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Cover Page Footnote

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Intergenous Protoplast Fusion between *Pichia manshurica* and *Rhodospordium paludigenum* to Increase the Production of Inulinase

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Abstract

The purposes of this study was to identify the optimum concentration of the lytic enzyme Glucanex for protoplast isolation and to conduct fusion for the purpose of increasing inulinase production. The study performs the protoplast fusion technique using *Pichia manshurica* and *Rhodospordium paludigenum*. Protoplast fusion consists of a series of stages: protoplast isolation, protoplast fusion, protoplast regeneration, and analysis of hybrid fusion results. Protoplast isolation and fusion success rate are determined by various factors, including age of the culture, media type, and type of lytic enzymes used. Hybrid results were analyzed using a fungicide as a marker and measuring specific growth rate (μ) of the hybrid compared with parental growth rates. Results demonstrated that a concentration of 4 mg/mL of Glucanex produces the greatest number of protoplasts, 7.2×10^{10} (cell/mL) for *P. manshurica* and 8.8×10^{10} (cell/mL) for *Rh. paludigenum*. The results of analysis of hybrid fusions indicate that the study has identified a new fusant, called fusant F4. Fusant F4 is capable of producing the highest inulinase, 0.6892 IU, compared with parentals *P. manshurica*, 0.557 IU, and *Rh. paludigenum*, 0.3263 IU. Fusant F4 has specific growth rate (μ) of 0.3360/h and generation time (g) of 2.0629 h.

Abstrak

Fusi Protoplas Intergenous antara *P. manshurica* dan *Rh. paludigenum* untuk Meningkatkan Produksi Inulinase. Teknik fusi protoplas yang dilakukan pada penelitian ini secara intergenous yaitu antara *Pichia manshurica* dan *Rhodotorula paludigenum*. Fusi protoplas dilakukan melalui serangkaian tahapan yaitu: isolasi protoplas, proses fusi protoplas, regenerasi protoplas dan analisis hibrid (*fusant*) hasil fusi. Tingkat keberhasilan isolasi dan fusi protoplas sangat ditentukan oleh berbagai faktor, diantaranya umur biakan, jenis medium, enzim litik, dan jenis fusogen yang dipergunakan. Hasil hibrid (*fusant*) yang diperoleh selanjutnya dianalisis dengan menggunakan fungisida sebagai marker dan kecepatan pertumbuhan spesifik (μ) hibrid (*fusant*) dibandingkan dengan parental. Tujuan penelitian adalah untuk memperoleh konsentrasi enzim Glucanex yang optimum untuk mengisolasi protoplas, dan untuk memperoleh *fusant* yang mampu menghasilkan inulinase tinggi. Hasil penelitian menunjukkan bahwa konsentrasi enzim litik Glucanex 4 mg/mL mampu menghasilkan protoplas terbaik, sebesar $7,2 \times 10^{10}$ (sel/mL) untuk *P. manshurica* dan $8,8 \times 10^{10}$ (sel/mL) untuk *Rh. paludigenum*. Hasil analisis hibrid (*fusant*) menunjukkan bahwa pada peneltiian ini berhasil diperoleh *fusant* baru, yaitu *fusant* F4. *Fusant* F4 mampu menghasilkan inulinase lebih tinggi 0,6892 IU, lebih baik dibandingkan dengan parental *P. manshurica* 0,557 IU, dan *Rh. paludigenum* 0,3263 IU, memiliki kecepatan pertumbuhan spesifik (μ) sebesar 0,3360/jam, dan waktu generasi (g) 2,0629 jam.

Keywords: fusant, inulinase, protoplast fusion, P. manshurica, Rh. paludigenum, specific growth rate

1. Introduction

Microbes, especially yeasts, can produce an enzyme that degrades inulin, called inulinase (EC. 3.2.1.7). The numbers of inulinase enzymes produced by microbial products and activities are usually few, and increasing

their production requires either optimizing the microbial growth environment or genetic manipulation. Naturally occurring yeast on dahlia tubers contains inulinase, the enzyme that converts inulin into fructose. Indigenous inulinolytic yeasts have been found in the wild plant *Dahlia* sp. in Bandungan-Ambarawa in central Java [1].

