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Characteristics of β-glucosidase production by *Paecilomyces variotii* and its potential application in bioassay system for boric acid determination

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This study reports the potential application of *Paecilomyces variotii* immobilized in calcium alginate beads as a sensing element in the analysis of boric acid. In the presence of boric acid, β-glucosidase production of *P. variotii* was inhibited and the changes of β-glucosidase concentration were correlated to the concentrations of boric acid. The optimum conditions of β-glucosidase production were observed when 6% (w/v) mycelia of *P. variotii* was immobilized in 2% (w/v) sodium alginate with 0.25 M calcium chloride, at initial pH 7 and temperature 45°C. The response time of less than 3 h exhibited by *P. variotii* towards boric acid was observed. A linear response range of boric acid concentration was obtained within the range of 0.05 to 0.215% (w/v), with a detection limit of 0.037% (w/v). The immobilized cell beads were considered reproducible with the relative standard deviation (RSD) of 4.96 and 4.81% in the presence (0.2% w/v) and absence of boric acid, respectively. From the results, *P. variotii* could be beneficially useful towards developing an alternative approach for boric acid analysis.

Key words: Microbial bioassay, boric acid, *Paecilomyces variotii*, inhibition, β-glucosidase.

INTRODUCTION

Boric acid is a boron compound that is normally used as a chemical preservative. This chemical has been used as pesticide and food additives, as well as compound in cleaning formulations (van Staden and Tsanwani, 2002). However, boric acid has been declared unsafe as food additive by the FAO/WHO expert committee as this chemical exhibits cumulative toxicity effect (Yiu et al. 2008). Boric acid is dangerous to human health if,

consumed in considerably large quantity (Yiu et al., 2008; van Staden and Tsanwani, 2002). In Malaysia, boric acid has been reported in several local food products such as yellow noodles and fish balls (Yiu et al., 2008; Siti-Mizura et al., 1991; See et al., 2010). Due to its toxicity to human, the detection of boric acid especially in food products is necessary for the prevention of boric acid poisoning incident.

Although, the importance of the determination of boric acid in the food industry was affirmed, only chemical methods are available for the determination of boric acid such as titration, spectrophotometric method based on curcumin, atomic absorption spectrophotometry (Yiu et

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al., 2008; Williams, 2000; Siti-Mizura et al., 1991), and gas chromatography-mass spectrometry (Zeng et al., 2009). These methods are reliable and well established, however some of them are affected by numerous interferences, expensive, and time consuming as they require complicated sample preparation steps (Zeng et al., 2009; van Staden and Tsanwani, 2002; Siti-Mizura et al., 1991). Additionally, these techniques increase the exposure of operators to chemicals such as sulphuric acid and chloroform, which are hazardous to health if handled improperly. Hence, development of a new method of boric acid determination for a simple, inexpensive and less chemically hazardous is desirable and thus prompted us to investigate this approach.

The use of microbes as biological sensing elements has recently been found to be an attractive approach (Odaci et al., 2009). Microorganisms are widely used in the construction of biosensor as they have unique abilities to adapt to adverse environmental conditions (D'Souza, 2001). In microbial sensor, the metabolic activity of the microorganisms is measured. The presence of toxic substances in the system would affect the microorganisms, where such effects can then be transformed into measureable signals (Frense et al., 1998). Apart from that using a whole cell system also offers several advantages such as simplicity in preparation and inexpensive construction of the system. The enzymes are also stable in the cells as they are within their natural environment (Tkac et al., 2003; Odaci et al., 2009; Frense et al., 1998; D'Souza, 2001).

Paecilomyces variotii is used as a biological component in this study since it tolerates certain concentrations of boric acid as shown in our previous study (Ang et al., 2011). The microorganism was then immobilized in calcium alginate beads as the matrix provides a gentle environment for the cells. Moreover, immobilization method using calcium alginate is inexpensive, easy to handle and non-toxic to cells (Idris and Suzana, 2006; Hsieh et al., 2007). This method has been used extensively either in cell or enzyme immobilization (Bhushan et al., 2008). By using the knowledge of biosensor and microbial biotechnology, we attempt to develop a bioassay system utilizing *P. variotii* which is simple and cost effective for determination of boric acid. In this study, optimization of β-glucosidase production was performed, followed by the investigation on the response of the cells towards boric acid. This study was based on the entrapment of boric acid sensitive *P. variotii* into calcium alginate beads. The inhibition effect of boric acid on β-glucosidase activity of *P. variotii* was measured and then correlated with the quantity of boric acid to investigate its potential application in bioassay system.

