Journal of Biological Researches: 18 (95-97), 2013

ISOLATION OF *Pichia manshurica* PROTOPLAST FROM *Dahlia* sp. PLANT

Wijanarka*, Endang Sutariningsih Soetarto**, Kumala Dewi*** and Ari Indrianto****
* Mathematic and Natural Science Faculty, Diponegoro University and Student of Doctoral Degree Gadjah Mada University
** Microbiology Laboratory, Biology Faculty, Gadjah Mada University
*** Plant Physiology Laboratory, Biology Faculty, Gadjah Mada University
**** Biotechnology Laboratory, Biology Faculty, Gadjah Mada University
Corresponing Author Email : wikasmara@yahoo.co.id

ABSTRACT

Isolation of protoplasts is an important step in the fusion process. Protoplasts are cells that have eliminated the cell wall, but the cell membranes and organs can still function properly. Pichia manshurica is one of indogenous yeast that derived from Dahlia's plants. The success rate protoplast isolation was determined by various factors, include the age of the culture and the used of lytic enzymes. The purpose of this research is to get the perfect age of yeast culture that is ready to be harvested and also to get the appropriate concentration of Glucanex lytic enzymes which used for protoplast isolation. The yeast of Pichia manshurica grown on YPD broth medium and growth observed in turbidimetry. Observation of the growth of yeasts performed every 6 hours for 42 hours. Glucanex lytic enzyme concentration used for the isolation of protoplasts is 0 mg / mL (L0 = control), 2 mg / mL (L2) and 4 mg / mL (L4). The results showed that the age of the culture is right and ready for harvest at the age of 24 hours and Glucanex lytic enzyme concentration of 4 mg / mL (L4) is able to produce the best of protoplasts at 7.2 x 1010.

Key words : Glucanex, Pichia manshurica, Protoplast isolation

ABSTRAK

Isolasi protoplas merupakan tahapan penting dalam proses fusi. Protoplas merupakan sel yang telah dihilangkan dinding selnya, akan tetapi membran dan organ selnya masih dapat berfungsi dengan baik. Pichia manshurica adalah salah satu khamir indogenous yang berasal dari tanaman bunga dahlia. Isolasi protoplas tingkat keberhasilannya sangat ditentukan oleh berbagai faktor, diantaranya umur kultur dan enzim litik yang dipergunakan. Tujuan penelitian ini adalah mendapatkan umur kultur khamir dalam jumlah yang maksimal sehingga tepat digunakan sebagai sumber protoplas serta mendapatkan konsentrasi enzim litik Glucanex yang cocok dalam menghasilkan protoplas. Khamir Pichia manshurica ditumbuhkan pada medium YPD cair dan dilakukan pengamatan pertumbuhan secara turbidimetri. Pengamatan pertumbuhan khamir dilakukan setiap 6 jam sekali selama 42 jam. Konsentrasi enzim litik Glucanex yang digunakan untuk isolasi protoplas adalah 0 mg/mL (L0 = kontrol), 2 mg/mL (L2) dan 4 mg/mL (L4). Hasil penelitian menunjukkan bahwa umur kultur yang tepat dan siap panen terjadi di fase eksponensial akhir pada umur 24 jam dengan jumlah 14,48 x 1010 sel/mL, konsentrasi enzim litik Glucanex terbaik pada konsentrasi 4 mg/mL (L4) dan mampu menghasilkan protoplas terbanyak yaitu 7.2 x 1010.

Kata kunci : Glucanex , Isolasi protoplas. Pichia manshurica

INTRODUCTION

Dahlia's plant is a herbaceous plant with the high about1.5 m or more. This plant establish the root clumps on the bottom of the trunk. The root tissue serves as a food storage. The shape of *Dahlia's* tuber roots was varied from small to large round and long to oval. The structure of *Dahlia's* tuber consists of tuber skin with yellowish white to brown on the color, the tuber flesh is thick, have white color, and have translucent buds (Rukmana, 2000). Most of *Dahlia's* tuber contain inulin, as one of carbohydrate. Inulin is a polysacharide that built by fructose monomer units through β-2-1-fructofuranocyde that preceeded by one of glucose molecule. In addition, in the rizosfer area and Dahlia's tube was found some microbe. Lunggani *et al* (2009), found a inunolitic yeast that produce inulinase. That yeast were identified as *Pichia manshurica*.

