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Research Article

Selection of *Bacillus subtilis* strains capable of producing β-glucanase supporting the hydrolysis of yeast cell walls

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Abstract

The selection of *B. subtilis* strains was carried out with 15 strains from the collections of the Vietnam National University of Agriculture and the University of Economics-Technology for Industries, Hanoi, Vietnam. To investigate the specific activity of β-glucanase in supporting the hydrolysis of beer yeast cells, CMC substrate in enzyme-activated test media of traditional methods was replaced by yeast cell walls in this study. The *B. subtilis* strains were activated on Nutrient Broth culture and then transplanted into MT3 culture for producing β-glucanase. Optical density (OD600nm) measurement was used to estimate the bacterial cell density. The β-glucanase activity formed by bacteria cells free supernatant was quantified by agar diffusion method on the enzyme-activated test media MT4. Two *B. subtilis* strains of BG21 and BG15 were selected based on their largest clear-zones on agar plates. By modifying the values of the affecting factors and keeping the remaining influencing factor sun changed, it was determined that the *B. subtilis* BG21 and BG15 strains produced the highest biomass at the conditions of the culture time of 24 and 28 hrs, at pH medium of 7.0, an orbital shaking incubator of 200 rpm and at 37ºC, respectively. Furthermore, the highest β-glucanase activity of the two strains BG21 and BG15 was exhibited at the culture time of 56 and 52 hrs, at pH medium of 7.0, an orbital shaking incubator of 200 rpm and at 37ºC, respectively. Practical applications: The highest β-glucanase producing *Bacillus subtilis* strains will be used for the production of biological products containing *B. subtilis* spores and crude β-glucanase which supports the hydrolysis of the beer yeast cells in animal feed production.

Keywords: *Bacillus subtilis*, clear-zone diameter, yeast cell wall, bacterial cell density, β-glucanase activity.

Abbreviations:

CMC - Carboxymethyl Cellulose
OD - Optical density

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Introduction

Beer yeast extract has been widely studied and applied on a global scale to produce microbiological cultures, food additives, functional foods, and animal feeds (Trinh Vinh Hien 2010). The cells hydrolysis can be carried out by autolysis or using enzyme preparations. Yeast cell wall is a gluco-manano-lipo-protein complex. The cell wall glucans are polymeric structures in which monomers are glucose bounded by β-1.3 and β-1.6 linkages (Popova 1992). β-glucan has been scientifically recognized as a

stimulant of the immune system with the ability to bind and activate specialized cells that are related to the immune system. By triggering the responses, β-glucan increases the body's immunity against infection and disease control ability (ICFOOD 2014). In order to break down the yeast cell walls, the degradation of glucan by the β-glucanase from microbial sources is considered to be the most commonly applied method. *B. subtilis* strains are distributed in the natural environment, mostly in the soil, the straw, and the grass, so it is referred to as "grass bacillus", which produces many enzymes that are beneficial to the digestive tract. Today, *B. subtilis* is widely used in the production of probiotic products added into pig feed (Ly Kim Huu 2005). The selection of safe *B. subtilis* strains capable of producing β-glucanase supporting the hydrolysis of β-glucan in the beer yeast cell walls was investigated in this paper.

Materials and Methods

Materials

Bacillus subtilis strains: The selection was carried out on 15 *Bacillus subtilis* strains from the collections of the Vietnam National University of Agriculture and the University of Economics-Technology for Industries. The *B. subtilis* strains were stored in glycerol stock 50% in a sterile Eppendorf Tube at -35°C.

Yeast cell walls: The beer yeast was cleaned off the residue of crude, suspended solids, bitter substances, and dead cells. The autolysis was carried out by adding water at a rate of yeast: water of 1:3, at 52°C, pH 5.8 for 22 hrs. Yeast cell wall was obtained after centrifugation and drying at 60°C (Nguyen Thi Thanh Thuy and Ho Tuan Anh 2017).

Culture media of microorganisms:

Basal culture media NB (g/l): Peptone (10), NaCl (5), and meat extract (5.5).

Basal culture media MT2 (Lisdiyanti et al. 2012) (g/l): Carboxymethyl cellulose (CMC) (10), D-glucose (1), yeast extract (2), KH_2PO_4 (0.5), $MgSO_4.7H_2O$ (0.2), $CaCl_2$ (0.04), $FeSO_4.7H_2O$ (0.02), and KNO_3 (0.75).