These inulinolytic yeasts are *P. manshurica* and *Rh. paludigenum*, both of which are capable of producing inulinase, albeit in small quantities [1]. Several research have created strains improved for inulinase production by manipulating growth environment or genetics [2]. One technique for improving the yeast strain is the protoplast fusion technique, which has been used intensively to improve yeast strains in a manner that overcomes the difficulties of conducting hybridization sexually. Protoplast fusion does not require cell trait or vector competence, and fusant testing is simpler and more systematic [3]. Given that inulinase enzymes produced by indigenous yeasts are few, this research seeks to increase the quality of the strain in enzyme production.

2. Methods

Yeast strains and media. *P. manshurica* and *Rh. Paludigenum* were obtained from laboratory at the Faculty of Mathematics and Natural Sciences, Universitas Diponegoro [1]. Both yeasts were grown at 27°C on yeast peptone dextrose (YPD) medium containing (w/v) yeast extract 1%, peptone 2%, and glucose 2%, with a pH of 5.5–6.0 [4]. Cultures were grown in liquid media in conical flasks containing 10% by volume of the broth and agitated on a rotary shaker at 120 rpm [5].

Protoplast preparation and fusion. *P. manshurica* and *Rh. paludigenum* were grown on YPD until the log phase, when cultures were harvested and suspended in sorbitol osmotic stabilizer 1 M in 0.2 M H₂PO₄/Na₂HPO₄ buffer with a pH of 5.8. [4]. After washing with the same buffer, cells were incubated at 27°C for 90 min with Glucanex 2 mg/mL and 4 mg/mL to achieve protoplast isolation. Protoplasts were collected from both types of cultures (0.5 mL *P. manshurica* and 0.5 mL *Rh. paludigenum*), and a fusion solution containing polyethylene glycol (PEG) 6000, CaCl₂, and glycine was prepared [6].

Fusions selection. Fusant hybrids were analyzed for minimum inhibitory concentration (MIC) value, reaction to the antifungal/fungicide Dithane M 45, inulinase production, specific growth rate (μ), and generation time (g) [6-7].

Growth medium and inulinase production. The medium for enzyme production contained *Dahlia* sp. powder 3%, NH₄NO₃ 0.23%, (NH₄)₂HPO₄ 0.37%, KH₂PO₄ 0.1%, MgSO₄·7H₂O 0.05%, and yeast extract 0.15% at a pH of 5–6. The medium was autoclaved at 121 °C 2 atm for 20 min. Following inoculation, the flask was incubated on a rotary shaker at 120 rpm [8].

Assay of inulinase activity. Inulinase activity was determined through the 3,5-dinitrosalicylic acid (DNS) method [9] using inulin as a substrate. One unit of inulinase activity was defined as the amount that liberates 1 μ mol of fructose equivalent from inulin per min [10].

3. Results and Discussion

Culture conditions and enzymatic isolation of protoplasts. In this study, *P. manshurica* (Pm) and *Rh. paludigenum* (Rhp) were grown on YPD medium and harvested after 24 h (Figure 1), in the late exponential (log) phase, when yeast cells are immature and their cell walls are easily dissolved by a lytic enzyme [4, 18].

Glucanex breaks down yeast cell walls so that they become round in both *P. manshurica* (Figure 2) and *Rh. paludigenum* (Figure 3) compared with the cells in controls, which are oval shaped.

Figure 4 shows that treatment with Glucanex 4 mg/mL (L4) rendered better results than treatment with 2 mg/mL (L2). Protoplast treatment with L4 produced 7.2×10^{10} (cell/mL) for *P. manshurica* and 8.8×10^{10} (cell/mL) for *Rh. paludigenum*. Treatment with L2 produced 5.2×10^{10} and 8.2×10^{10} , respectively. Therefore, in subsequent research, L4 will be used in the fusion process.