Pichia manshurica is a yeast that produce inulinase. Inulinase enzyme's able to reorganize inulin molecules into simpler units that is fructose. The applications product of inulinase are High Fructose Syrup (HFS, fructose syrup), Fruktooligosakarida (FOS) and Inulooligosakarida (IOS) (Singh and Prabhjot, 2006; Bonciu and Gabriela, 2011). However, inulinase that produced by the yeast is very low. Therefore, we need some genetic manipulation to improve the activity that Pichia manshurica. One of technique that used to increase the activity of inulinase production by using the protoplast fusion technique. Hopefully with this genetic manipulation will find new fusan that having a higher inulinase activity compared with the parental.

In this technique, there are several steps that must be passed, one of them is the isolation of protoplasts. Protoplast isolation process that produces viable and large amount of protoplast is need for the fusion process at a later stage, therefore the protoplast isolation stage is very important. The number of protoplasts thet resulting in the isolation process is influenced by several factors, including the age of the culture as a source of protoplasts and the concentration of lytic enzymes. Therefore, this study aimed to obtain the maximum age of yeast culture so that can be used as a source of protoplasts and to know the Glucanex lytic enzyme concentration that suitable for protoplast isolation.

MATERIAL AND METHOD

Microorganism and culture medium

The primary culture of *Pichia manshurica* were grown on PDA (Potato Dextrose Agar) medium. And YPDB (Yeast Peptone Dextrose Agar) medium were used for the isolation of Protoplast. YPDB medium consist of 10 g/L yeast exract, 20 g/L peptoe, 20 g/L glucose, and pH 4.5 – 5.5 (Santopietro *et al*., 1997).

Lytic enzyme and osmotic stabilizer

The lytic enzyme that used in this experiment is Glucanex (Sigma Chemicals Co™) with concentration 2 mg/ml (L2), 4 mg/ml (L4) and control (L0, without Glucanex). That enzyme were dissolved in sorbitol osmotic stabilizer 1.0 mol/L in 4 mg/ml phosphate buffer 0.2 M pH 5.8.

Growth of *Pichia manshurica*

Pichia manshurica were grown on YPDB medium. The growth were observed every 6 hours for 42 hours at room temperature. The measurement of growth were observed

through optical density (OD) with 520 nm wavelength by using turbidimetry method. The cell of Pichia mashurica were harvested at late log phase (Santopietro *et al.*, 1997).

Protoplast Isolation

1 ml of yeast culture that had been harvested subsequently put in ependorf tube. The sample were centrifugated for 10 minutes at 3000 rpm. The pellet were washed in a osmotic stabilizer solution, then given 2 mg/ml and 4 mg/ml of stabilizer solution that contain lytic enzyme. Then the sample were incubated for 90 minutes. The observation of the protoplast were counted by using haemocytometer.

RESULT

Growth of *Pichia manshurica*

The results showed that the pattern of growth of the *Pichia manshurica* grown on YPDB media can be seen in Figure 1, while the growth phase that occurs during 6 consecutive intervals can be seen in Table 1.

Table 1. *Pichia manshurica* growth based on the value of OD

Incubation time (hour)	**OD (Optical Dencity)**
0	0.0188
6	0.2771
12	0.5524
18	0.7094
24	0.8011
30	0.8512
36	0.8435
42	0.8382

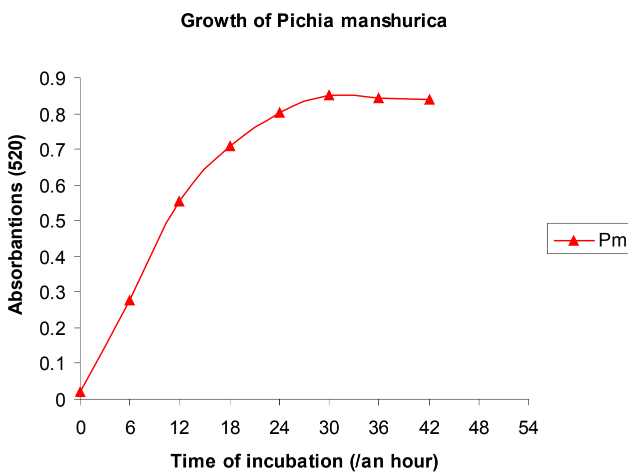

Figure 1. Growth of *Pichia manshurica*

Based on figure 1, show that the *Pichia manshurica* has entered at the late of logarithmic phase in the life of 24 hours without a lag phase, this can happen because of the addition of starter *P. manshurica*. The addition of starter serves to negate the lag phase, because *P. manshurica* does not require the adaptation to the environment. Log phase starting from 0 hour incubation time (t0) until 24 hours (t24), this is evidenced by qualitative data from the OD (turbidimetry) is 0.0188 and 0.8011 at t0 and t24, respectively.

