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# Influence of additives on *Saccharomyces cerevisiae* β-glucan production

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**Abstract**

In *Saccharomyces cerevisiae*, many changes in carbohydrate composition of cell wall occur when cells grow under various conditions. In this study, effects of sole additives (SDS, EDTA, and NaCl) including SDS in combination with NaCl and EDTA were studied in order to enhance β-glucan production in *S. cerevisiae* Angel®. The results showed that all media supplemented with the studied additives could be able to enhance b-glucan production in different levels (7-40%). Yeast cultured in YPD medium supplemented with 100 ppm SDS produced the highest β-glucan content, followed by one supplemented with a combination of 20 ppm SDS and 3000 ppm NaCl. This indicated that sole SDS was the best additive to enhance β-glucan production, compared with control. Consequently, the β-glucan from yeast cultured with this condition was extracted and characterized. Its chemical structure was revealed by $^{13}$C-NMR. Not only the highest β-glucan content but also the lowest proteins determined using FTIR were found in yeast cells cultured with this condition. In addition, it was found that β-glucan from all additives addition cultures seemed to possess higher branching chains than control, with exception in EDTA 50 ppm. Apart from the composition, changes in morphology of yeast cells when cultured in various studied conditions were investigated by confocal microscope and SEM. Furthermore, based on aspect ratio, cells cultured with sole SDS were slightly more spherical than other treatments. Overall, the obtained results implied that the additives could activate cell wall synthesis of yeast cell, relevant to β-glucan content and β-glucan branching formation.



## Introduction

β-Glucans are group of polysaccharides that are composed of glucose units linked together with b-glycosidic bonds (Klis *et al*., 2002). They have been used in many industries, such as the pharmaceutical, food, feed, and cosmetics industries (Reed and Nagodawithana, 1991; Suphantharika *et al*., 2003; Satrapai and Suphantharika, 2007). β-Glucans also exhibit medicinal properties such as antitumor, antimicrobial and antioxidant activities plus mycotoxin absorption (Ross *et al*., 1999; Chen and Seviour, 2007; Magnani *et al.*, 2009, 2011) as well as uses in stimulation of the immune response in animals, such as shrimps, weaned pigs and mice, and the reduction of blood cholesterol and glucose levels (Ostroff, 1997; Nicolosi *et al*., 1999; Hayen and Pollmann, 2001; Ortuno *et al*., 2002).

Generally, cell wall of *Saccharomyces cerevisiae*, being about 70 nm thick, accounts for 20% of the cell's weight (Walker, 1999). The composition of the cell wall is mainly mannoproteins, β-glucan (85-90% of cell dry mass), small amount of chitin (1-3 %) and lipid (2-5%) (Klis, 1994). Thus, *S. cerevisiae* has been considered as an interesting source of β-glucan production since the β-glucan from *S. cerevisiae* has various properties that are more preferable to those found in other sources (Nguyen *et al.*, 1998). The β-glucan has been made up about 55 - 65% w/w of yeast cell walls, consisting of both long chains of β-1,3-glucan (about 85% of the total) and short chains of β-1,6-glucan (Klis *et al.*, 2002). *S. cerevisiae* can be easily and rapidly grown in a diverse array of culture media at a low production cost and its whole genome is already known. Thus, *S. cerevisiae* is a good natural choice for β-glucan production. However, in terms of improving b-glucan production from microbial cells there appears to be little, and no specific information