MATERIALS AND METHODS

Materials

Potato dextrose agar, sodium chloride, boric acid and Tween 80

were purchased from Merck. Cellobiose, 4-nitrophenyl-b-D-glucoside, calcium chloride and sodium alginate were acquired from Sigma. Potassium chloride, potassium dihydrogen phosphate, ferrum sulphite, magnesium sulphate, sodium nitrate and sodium hydrogen phosphate were obtained from Fisher Scientific.

Microorganism

Paecilomyces variotii used in this research was obtained from American type culture collection (ATCC 62398). The culture was maintained on potato dextrose agar at 4°C. Stocks were sub-cultured at 3 month intervals.

Preparation of inoculums and cell harvesting

Spore suspension was prepared by transferring the spores from a 5-day-old growing colony on potato dextrose agar (PDA) into 50 ml sterile distilled water containing 0.1% (v/v) Tween 80. One ml (1 x 10^7 spore/ml) of the spore suspension was transferred aseptically into 250 ml Erlenmeyer flask containing 49 ml sterile production medium. The medium consisted of (g/l): 0.4% (w/v) cellobiose, 1.5 g KH_2PO_4, 0.5 g $MgSO_4·7H_2O$, 0.5 g KCl, 0.01 g $FeSO_4·7H_2O$, 2 g $NaNO_3$. The cells were grown for 48 h at 37°C on a rotary shaker at 150 rpm. The mycelia cells were then harvested by centrifugation at 10,000 x g for 10 min and washed with 0.85% (w/v) NaCl saline solution. The harvested mycelia were used for immobilization.

Cell immobilization in alginate beads

Two days old culture at the exponential growth phase (Figure 1) was used for cell immobilization. The procedures proposed by Anisha and Prema (2008) and Hsieh et al. (2007) were used in cell immobilization with slight modification. Approximately 1.5 g of mycelia (wet weight) were mixed thoroughly with 3 ml of sodium alginate solution and stirred to obtain a homogeneous mixture. The mixture was then placed into a syringe and allowed to drop into 100 ml 0.25 M $CaCl_2$ solution at room temperature. The beads formed were allowed to cure in the $CaCl_2$ solution overnight at 4°C and then washed with saline solution to remove the excess of $CaCl_2$ and free cells. The average diameter of beads was 3 to 4 mm. All procedures were carried out under sterile conditions.

β- Glucosidase assay

The β- glucosidase assay was based on the procedure described by Parker et al. (1999). Enzyme activity was determined by using 1 ml of 10 mM 4-nitrophenyl-b-D-glucoside in 50 mM acetate buffer pH 4.5 as substrate and 500 µl of enzyme extract. The mixture was incubated at 40°C for 10 min. Then, 2 ml of 1 M $NaHCO_3$ was added to stop the reaction. Absorbance of 4-nitrophenol at 410 nm was measured.