Culture media MT3: The culture was similar to MT2 but CMC was replaced with the yeast cell wall as a substrate.

Basal culture agar media MT4 for testing the activity of β-glucanase according to the method of Quyen Dinh Thi and Do Thi Tuyen (2014) with small modifications (g/l): yeast cell wall replaced of CMC (2), yeast extract (0.25), KH_2PO_4 (3.5), $MgSO_4$ (6.25), KNO_3 (5), and Agar (20).

In this study, we investigated the capacity of producing β-glucanase of *B. subtilis* strains which supports the hydrolysis of beer yeast cells, CMC substrate was replaced by yeast cell walls. Basal culture media MT4 was adjusted to pH 7.0 and autoclaved at 121°C for 20 min before being poured into a petri plate with ~ 4 mm in thickness. Each plate was punched with round holes of 7 mm in diameter.

Methods

Selection of *B. subtilis* strains produced high β-glucanase activity supporting the hydrolysis of yeast cell walls (as a substrate). The *B. subtilis* strains were activated on NB basal culture media and transplanted into culture media MT3 for producing β-glucanase at 200 rpm, 37°C for 50 hrs with inoculation ratio of 1% (v/v). After centrifuging for 15 min at 8000 rpm, the cell-free supernatant was introduced into media MT4 agar to examine the β-glucanase activity according to the agar diffusion method. The volume of 100 µl of the supernatant was poured into the 7 mm-diameter holes on the MT4 agar plates. The plates were then incubated at

4°C for 30 min to diffuse the enzyme into the agar holes, and incubated at 55°C for 12 hrs. After incubation, the plates were flooded by Lugol solution of 1%, left for 30 min. Following that, Lugol was removed to observe clear zone. A clear zone was formed in the β-glucan degradation area around the hole due to no coloration with Lugol. The β-glucanase activity was expressed by the difference of clear zone diameter and the diameter of the hole D-d (mm) (D was the clear-zone diameter and d was the hole diameter).

Determination of cell density in the suspension of *B. subtilis* by OD measurement. The OD600nm of the suspension is proportional to the cell density. After being activated on NB culture, the *B. subtilis* strains were incubated in MT3 media. The bacterial density was evaluated by the OD600nm measurement.

Determination of factors affecting the biomass growth of *B. subtilis.* *B. subtilis* strains were incubated on NB media at a rate of 200 rpm for 24 h at 37°C, 1% transplanting rate and transplanted into the β-glucanase producing culture media MT3. In various experimental series, the influenced factors were tested:

- Culture time at 16, 20, 24, 28, and 32 hrs;
- pH culture media of 5.0, 6.0, 7.0, 8.0 and 9.0;
- Incubation temperature of 23, 30, 37, 44 and 51°C.

Determination of factors affecting the β-glucanase production of *B. subtilis*. According to the above mentioned method, *B. subtilis* strains were incubated in culture media MT3. In different experiments, the experimental factors included the following:

- Culture time: 40, 44, 48, 52, 56 and 60 hrs;
- pH of the culture media: 5.0, 6.0, 7.0, 8.0, and 9.0;
- Incubation temperature: 23, 30, 37, 44, and 51°C.

Results and Discussion

The ability of *B. subtilis* strains to produce β-glucanase was based on the principle that degraded β-glucan by endo-β-glucanase present in the agar plate will not form coloration with Lugol.

1. Selection of *B. subtilis* strains produced β-glucanase for hydrolyzing of yeast cell walls.

B. subtilis strains of the collection have been identified based on the morphological, physiological and biochemical characteristics. After activation and cultivation in the enzyme-producing media MT3, the activity of the β-glucanase was expressed by the difference of clear-zone diameter and the diameter of the hole formed on the agar plate in the culture media MT4. The results of the selection of *B. subtilis* strains producing β-glucanase for hydrolysing of yeast cell walls are presented in Table 1.