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on the effect of additives on β-glucan production in *S. cerevisiae* compared with other organisms. For instance, *Botryosphaeria rhodina* was stimulated to produce β-glucan by the use of emulsified media (Crognale *et al*., 2007), whilst induction of β-glucan synthetase in mushrooms was attained by culturing them in olive mill wastewater (Reverberi *et al*., 2004). Several factors including the type of culture medium, carbon source, pH, temperature, aeration rate and culturing conditions influence the morphology and composition of the cell wall during growth process. β-Glucan production by S. cerevisiae in a batch fermenter was found to be optimal when grown in Yeast Peptone Dextrose (YPD) pH 4.0, with galactose as the carbon source, at 37°C and well-aerated conditions of $pO_2$ > 50% saturation (Aguilar-Uscanga and Francois, 2003). Galichet *et al.* (2001) studied the structural modifications in the cell wall of *S. cerevisiae* TCY 70 mutant strains. The results indicated that S. cerevisiae mutant TCY 70 was exhibited 30% decreased in mannoprotein and increased in β-glucan. Nevertheless, there is currently no information about the effect of additives (EDTA, SDS and NaCl) on structural modification and β-glucan production. Therefore, in this study, we investigated the effect of EDTA, SDS and NaCl on β-glucan production and cell wall composition of *S. cerevisiae.*

## Materials and Methods

*Yeast cultivation*

From our previous work (Mongkontanawat *et al.*, 2011), *S. cerevisiae* Angel® produced the highest β-glucan yield among other two commercial baker's yeasts. Thus, *S. cerevisiae* Angel® was selected for further studies in this work. Starter culture (5 ml) was inoculated into flask containing 500 ml YPD medium (1% (w/v) yeast extract, 2% (w/v) peptone and 2% (w/v) dextrose (Himedia, India) with three different additives (EDTA, SDS and NaCl) and their combination. Moreover, it should be noted that concentration of the studied additives as shown in Table 1 were previously obtained from optimization growth of *S. cerevisiae* Angle®. Cells were incubated at 30°C, pH 4 in reciprocal shaking 200 rpm for 24 h. The yeast cultures were collected by using centrifugation at 8000 x g for 10 min, freeze dried and analyzed as follows.

*Yeast cell morphology*

Yeast was cultured in YPD medium, adjusted to pH 4.0, and supplemented with an optimum concentration of the three additives (EDTA, SDS and NaCl), as shown in Table 1, incubated at 30°C with shaking at 200 rpm for 24 h. Yeast cells were then sampled from the culture and photographed with a confocal microscope in differential interference contrast (DIC) mode at 400X magnification (Nikon C1 Digital Eclipse, Japan). The images were taken with ImageJ software (NIH Image, USA.) to determine an aspect ratio (length / width) of each single cell, for at least 45 cells per treatment (Coelho et al., 2004).

Table 1. YPD (Yeast Peptone Dextrose) media supplemented with various additives

| Treatment | EDTA (ppm) | SDS (ppm) | NaCl(ppm) |
|---|---|---|---|
| 1(control) | 0 | 0 | 0 |
| 2 | 0 | 100 | 0 |
| 3 | 50 | 0 | 0 |
| 4 | 0 | 0 | 30,000 |
| 5 | 5 | 10 | - |
| 6 | 0 | 20 | 3,000 |
| 7 | 5 | 10 | 3,000 |

Furthermore, scanning electron microscopy (SEM) (JEOL, model JSM-5410LV, Japan) at a magnification of 5,000 X and 20,000 X was used to capture images of whole cell shape and cell wall surface of yeasts grown under the studied culture conditions.

*Polysaccharide, protein and lipid ratio and b-(1,6) : β-(1,3) ratio*

Powdered yeast (about 1-2 mg) was used in this study. FTIR spectra were obtained in absorption mode at room temperature. Spectra were recorded from 600-4500 $cm^{-1}$ by IR Prestige 21(Japan). Second derivative and Fourier self-deconvolution of the spectra were made using a computer software in the instrument in order to obtain the positions of the overlapped bands. The curve-fitting used to quantify ratio of polysaccharides, proteins and lipid was based on a least-square method using Gaussian bands. The accuracy of the fit is given by the chi-square value, which the lower the value of $\chi^2$, the better fit (Galichet *et al*., 2001; Adt *et al*., 2006).

*Glucan content determination*

Glucan contents in yeast powder were analyzed using a Yeast Beta-Glucan Assay Kit (Megazyme, Ireland) as follows. For total glucan content, 10 mg of yeast cell were placed in eppendorf tube then 0.15 ml of 37% hydrochloric acid was added. The solution was mixed and incubated at 30°C for 45 min (vortexed every 15 min). Then, 1 ml of distilled water was added, mixed and incubated at 100°C for 2 h before added with 0.5 ml of 4 M KOH. The 200 µl solution was taken, adjusted volume to 1 ml with sodium acetate buffer pH 5 (800 µl) and mixed.

