.4 ± 0.4	0.2	12.08 ± 0.18
D2-2	3.7 ± 0.2	0.23	8.14 ± 0.28
R. oryzae R-15	3.0 ± 0.05	0.18	10.4 ± 0.42
R. microsporus R-67	3.3 ± 0.03	0.19	8.65 ± 0.67

[1] in dm^{-3} of production medium
[2] mean ± std. dev.

2.0% (w/v) of glycerol, with the yield for the most effective fusant ranging from 0.2 to 0.27 g of fumaric acid per g of glycerol (Table 2). In comparison, the parental strains *Rhizopus oryzae* R15 and *Rhizopus microsporus* R67 produced, after seven days of culture, 0.3% (w/v) and 0.33% (w/v) of fumaric acid, respectively, with a yield of 0.18-0.19 g per g of glycerol. Double fusion rounds led to a 1.46-fold increase in fumaric acid productivity relative to the parental strains. Studied strains and fusants produced biomass in the range of 7.81-12.08 $g \cdot dm^{-3}$. They also secreted lactic acid as byproduct at concentration below 0.012% (w/v). To date, no studies have been carried out on improvement of fumaric acid production by *Rhizopus* with the use of glycerol as the only carbon source in the medium. Kang et al. (2010), who cultured a *Rhizopus oryzae* mutant on an optimized medium with glucose, reported 0.45 $g \cdot g^{-1}$ of fumaric acid production, which was 1.9 fold higher than for the parental strain. Our results show that glycerol, which is a by-product of many biotechnological processes, may be a low-cost substrate for an increased fumaric acid production by *Rhizopus*. Considering the increasing demand for biological fumaric acid, it is necessary to continue studies on improvement of *Rhizopus* strains by mutagenesis, genome shuffling (Gong et al., 2009), protoplast fusion, or polyploidization (El-Bondkly et al., 2011).

Conclusions

This study is the first report on protoplast fusion of *Rhizopus oryzae* and *Rhizopus microsporus* strains for an improved production of fumaric acid using glycerol as the only carbon source. The results show that the fusion method enhances the production of fumaric acid, and as result glycerol can be successfully used as a component of the productive media for *Rhizopus*. The possibility of a further increase in the yield of fumaric acid should be explored in

an optimization study on medium components and growth conditions.

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