Table 1. Clear zone diameter of β-glucanase from different *B. subtilis* strains

Code of strains	Clear zone diameter D-d (mm)	Code of strains	Clear zone diameter D-d (mm)
BG8	10.2^{e*}	BG16	9.3^{f}
BG9	9.2^{f}	BG17	6.3^{i}
BG10	11.2^{d}	BG18	11.1^{d}
BG11	12.1^{c}	BG19	8.2^{g}
BG12	8.1^{g}	BG20	12.1^{c}
BG13	10.1^{e}	**BG21**	$\mathbf{14.2^{b}}$
BG14	7.3^{h}	BG22	11.1^{d}
BG15	$\mathbf{16.2^{a}}$		

*Means with the same letter are not significantly different from each other ($p<0.05$).

As illustrated by Table 1, the clear zone diameter of the collected strains ranged widely from 6 to 16 mm. The strains BG15 and BG21 produced the highest enzyme activity for the substrate is the yeast cell

walls. For the substrate of yeast cell walls, this is a completely new substrate that has not been previously published when testing the activity of β-glucanases, which was evident by the largest clear zone diameters of 16.2 and 14.2 mm, respectively. The two strains, BG15 and BG21, were selected for further experiments.

2. Determination of suitable conditions for the highest biomass growth of selected *B. subtilis* strains.

Factors influencing the biomass growth of *B. subtilis* during incubation consist of temperature, pH, and incubation time, etc. The examination of biomass growth was carried out by measuring the OD of the microbial suspension when modifying the values of the experimental factor and keeping the remaining influencing factors unchanged.

2.1. Influence of culture time on biomass growth of the selected *B. subtilis* strains.

In order to identify the suitable conditions for biomass growth of selected *B. subtilis*, the selected *B. subtilis* strains were incubated at 200 rpm on basal culture media NB at 37°C for 24 hrs, and then transferred into media MT3 to be cultured at 200 rpm, pH 7.0, at 37°C, the experimental factor was culture time which varied from 16, 20, 24, 28, to 32 hrs. The measured OD of the suspension of *B. subtilis* during incubation was shown in Figure 1.

The incubation time of *B. subtilis* BG15 strain at 28 hrs and its BG21 strain at 24 hrs, the value of OD600nm was higher than that of other culture times with the OD of 2.23 and 1.86, respectively. The increase in the culture time led to insignificantly changes of the OD value. Thus, the suitable culture time was determined at 28 hrs and 24hrs for BG15 strain and BG21 strain, respectively.

2.2. Influence of pH on the biomass growth of the selected *B. subtilis* strains.

According to the method described in section Materials and Methods and obtained results from 2.1, in order to examine the effect of pH of culture media MT3 on the biomass growth, the pH of culture varied from 5.0 to 9.0, with an interval of 1.0. The values of OD during incubation at different pH were shown in Figure 2.

As can be seen from Figure 2, the pH of the culture media affects the biomass growth ability of *B. subtilis*. The measured results showed that the OD values varied in a large interval, which revealed the influence of the pH on the metabolism and reproduction of bacteria. Both strains were grown in the almost examined pH medium, indicating that two *B. subtilis* strains can grow at wide pH range. At pH 7.0, the OD value was the highest of 2.42 and 1.97 for the strains BG15 and BG21, respectively. Therefore, pH 7.0 was selected for the later experiments. The studied results were similar to the findings of Nguyen Thi Thanh Thuy et al. (2016) who studied the influence of pH on the biomass production ability of *B. subtilis*.

2.3. Influence of incubation temperature on biomass growth of the selected *B. subtilis* strains.

In this series of experiments, *B. subtilis* strains were incubated on media MT3 for biomass production, the incubation temperature was experimented in different values of 23, 30, 37, 44 and 51°C. After the incubation, the OD of the bacterial cell suspension was measured in order to evaluate the biomass production of the selected strains (Fig. 3).

The results in Figure 3 showed that the incubation temperature had a considerable influence on the biomass production of *B. subtilis*. At 37°C, two *B. subtilis* strains BG15 and BG21 had the highest biomass, with the OD value of 2.37 and 1.90, respectively. At 51°C, the growth rate of both strains decreased significantly. The obtained results were similar to the findings of Ly Kim Huu (2005) in a study on the effects of temperature, pH, and incubation time on the growth of *B. subtilis*.