Protoplast fusion using PEG. This study used the fusogen PEG 6000 at concentrations of 30% and 35%. The 30% concentration of PEG 6000 formed aggregates of 2–3 (Figure 5), while the 35% concentration formed aggregates of more than 3.

Fusions selection. In fungicide testing on all four selected fusants (F1, F3, F4, and F 7), Fusant F1 (white) and F7 (white) had an MIC value of 200 ppm of Dithane M45 (Table 1). This MIC value was slightly lower than that of the parental *P. manshurica* (white), which was 300 ppm.

The parental *Rh. paludigenum* (red; Rhp) had the same MIC value (200 ppm). Fusant F3 had an MIC value identical to the parent Rhp, 200 ppm. Fusant F4 (white) had an MIC value of 50 ppm, below those for both parents.

Test of fusants F1, F4, and F7 based on inulinase production. Fusants F1, F4, and F7 were grown on ISM medium, in which inulin is used as the sole carbon

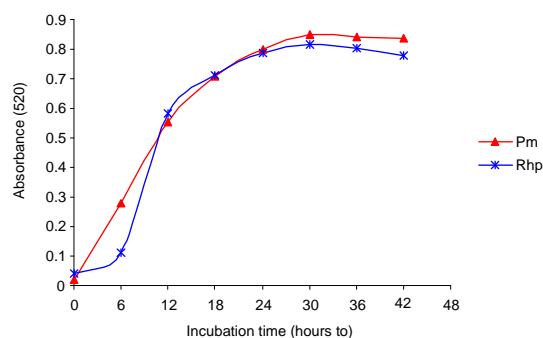


Figure 1. Growth Curve Showing Number of Cells of *P. manshurica* and *Rh. paludigenum* for Protoplast Isolation on YPD Medium

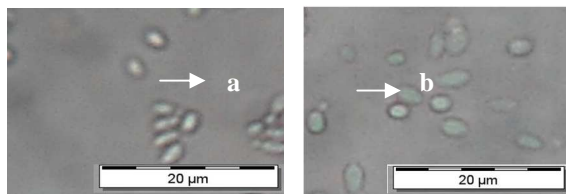


Figure 2. *Pichia manshurica*. (a) Cell Normal (oval) (b) Protoplasts (rounded)

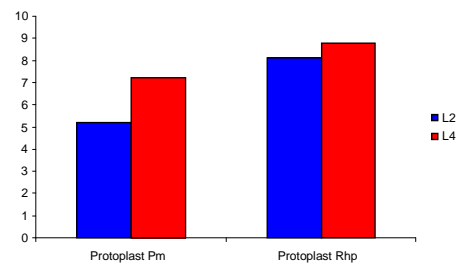


Figure 4. Effect of Enzyme Concentrations on Protoplasts

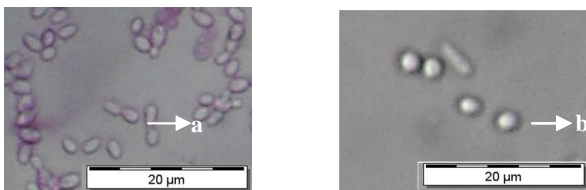


Figure 3. *Rhodospiridium paludigenum*. (a) Cell Normal (Oval) (b) Protoplasts (Rounded)

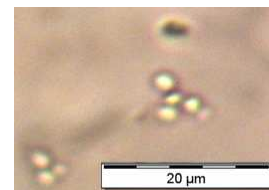


Figure 5. Protoplast Aggregation at PEG 6000 Concentration of 30%

Table 1. Test Results of Antifungal Dithane M45 (ppm) on Parentals Pm and Rhp and on Fusants