Optimization of β-glucosidase production

In order to obtain the optimum conditions of β-glucosidase production by *P. variotii*, several important factors such as initial pH of medium, temperature, amount of cell loading, concentration of sodium alginate and calcium chloride were investigated for this purpose. The study was performed in the medium at pH 4, 5, 6, 7, 8, and 9; temperature 25, 30, 35, 37, 40, 45, 50, and 55°C; concentration of sodium alginate at 1, 2, 3, 4 and 5% (w/v); concentration of calcium alginate at 0.05, 0.1, 0.25, 0.5, 1.0, and 1.5 M; and cell biomass loading at 2, 4, 6, 8, 10, 12 and 14% (w/v).
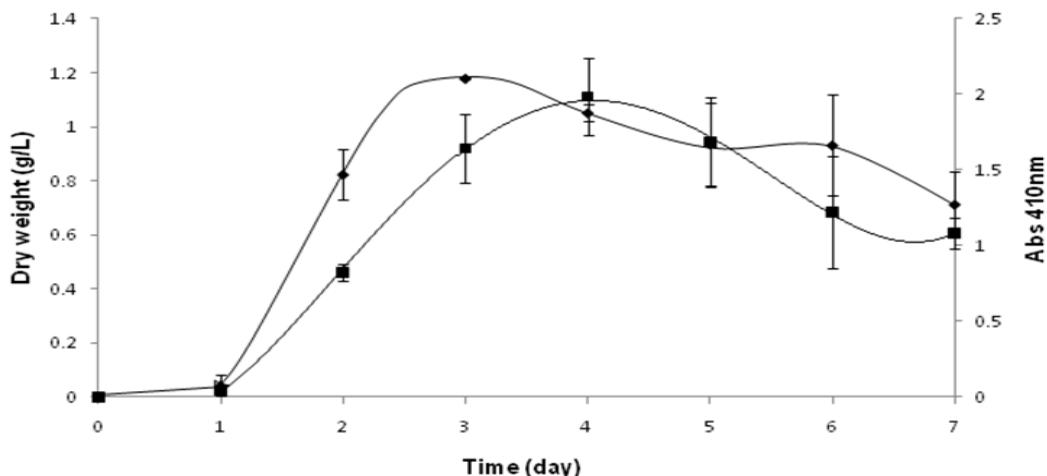
Figure 1. Growth curve and time course of ß-glucosidase production of *P. variotii* in liquid medium containing cellobiose at 37°C for 7 days. ♦: Dry cell weight; ■: Production of β-glucosidase. Each point represents mean value with the standard deviation of three measurements.

Then, the absorbance 410 nm was measured after 3 h of incubation.

Measurement of cells performance towards boric acid

Response time of the β-glucosidase production by *P. variotii* towards boric acid was investigated within seven hours by addition of 0.2% (w/v) boric acid. The study was carried out using spectrophotometer at designed time interval. Response range of boric acid was determined by subjecting the immobilized cell into different concentrations of boric acid solution ranging from 0 to 1.2% (w/v) boric acid. The production of β-glucosidase towards different concentrations of boric acid was observed after three hours. Reproducibility test of the immobilized cells towards 0 and 0.2% (w/v) boric acid was performed. Ten different sets of immobilized cells were tested in the same concentration of boric acid solution. The production of β-glucosidase was observed after three hours.

In order to study the possibility of reusing the entrapped cell, a single set of immobilized cells was tested for its repeatability at 0 and 0.2% (w/v) boric acid under optimum conditions for 10 times. The production of β-glucosidase was observed after three hours and then compared with its first cycle's response. Effect of potential interferences on the production of β-glucosidase was also tested with other acidic preservatives such as benzoic acid and sorbic acid. The degree of interference from these preservatives was evaluated by measuring the absorbance. The changes of β-glucosidase production caused by the preservatives were compared with the response of control and boric acid after three hours of incubation.

Statistical analysis

The statistical analysis was performed using SPSS software version 15.0 for windows (SPSS Inc., Chicago, USA). All the experiments were conducted in triplicates and the results given here are the mean value with the standard deviation of three measurements. The data were analyzed using Analysis of Variance (ANOVA) followed with Duncan test at 95% level of significance.