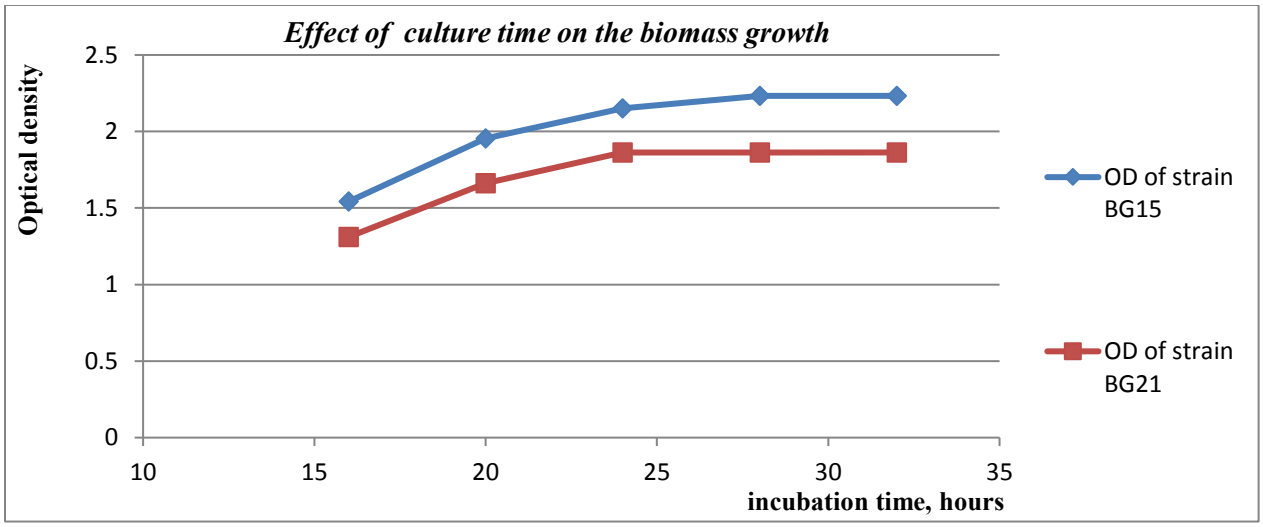

Figure 1. Optical density (OD600nm) of *B. subtilis* cell suspension during culture time (hours)

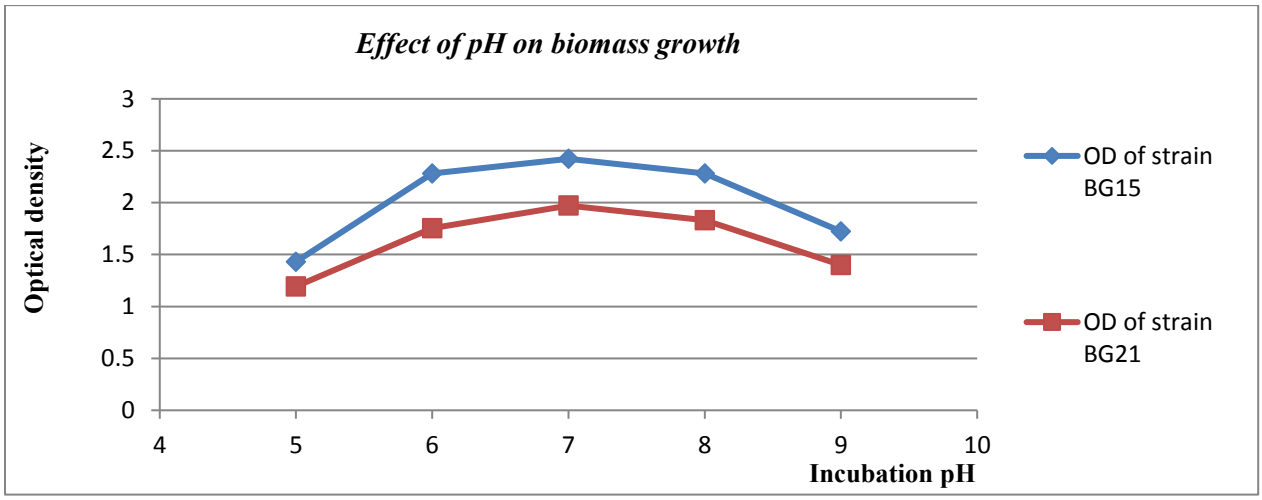

Figure 2. Optical density (OD600nm) of *B. subtilis* cell suspension at pH 5.0-9.0