Strain	Antifungal	ppm					
		25	50	100	200	300	
Parental	Pm (white)	Dithane M45	+	+	+	+	-
	Rhp (red)	Dithane M45	+	+	+	-	-
Fusant	F1 (white)	Dithane M45	+	+	+	-	-
	F3 (red)	Dithane M45	+	+	+	-	-
	F4 (white)	Dithane M45	+	-	-	-	-
	F7 (white)	Dithane M45	+	+	+	-	-

Description: - : No growth; + : Growth; Pm : *P. manshurica*; Rhp : *Rh. paludigenum*

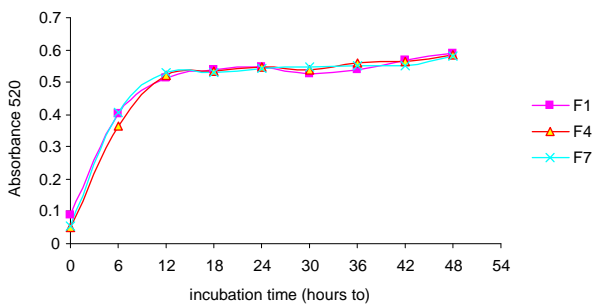


Figure 6. Turbidity Growth Patterns of Fusants F1, F4, and F7

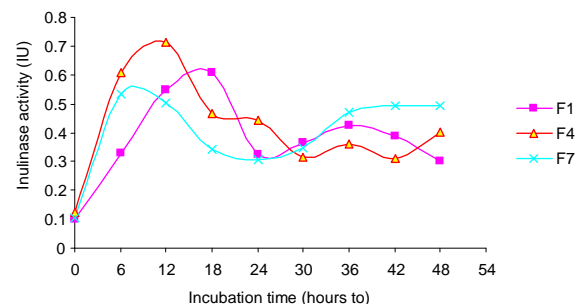


Figure 7. Production of Inulinase by Fusants F1, F4, and F7

source [8]. This study observed growth patterns and inulinase production every six h for 48 h (Figures 6 and 7).

Protoplasts are cells in which the cell wall has been removed, but which have their cell membranes and internal structures intact and are able to perform normal physiological functions. Protoplast isolation must be conducted carefully and in an osmotic stabilizer solution to prevent excessive intake of water into the cells, which

can lead to cell rupture [11]. Osmotic stabilizer solutions used to isolate protoplasts contain sugar and sugar alcohols [5].

In Figure 1, it is clear that *P. manshurica* and *Rh. paludigenum* have entered the late log phase at the age of 24 h without a lag phase. This can occur due to the addition of starter, which serves to negate the lag phase [12]. In this study, the starter reduced the lag phase, and the log phase was reached quickly.

Cells were isolated through centrifugation. Pellets obtained were immersed in osmotic stabilizer solution containing 1 M sorbitol Glucanex 2 mg/mL (L2) and 4 mg/mL (L4). This solution served to maintain the stability of the protoplasts. Osmotic stabilizer plays a very important role in protoplast isolation, as it also increases protoplast release [6].

The lytic enzyme, containing enzyme hydrolyzed glucan, chitin, protein, and cellulose, causes protoplasts to be round. The lytic enzyme used in protoplast isolation depends on the composition of the microbial cell walls in the yeast. The main constituents of yeast cell walls are β glucan, chitin, and mannoprotein. Therefore, a suitable lytic enzyme by which to hydrolyze yeast cell walls is Glucanex, or novozim, because it contains enzymes β glucanase, cellulase, protease, and chitinase [11] to destroy yeast cell walls. When cell walls have been destroyed by Glucanex, the protoplasts appear more rounded, an effect enhanced by the osmotic stabilizer solution [19].

This result is in accordance with previous research, which found that the best concentration of PEG is 25–33% [13]. If the concentration of PEG is too high, it will be toxic to protoplasts [14]. In addition, protoplast fusion using high PEG concentrations can be toxic to recombinants [15]. However, if the concentration of PEG is too low, it will not aggregate protoplasts, and if the concentration is too high to form aggregates, there will be no fusion, only toxicity [13]. The protoplast fusion process results in cell membrane activation, causing cells to adhere to other cells. These attachments result in aggregate formation.