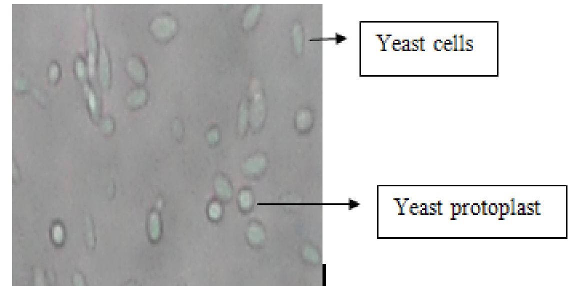

Figure 2. *Pichia manshurica* at 400x magnification

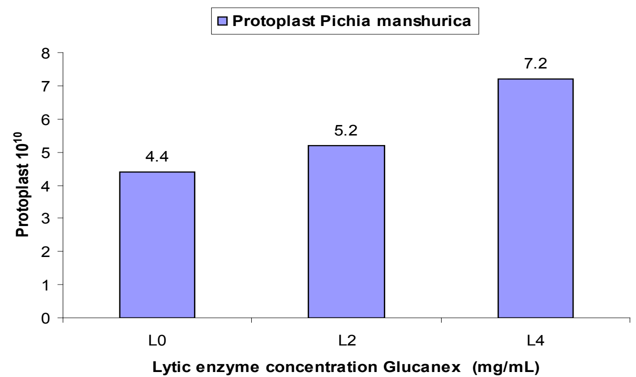

Figure 3. The effect of Glucanex enzyme to the number of protoplast.

Protoplast Isolation

Based on the observations of the growth curve, the protoplast that used in this experiment were isolated from yeast cells with 24 hours of culture age. In protoplast isolation, it is clear that yeast cells are given a lytic enzyme gives a rounded shape, whereas the control gives shape to oval cells. (Figure 2).

The effect of Glucanex lytic enzyme to the number of *Pichia mashurica* protoplast shown in figure 3. Based on figure 3, on glucanex lytic enzyme is able to liberate protoplasts as 5.2 x 1010; 7.2 x 1010 and 4.4 x 1010 at 2 mg/ml (L2); 4 mg / ml (L4) and control, respectively (Figure 3). Based on Figure 3 it is clear that the increase of Glucanex lytic enzymes concentration that added, it will increase the release of protoplast.

DISCUSSION

On the growth of the *P. manshurica* yeast is clear that the log phase (exponential) begins at the 0 (t0) up to 24 hours (t24) without adaptation phase. This can occur because of the addition of the starter. The addition of starter serves to negate the lag phase. This is in accordance with the opinion of Alexander and Jeffries (1990) that the starter works to reduce the lag phase and is expected to quickly reach log phase.

Based on the pattern growth of *Pichia mashurica*, the harvesting of cells for isolation of protoplasts done at the time of the growth of *P. manshurica* entering the late log phase (24

hours). It is due because the yeast cells in the most active phase of metabolism to cell division, so that the yeast cells are not yet old enough. In addition, the structure of the yeast cell wall is still not so strong, so it is easily solved by lytic enzymes.

For the isolation of protoplas was done by centrifugation of *Pichia mansurica* culture so it will get the pellet. Then, the pellet were incubated at sorbitol osmotic stabilizer solution 1M. The sorbitol osmotic stabilizer solution 1M serve to maintain the stability of protoplast, so we can get the protoplast with good quality. This result accordance with Varavallo *et al.* (2004) that the osmotic stabilizer have an important role for protoplast isolation and able to increase the release of protoplast (Balasubramanian and Damodaran, 2008). Without osmotic stabilizer, the protoplast will lysis and become die. Ezeronye and Okerentugba (2001) said that the protoplast can be lysis because the absence of osmotic stabilizer. This is because the osmotic pressure on outer cell is smaller that in inner cell so that the water will entering the cell and causing bulging and rupture. Judoamidjojo *et al* (1990) to avoid the protoplast lysis so the nprotoplast should be treated with a solution which has a same osmotic pressure of the cell.