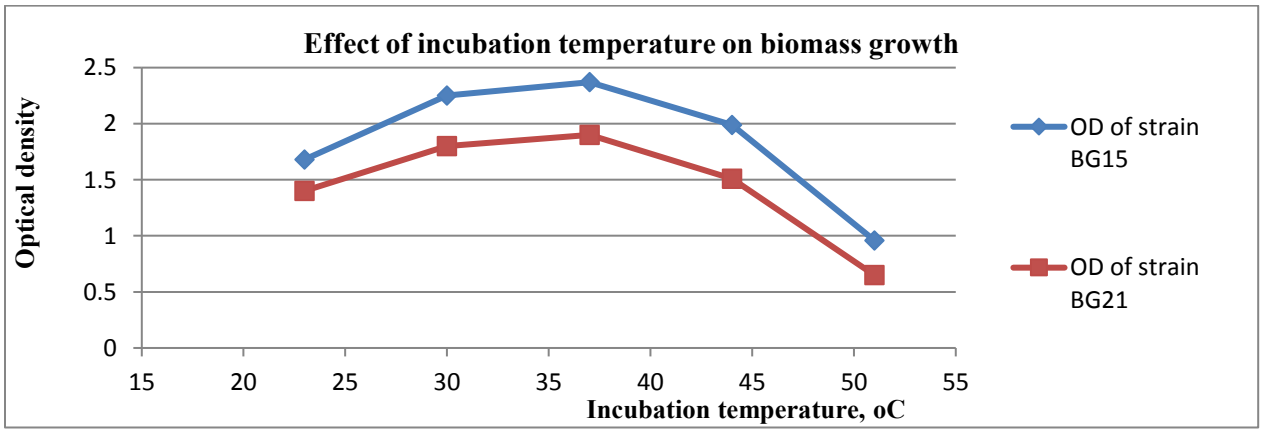

Figure 3. Optical density (OD600nm) of *B. subtilis* cell suspension at incubation temperature of 23-51^{o}C

From the obtained results of the investigation of the effects of time, pH and temperature during incubation, the suitable conditions for the highest biomass production of the *B. subtilis* strains BG15 and BG21 were culture media MT3, at pH 7.0, 200 rpm, incubation temperature of 37°C, culture time of 28 hrs (BG15) and 24 hrs (BG21).

3. Identification of the suitable conditions for β-glucanase production by the selected *B. subtilis* strains.

The *B. subtilis* strains were cultured following the described method, β-glucanase was produced in media MT3. The β-glucanase activity was evaluated by the method of clear zones on agar plates of media MT4.

3.1. Effect of incubation time on β-glucanase production ability of *B. subtilis* strains.

In this series of experiments, *B. subtilis* strains were cultured on media MT3, at an orbital shaking incubator of 200 rpm, at pH 7.0 and at 37ºC. The experimental factor was incubation time which varied in the interval of 40, 44, 48, 52, 56, and 60 hrs. The β-glucanase activity was evaluated by the agar diffusion method on the media MT4, the results were shown in Table 2.

Table 2. Effect of incubation time on clear zone diameter of β-glucanase

Incubation time (h)	Clear zone diameter D-d (mm)	
	BG15	**BG21**
40	12.3[d]*	9.3[e]
44	14.2[c]	11.1[d]
48	16.1[b]	13.1[c]
52	**17.1[a]**	14.1[b]
56	17.2[a]	**15.2[a]**
60	16.2[b]	15.1[a]

*Means with the same letter in the same column are not significantly different from each other *($p<0.05$)*.

The obtained data in Table 2 showed that, after 52 and 56 hrs of incubation, the two strains of B. *subtilis* produced the higher levels of β-glucanase than that of previous incubation times, as can be seen from the clear zone diameter of 17.1 mm (BG15) and 15.2 mm (BG21), respectively. The clear zone of 17.1 mm in diameter of BG15 indicated a higher enzyme activity than those of Do Thu Ha (2004). Therefore, it can be concluded that the suitable incubation time was established at 52 and 56 hrs for BG15 and BG21, respectively. Compared to previous research by Dewi et al. (2016) reported that the strain *B. subtilis* SAHA 32.6 produced the highest β-glucanase activity in the medium containing 1% β-glucan after 12 hrs of incubation, the culture time of strain BG15 and BG21 was significantly longer.