The addition of Ca^{2+} ions during the fusion process results in an increase in fusion frequency resulting from PEG's ability to bind to Ca^{2+} ions, thereby forming a bridge between the membrane and the PEG. In water, PEG (polyethylene glycol, $\text{HOCH}_2(\text{CH}_2\text{-O-CH}_2)_n\text{CH}_2\text{OH}$) has a slightly negative charge and is able to form hydrogen bonds with the plasma membrane in protoplasts. In protoplast fusion, PEG acts as a bridge between two or more protoplasts, resulting in aggregates [16].

The fusion pellets obtained using 30% PEG on *P. manshurica* and *Rh. paludigenum* were grown in osmotic stabilizer on YPD medium for 24 h and then poured onto plates of selective medium containing inulin as the sole carbon source. All fusants (F1, F3, F4, and F 7) grew on this Inulin Selecting Media (ISM), proving that fusants are capable of producing the enzyme inulinase.

Based on MIC measurements, parental *P. manshurica* (Pm) can be inhibited by the fungicide Dithane M 45 at a minimum concentration of 300 ppm, while *Rh. paludigenum* (Rhp) requires a minimum concentration of only 200 ppm. The extent of resistance to a microbial fungicide/antifungal can be used as a marker. Therefore,

resistance to Dithane M 45 was used as a marker, with the mark being at the concentration of 300 ppm for *P. manshurica* and at 200 ppm for *Rh. paludigenum*. These values are based on the pattern of the fungicide's attack on microbial cells at the time of transcription, translation, protein synthesis, and cell wall synthesis [6, 7].

These results indicate that fusion has occurred between parentals *P. manshurica* and *Rh. paludigenum*. In other words, fusants F1, F4, and F7 were selected. Fusant F3 was not selected, because it has properties similar to the parent *Rh. paludigenum*. Testing of fusants F1, F4, and F7 involved continuing to select one of them based on its ability to produce inulinase.

The log phase growth pattern occurred from incubation time T_0 to T_{12} (Figure 6), during which time inulinase was produced. At 12 h, fusant F4 had produced 0.6892 IU of inulinase. During the same time period, parentals *P. manshurica* and *Rh. paludigenum* had generated 0.3263 IU and 0.557 IU, respectively. Of the four fusants, fusant F4 produced the highest amount of inulinase compared with the parentals (Figure 7). In addition, fusant F4 produced a great deal of inulinase compared with *Debaryomyces hansenii* DUCC-W8 (0.639 IU) [17]. Inulinase is categorized as a primary metabolite and is produced only in the log phase due to induction of inulin from the environment (in this case, from the medium). Fusant F4 had a specific growth rate (μ) of 0.3360/h and a generation time (g) of 2.0629 h. These values lie between those of the two parentals. *P. manshurica* had specific growth rate (μ) of 0.2793/hour and generation time (g) of 2.4815 h and *Rh. paludigenum* a (μ) of 0.3787/h and a (g) of 1.8304 h.

4. Conclusions

A Glucanex concentration of 4 mg/mL produces the greatest number of protoplasts: 7.2×10^{10} (cell/mL) for *P. manshurica* and 8.8×10^{10} (cell/mL) for *Rh. paludigenum*. Results of analyses of the hybrid (fusant) based on MIC values showed that a new fusant, fusant F4, is the best. This fusant is capable of producing the highest inulinase, 0.6892 IU, compared with parentals *P. manshurica*, at 0.557 IU, and *Rh. paludigenum*, at 0.3263 IU. Fusant F4's MIC value for Dithane M45 is 50 ppm, while the parental MICs are 300 ppm and 200 ppm for *P. manshurica* and *Rh. paludigenum*, respectively. Fusant F4 is a combination of the two parental, with a (μ) of 0.3360/h and a (g) of 2.0629 h.

Acknowledgements

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