After that, the mixtures were centrifuged at 13,000 x g for 5 min. Samples (20 µl) were taken to each well (in duplicates) before added with 10 µl of a mixture of exo-1,3-β-glucanase plus β-glucosidase and then incubated at 37°C for 90 min. Finally, 200 µl of glucose oxidase/peroxidase were added and incubated at 37°C for 30 min. The absorbance was measured at 490 nm with microtiter plate reader. The concentration of glucose in the sample was calculated from D-glucose standard curve.

For β-glucan content, 100 mg of milled yeast were placed in test tubes. Then 2 M KOH (2 ml) was added and the pellets were stirred with magnetic stirrer in ice bath for 20 min. Next, 8 ml of 1.2 M sodium acetate buffer (pH 3.8) were added to the mixture. Then, 1 ml of sample was taken to an eppendorf tube. Amyloglucosidase plus invertase (20 µl) were added and incubated at 40°C for 30 min. After that, the mixture was centrifuged at 13,000 x g for 5 min. Supernatant (20 µl) were taken to microtiter plate. Glucose oxidase / peroxidase (200 µl) were added to each well and incubated at 37°C for 30 min. The absorbance was measured at 490 nm with microtiter plate reader. The concentration of glucose in the sample was calculated from the standard curve of D-glucose. For β-glucan content, it was calculated from total glucan minus β-glucan.

*β-Glucan extraction and characterization*

β-Glucan from yeast cells cultured with 100 ppm SDS was extracted. NaCl (3% w/v) was added to make 30% w/w cell suspension and incubated at 55°C, pH 5 for 24 h with mild agitation (120 rpm). Then, the mixture was heated to 85°C, maintained at this temperature for 15 min and cooled down to 25°C. The mixture was centrifuged at 4,500 x g for 10 min. Next, the cell debris was adjusted to 30% w/v with sodium phosphate buffer, 0.02 M, pH 7.5, with glass spheres (0.4 mm). The suspension was autoclaved at 121°C for 4 h. The insoluble cell wall residue was separated, washed three times, and centrifuged at 4,500 x g for 7 min at room temperature. After that, the residue (10 %w/v) in distilled water was sonicated at 20 KHz, 150 W in an ice batch for 6 min and centrifuged at 4,500 x g for 15 min at 10°C. Lipid was extracted with acetone by using cell wall residue:acetone as 1:1 (w/v) for 2 h and centrifuged at 4,500 x g for 7 min. Cell wall residue without lipid was washed with distilled water for 4 times and separated by centrifugation. Finally, 0.3% v/v of the cell wall residue was treated with 0.3% (w/v) of Savinase 16L type Ex (EC. 3.4.21.62) at 55°C pH 10 for 4 h to remove protein (Freimund et al., 2003; Pornchalearn, 2006; Liu et al., 2008; Magnani et al., 2009). The obtained β-glucan was freeze-dried, and then dissolved in DMSO for structural analysis using $^{13}$C-Nuclear Magnetic Resonance (NMR).

*Statistical analysis*

The data were collected from triplicates. Analysis was performed by statistical package SPSS 17 for windows, $p < 0.05$ (two-tailed) was considered as statistically significant. All of data were analyzed with Analysis of Variance (ANOVA) and multiple comparision T-test.

## Results and Discussion

*Yeast cell morphology*

The result of scanning electron microscopy showed that rounder cell shape and the highest number of bud scars was found when yeast was cultivated with 100 ppm SDS (treatment 2). The result corresponded with the aspect ratios from confocal microscope which were ca. 1.14 – 1.35 in all treatments and the lowest aspect ratio was also found when yeast was cultured with 100 ppm SDS (1.14 ± 0.05). This effect could be explained that the certain concentrations of SDS could activate FKS1 and PIM1 which are the gene that activate actin cytoskeleton and control cell size, respectively (Delley and Hall, 1999; Casano *et al.*, 2001). During this process, actin patches and actin cables synthesis are randomly distributed before budding process commence (Motizuki *et al.*, 2008). Thus, rounder cells increased due to actin cytoskeleton.