RESULTS AND DISCUSSION

Growth curve and time course of β- glucosidase production

The growth profile and time course of β-glucosidase production of *P. variotii* during 7 days of incubation is presented in Figure 1. The growth curve of *P.variotii* indicates the four phases normally found in microbial growth profile. The lag phase of *P.variotii* was observed at the first day and was followed by the log phase at the second day after inoculation. At second day of incubation, the growth of *P.variotii* progressively increased with approximately 95% higher in dry weight than that at day one. The stationary phase occurred afterward at the third day of inoculation, remained for a half day and then, entering the death phase as the cell number begun to decrease. On the other hand, the result shows the time course for the production of β-glucosidase depicting *P.variotii* actively producing β-glucosidase at second to third day and reached the maximum production at day four during late stationary phase. The result indicates that *P.variotii* started to produce β- glucosidase at log phase and continued to increase until day four. Similar result was also reported by Sohail et al. (2009) who studied on the growth of *Aspergilus niger* MS82 as well as its cellulose production. They revealed that the maximum production of ß-glucosidase occurred during the stationary phase of *A. niger* MS82. Moreover, *Penicillium purpurogenum* has produced β- glucosidase after twenty four hours and reached its maximum level within four days of growth (Dhake and Patil, 2005). Due to the relationship found between growth time of *P. variotii* and production of β-glucosidase, two days old mycelia were harvested and used in subsequent experiments. At this stage, *P.variotii*
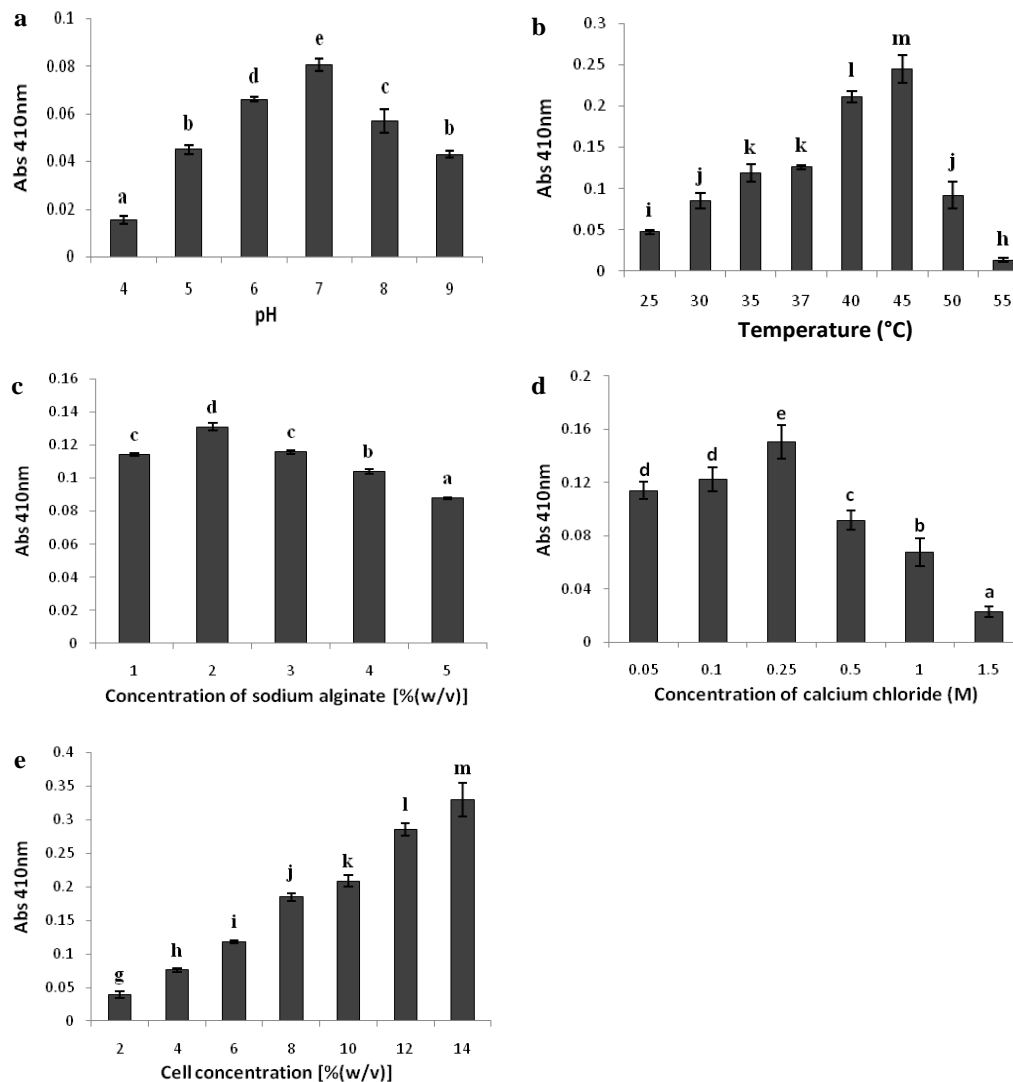
Figure 2. Optimization of factors influencing the production of β-glucosidase by *P. variotii*: **a**) pH; **b**) temperature; **c**) concentration of sodium alginate; **d**) concentration of calcium chloride; **e**) different cell concentrations. Each bar represents mean value with the standard deviation of three measurements. The bars marked with different letters are significantly different in Duncan test (p < 0.05).

exhibited active growth and optimum enzyme production.