3.2. Effects of pH on the enzyme production ability of *B. subtilis* strains.

By the obtained results from 3.1, in this series of experiments, the experimental factor was pH.

At different pH values, β-glucanase activity of the two strains of *B. subtilis* was variable (Table 3).

The obtained results showed that both strains produced β-glucanase at different pH values. At pH 5.0 and at pH 9.0, the clear zone diameter significantly decreased in comparison with pH 7.0. This finding was consistent with the previously published study on the effect of pH on the metabolism of *B. subtilis* (Ly Kim Huu 2005).

At pH 7.0, the clear zone diameter of *B. subtilis* BG15 and BG21 reached the maximum values of 16.1 and 15.2 mm, respectively. The optimum pH 7.0 value was used for further experiments.

3.3. Effects of incubation temperature on enzyme production ability of *B. subtilis* strains.

The *B. subtilis* strains were cultured at pH 7.0, incubation time of 52 hrs (strain BG15) and 56 hrs (strain BG21). The incubation time was varied of 23, 30, 37, 44, and 51ºC. The measured clear zone diameters after incubation at different temperatures were shown in Table 4.

Table 3. Effect of pH on clear zone diameter of β-glucanase

pH	Clear zone diameter D-d (mm)	
	BG15	**BG21**
5.0	7.1[d*]	6.2[d]
6.0	14.1[b]	13.2[b]
7.0	**16.1[a]**	**15.2[a]**
8.0	14.2[b]	13.2[b]
9.0	9.1[c]	9.2[c]

*Means with the same letter in the same column are not significantly different from each other *(p<0.05)*.

Table 4. Effect of incubation temperature on clear zone diameter of β-glucanase

Incubation temperature (°C)	Clear zone diameter D-d (mm)	
	BG15	**BG21**
23	8.1[d*]	6.1[d]
30	12.1[b]	11.3[b]
37	**17.1[a]**	**16.3[a]**
44	10.2[c]	9.2[c]
51	5.2[e]	3.1[e]

*Means with the same letter in the same column are not significantly different from each other *(p<0.05)*.

At 37°C, the clear zone diameters of the two strains BG15 and BG21 reached the highest value as compared to other culture temperatures, 17.1 and 16.3 mm, respectively. The decreasing enzyme production was observed at 44°C. At 51°C, the obtained enzyme yield of both strains decreased significantly. The suitable temperature of 37°C for both *B. subtilis* BG15 and BG21 to produce high β-glucanase was chosen. With the use of the CMC substrate, under the influence of similar conditions of incubation temperature and pH, the clear zone diameter was higher than the obtained results in Table 4 (Nguyen Thi Thanh Thuy et al. 2016). It is believed that the yeast cell walls are more difficult to be hydrolysed than CMC because of the enzymes formed during the incubation of *B. subtilis*.

From the results of the investigation of the effects of incubation time, pH, and incubation temperatures on the β-glucanase production ability of both *B. subtilis* BG15 and BG21 strains, the suitable conditions for the production of β-glucanase were media MT3, pH 7.0, 200 rpm at 37°C, and for an incubation time of 52 hrs (BG15) or 56 hrs (BG21).

The strain BG15 exhibited the largest clear zone diameter on agar plate containing yeast cell walls (as a substrate) was selected for subsequent studies (Figure 4).

Figure 4. The β-glucanase activity against yeast cell walls from strain of BG15 and BG21

Conclusions

The highest biomass of *B. subtilis* strains BG21 and BG15 could be obtained after culture time of 24 and 28 h, respectively, in the same culture condition of culture media MT3, at pH 7, 200 rpm and at 37°C. Moreover, the highest yield of β-glucanase was found at the incubation time of 52 and 56 hrs, respectively, at pH 7.0, 200 rpm and at 37°C in the same medium. The *B. subtilis* strain BG15 was selected for further studies based on the highest clear zone diameter of β-glucanase against yeast cell walls.

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