*Polysaccharide, protein and lipid ratio and β-(1,6) : (1,3) ratio*

From the FTIR spectrum of yeast cell, three main regions corresponding to polysaccharides (950 – 1,185 cm$^{-1}$), proteins (1,480 – 1,700 cm$^{-1}$) and lipid (2,840 – 3,000 cm$^{-1}$) were observed, which are in good agreement with the previous works (Adt *et al.*, 2006). It is worth noting that the spectra of yeast cell from all treatments including control possess a similar spectral pattern, indicating similar chemical composition but the absorbance intensity of each treatment looks slightly different. The attributions of main absorptions are characteristic of glucan structure (Šandula *et al.*, 1999; Burattini *et al.*, 2008), which related to OH stretching (3,000-3,500 cm$^{-1}$); CO stretching (1,028 cm$^{-1}$); C-O-C stretching (glycosidic linkages, 1,150 cm$^{-1}$); and β-anomeric carbon (890 cm$^{-1}$). Characteristic absorption of proteins was also observed at 1,654 (amide I) and 1,541 (amide II) cm$^{-1}$. N-H vibration of proteins which generally

expected at 3,400 $cm^{-1}$ could be overlapped by OH vibration as well as C-H stretching ($CH_2$ and $CH_3$) of lipid at about 2,925 $cm^{-1}$ could be overlapped by CH stretching of glucan.

The similar spectra were obtained from other treatments. The three regions observed were then analyzed separately using Fourier deconvolution, second derivative and curve fitting for extracting the hidden bands (data not shown). The following absorption bands were assigned according to previous works (Adt *et al*., 2006; Galichet *et al*., 2001). The band at 990 $cm^{-1}$ is assigned to β-1,6-glucan while two bands at 1,078 and 1,097 $cm^{-1}$ belongs to β-1,3-glucan. Mannan in cell wall mannoproteins and glycosidic linkage (C-O-C) appears at 969 and 1,150 $cm^{-1}$, respectively (Adt *et al.*, 2006; Burattini *et al*., 2008). Twelve bands were obtained from curve-fitting the data in protein regions (1,480-1,700 $cm^{-1}$). The band ranging approximately 1,500 $cm^{-1}$ are assigned to amide II (C=O) of protein and/or chitin whereas amide I (N-H) is identified at ca. 1,600 $cm^{-1}$. For lipid region (2,840-3,000 $cm^{-1}$), peaks in this region are attributed to C-H stretching modes; asymmetric $–CH_2$ and $–CH_3$ assigned at 2,925 and 2,955 $cm^{-1}$, respectively while symmetric ones located at 2,852 and 2,868 $cm^{-1}$, respectively. Peak at 2,896 $cm^{-1}$ corresponded to C-H deformation of $CH_3$ (Burattini *et al*., 2008). Then the normalized total area from curve-fitting of polysaccharide, protein and lipid regions was carried out and calculated as a ratio of three main components (Table 2). The results corresponded well with absorbance intensity of the FTIR spectra showing the highest intensity in polysaccharide region, followed by protein and lipid regions, respectively. Based on the same amount of lipid, the polysaccharide was shown approximately 2 fold higher than protein in all of treatments. The ratio of polysaccharide to protein showed higher than control and other treatments when yeast was cultivated with 100 ppm SDS (treatment 2), implying lower protein content in yeast cell. Because some protein in cell wall can be extracted with SDS, so the low protein was observed (Montijn *et al*., 1994). The higher amount of polysaccharide found when yeast cultured with some additives could possibly result from the stress. Since the stress could increase the cell wall formation, making polysaccharide increase. However, the lower polysaccharide than control were found when yeast was cultured with 30,000 ppm NaCl (treatment 4) and combination of 20 ppm SDS and 3,000 ppm NaCl (treatment 6). Because in hyperosmotic condition of NaCl resulted in cell shrinkage, loss of viability cell and thus stimulated trehalose turnovered (Attfield, 1977; Parrou *et al.*, 1997).