In the Glucanex lytic enzyme treatment L2 and L4 can break down the cell walls of yeast that will shape roundly compared with control (L0 , without Glucanex) that have oval shape (Figure 2). This is due because to the work of Glucanex lytic enzyme that containing enzyme that can break glucan, chitin, protein, cellulose so that the protoplasts are round. The lytic enzymes that used in the protoplasts isolation depends on the composition of the cell wall of the yeast. The major composition of yeast cell wall are β glucan, chitin and manoprotein. Thus. the suitable lytic enzymes used to break down the cell wall of yeast is glucanex.

Glucanex used as a lytic enzyme in yeast because that enzyme containing β glucanase, cellulase, protease and chitinase, so that the yeast cell wall can be destroyed / lysed (Verma *et al*., 2004; Varavallo *et al*., 2004). Based on the results (figure 3) that Glucanex lytic enzyme treatment 4 mg/ml (L4) has better results compare with 2 mg/ml (L2) and control (L0). It is shown that the L4 treatment of protoplasts generated amount of 7.2 x 1010 for *P. manshurica* and L2 of 5.2 x 1010 and 4.4 x 1010 for control. The number of protoplasts generated is directly proportional to the concentrations of lytic enzymes Glucanex and lytic enzyme given, then the number of protoplasts released also more and more (Figure 3).

Based on the result, it can be concluded that *P. manshurica* at 24-hour culture age and Glucanex lytic enzyme treatment with concentration of 4 mg / ml (L4) better when compared with the treatment of L2 and L0 being able to release protoplasts (protoplast isolation).

ACKNOWLEDGEMENT

A big thank you to the Directorate General of Higher Education of the Republic Indonesia, which has finance writer through BPPS scholarship to pursue the doctoral education .

REFERENCES

Alexander, M.A. and T.W. Jeffries. 1990. Respiratory efficiency and metabolize artitioning as regulatory phenomena in yeasts". *Enzyme Micobe. Technol.* 12: 2-29.

Balasubramanian, N. and Damodaran, L. 2008. Characteristics of Protoplast inter, intra-fusan and regeneration of antagonistic fungi *Trichoderma harzianum* and *Trichoderma viridae. African Journal of Biotechnology*. 7 (18): 3235-3243

Ezeronye, O.U and Okerentugba, P.O. 2001. Optimum Conditions for Yeast Protoplast Release and Regeneration in *S. cerevisiae* and C. *tropicalis* Using Gut Enzyme of The Giant African Snail Achatina achatina. *Letters in Applied Microbiology.* 32: 190-193.

Judoamidjojo M.,, Abdul A.D dan Endang, G.S. 1990. Teknologi Fermentasi. Rajawali Press. Jakarta.

Lunggani, A. T., Wijanarka anda Endang K., 2009. Produksi IOS Prebiotik Berbasis Pemanfaatan Umbi Dahlia (*Dahlia variabilis*) Oleh Khamir Inulinolitik Dan Pengujian Antimikrobanya Secara Invitro. Penelitian Hibah Multiyears Desentralisasi.

Rukmana, R. 2000. Dahlia: Prospek Agribisnis dan Teknik Budi Daya. Penerbit Kanisius. Jogyakarta.

Santopietro, L.M.D., JFT. Spencer, D.M. Spencer and Sineriz. 1997. Characterization of intergeneric hybrids obtained by protoplast fusion between *Phaffia rhodozyma, Cryptococcus laurentii* and *S. cerevisiae*. *Biotechnology technique*. 11 (10): 769-771.

Singh, P and Prabhjot. 2006. Production of Inulinase : Recent Advances. F*ood Technol. Biotechnol*. 44 (2): 151-162

Varavallo MA, Marisa VdQ, Jorge FP and Elza FdA. 2004. Isolation and Generation of *Penicillium brevicompactum* Protoplas Fusion. *Maringa.* 26 (4).: 475-479.

Verma, N., Bansal and Vivek, K. 2004. Protoplast Fusion Technology and Its Biotechnology Applications