Table 2. Polysaccharide:protein:lipid ratio in cells of *S. cerevisiae* Angel® cultured in YPD medium supplemented with additives at different concentrations for 24 h

| Treatments | Normalized total area of different regions | | | Polysaccharide: protein: lipid ratio | β-(1,6) : β-1,3) ratio |
|---|---|---|---|---|---|
| | Polysaccharide | Protein | Lipid | | |
| 1(control) | 0.37 | 0.19 | 0.03 | 12.3:6.3:1.0 | 1.63 |
| 2 | 0.38 | 0.18 | 0.03 | 12.7:6.0:1.0 | 2.08 |
| 3 | 0.38 | 0.20 | 0.03 | 12.7:6.7:1.0 | 1.59 |
| 4 | 0.35 | 0.19 | 0.03 | 11.7:6.3:1.0 | 2.57 |
| 5 | 0.37 | 0.19 | 0.03 | 12.3:6.3:1.0 | 1.84 |
| 6 | 0.39 | 0.20 | 0.04 | 9.8:5.0:1.0 | 2.24 |
| 7 | 0.37 | 0.20 | 0.03 | 12.3:6.7:1.0 | 2.04 |

From deeply quantitative investigation of three principal components in yeast cell wall, it is interesting that mannan decreased when yeast cultured with additives, in contrast, β-(1,6)-glucan increased, implying more branching occurred in the glucan structure and less mannoproteins when yeast cultivated with studied additives. When focusing on β-(1,6) : β-(1,3) ratio in cells of *S. cerevisiae* Angel® cultured in YPD medium supplemented with additives (Table 2), the two bands identified at ca. 990 $cm^{-1}$ and ca.1,078 $cm^{-1}$ which assigned to β-(1,6)-glucan and β-(1,3)-glucan, respectively were used for evaluation. The result showed that the β-(1,6)-glucan to β-(1,3)-glucan ratio was higher than control when yeast were cultivated with additives. The highest β-(1,6)-glucan to β-(1,3)-glucan ratio (ca. 1.6 times higher than control) was observed when yeast was cultivated with 30,000 ppm NaCl (treatment 4), followed by combination of 20 ppm SDS and 3,000 ppm NaCl (treatment 6) (ca. 1.4 times higher than control). This could be explained that cells probably try to adjust themselves to the stress condition by synthesizing the highly branching cell wall. Since the higher β-(1,6)-glucan is expected to increase the rigidity of the glucan matrix and make cell wall stronger (Jamas, Rha and Sinskey, 1986; Smits and Brul, 2005). With exception when yeast was cultivated with 50 ppm EDTA (treatment 3), the β-(1,6)-glucan to β-(1,3)-glucan ratio was slightly lower than control. This result can be explained that EDTA stimulates β-(1,3)-glucan synthase, so the β-(1,3)-glucan is higher therefore the lowest ratio of β-(1,6)-glucan to β-(1,3)-glucan was observed (Leal *et al*., 1984; Guillen *et al*., 1985).

### β-*Glucan production*

Compared with control (treatment 1), yeasts were firstly cultured in media supplemented with sole additives such as EDTA, SDS, and NaCl with 50, 100, and 30,000 ppm, respectively (treatment 2-4).

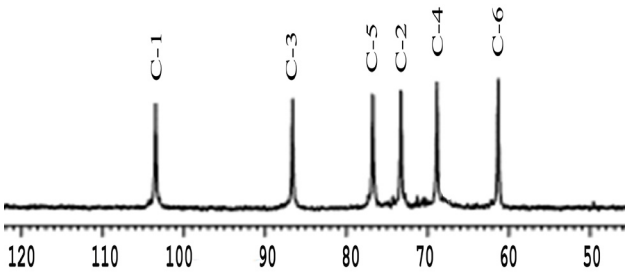


Figure 1. $^{13}C$ NMR spectrum of β-glucan extracted from yeast when cultured with 100 ppm SDS (treatment 2)

It was found that β-glucan contents of treatments 2-4 were higher than that of control. In particular, treatment 2 which is YPD medium supplemented with 100 ppm SDS showed significantly highest β-glucan content (8.16 %w/w, ca. 39% higher than control) while the β-glucan contents in medium supplemented with 50 ppm EDTA (treatment 3) and 30,000 ppm NaCl (treatment 4) were not significant different (ca. 6 %w/w). The result corresponded well with the previous studies which reported that a detergent, SDS, could act on the plasma membrane, which could increase β-1,3-glucan synthase activity and stimulate depolarizing of β-glucan synthase gene (FKS1) (Frost *et al*., 1992; Frost *et al*., 1994; Delley and Hall, 1999; Casano *et al.*, 2001; Lesage and Bussey, 2006). Consequently, the budding process, cell wall synthesis, the number of cells and β-glucan content were increased (as well as found in this study). Cell wall is biosynthesized mostly during budding process (Frost *et al*., 1994; Cabib, 2001).

From this point, effects of SDS combination with EDTA and NaCl (treatment 5-7) were further monitored. Since it has been reported that the addition of EDTA, fluoride and GTP to culture medium stimulates β-1,3-glucan synthase activity in *S. cerevisiae* (Leal *et al*., 1984). Furthermore, several reports stated that NaCl could stimulate phosphoglucomutase (PGM2) and uridine diphosphoglucose pyrophosphorylase (UGPase) activity, which are enzymes involved in the UDP-glucose synthesis and trehalose accumulation. In addition, increasing of the saline level also increased ACT1 gene translation (actin biosynthesis) (Blomberg, 1995, 2000). It clearly showed that β-glucan content in yeast cultivated in media supplemented with SDS combinations slightly decreased, compared with sole SDS condition. However, they are still higher than control. In conclusion, when compared with b-glucan content in control, there were only 3 treatments which could significantly enhance the β-glucan production: 100 ppm SDS (treatment 2, ca. 40%), a combination of 20 ppm SDS and 3,000 ppm NaCl (treatment 6, ca. 27%) and a combination of three additives; SDS, EDTA, and NaCl (treatment 7, ca. 15 %).

Thus, it is clear that additives could activate β-glucan formation and cell wall synthesis of yeast cell, which could be explained that yeast under stress conditions tried to produce larger amount of cell wall component to protect cell from stress. Therefore, β-glucan synthase is redistributed in response to cell wall stress to repair general cell wall damage (Delley and Hall, 1999; Smits *et al*., 2001, Klis *et al*., 2002; Hohmann, 2003).

β-*Glucan extraction and characterization*

From the obtained results, it was found that *S. cerevisiae* Angel® when cultured with 100 ppm SDS (treatment 2) could give the highest β-glucan. As a consequence, the yeast cells from this treatment were used as a starting material for β-glucan isolation. Then, the extracted β-glucan was characterized using $^{13}C$-NMR. Figure 1 shows 13C-NMR spectrum of β-glucan extracted from yeast when cultured with 100 ppm SDS and the peak assignments are as follows: signals at 103, 86, 76, 73, 68, and 61 ppm corresponding to C1 (β-configuration), C3, C5, C2, C4, and C6, respectively (Tada *et al*., 2007). Moreover, it should be noted that no protein and lipid was detected since they were eliminated before extraction.

## Conclusions

β-Glucan production in *S. cerevisiae* Angel® increased when yeast was cultured in a medium supplemented with the studied additives (EDTA, SDS, and NaCl). The best condition was revealed at 100 ppm of SDS, giving the highest β-glucan content (40% higher than control). Moreover, in this treatment, significant change in cell morphology was found, which cells became more spherical and possessed a high number of bud scars, compared with control and other treatments. Concerning composition of cell wall, an increase in β-glucan content with also high β-(1,6)-glucan branching but less extent in mannans and proteins were found. These results indicated that additives could activate cell wall productions, branching and β-glucan synthesis in yeast cells.

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