Optimization of β-glucosidase production

Figure 2 represents the various optimization conditions for the production of β-glucosidase by *P. variotii*. The effect of different initial pHs of the medium on the production of β-glucosidase by immobilized cells is shown in Figure 2a. The optimum ß-glucosidase production was observed at pH 7, displaying an almost bell-shaped enzyme production within the pH range of 5 to 9. A significant reduction of ß-glucosidase production was recorded at pH 4 and 9 in which the production of ß-glucosidase decreased 81 and 47%, respectively as

compared to optimum condition. This proved that the system was not suitable in acidic and alkaline environment. At the extreme acidic conditions, catalytic domain of ß-glucosidase changed due to the irreversible transformation of protein conformation (Wang et al., 2009). However, neutral medium was more suitable for ß-glucosidase production by *P. variotii* as the highest activity was achieved at pH 7. Hence, pH 7 was used for subsequent experiments. Moreover, the optimum activity of β-glucosidase was also observed at pH 7 when the free form of cells was used in the system (data not shown).

Figure 2b depicts the effect of various temperatures on the production of β-glucosidase. At the temperature range from 25 to 55°C, the enzyme activity was found to

be significantly (P < 0.05) higher at temperature of 45°C than the rest of the temperature range. As the temperature increased, the production of β-glucosidase increased gradually, reaching a maximum at temperature of 45°C and then decreased thereafter. This pheno-menon might be due to the increase of mass transport and enzyme reaction rate with temperature (Mulchandani et al., 2002; Banik et al., 2008). Nevertheless, the activity decreased at temperature above 45°C presumably due to enzyme inactivation and denaturation. High temperature resulted in the loss of enzyme activity (Timur et al., 2007). Therefore, the optimum temperature 45°C was then used for the subsequent experiment.

The effect of sodium alginate on the production of β-glucosidase is displayed in Figure 2c. The result indicates that the production of β-glucosidase increased in accordance with the increase of sodium alginate concentration, reaching a maximum at concentration of 2% (w/v), and then decreased thereafter. The production of β-glucosidase significantly reduced for about 33% at medium containing 5% (w/v) sodium alginate. Similarly, the production of β-glucosidase reduced approximately 13% at concentration of sodium alginate 1% (w/v). As shown in Figure 2c, beads prepared from alginate of 1% (w/v) concentration had less enzyme activity. The beads that had been formed were too fragile and soft to hold the cells. Due to low mechanical strength, the mycelia were released out from the matrix during the process of immobilization (Idris and Suzana, 2006; Fraser and Bickerstaff, 1997). Apart from that high amount of sodium alginate increased the rigidity of the bead therefore, the production of enzyme decreased. The microbial cells in the beads were unable to obtain adequate nutrient from the medium owing to mass diffusion barrier (Idris and Suzana, 2006; Anisha and Prema, 2008). Similar behavior of the gel beads was also observed on α-amylase (Konsoula and Liakopoulou-Kyriakides, 2006) as well as lactic acid (Idris and Suzana, 2006) production using the same polymer. Hence, the maximum concentration of sodium alginate of 2% (w/v) was sufficient for cell immobilization and used in subsequent experiments.

Another factor that may affect the efficiency of the immobilized system was calcium chloride that was used as a cross linker. It may affect the mechanical strength of alginate and also the live cells and hence, affect the efficiency of the enzyme production (Konsoula and Liakopoulou-Kyriakides, 2006). Six different enzyme production concen-trations of calcium chloride (0.05, 0.1, 0.25, 0.5, 1.0 and 1.5 M) were tested for their effect on the hardness of the beads. As shown in Figure 2d, the maximum enzyme activity was obtained with beads prepared by using 0.25 M calcium chloride. The increase in calcium chloride concentration from 0.5 to 1.5 M led to a decrease in enzyme activity due to the formation of hard beads. Hardened beads prevented the enzyme from leaking out from the matrix into the production medium

(Ertan et al., 2007). Besides that, negligible amount of calcium chloride increased the vulnerability of the cell to leak out from the gel as the gel was fragile (Won et al., 2005; Anisha and Prema, 2008). Hence, alginate beads prepared by using 0.25 M calcium chloride were used in subsequent analysis. The optimum concentration of calcium chloride was similar to those obtained for the production of L-glutamic acid (Sunitha et al., 1998).

Figure 2e illustrates the effect of different cell concentrations on β-glucosidase production. Result displays that the enzyme activity increased 88% by the increment of entrapped cell from 2% to 14% (w/v). This was attributed to the increase of β-glucosidase production with the increase of cell amount and catalytic activity. Similar result was also obtained for free cell (data not shown). However, when the cell amount was increased up to 8%, the spherical shape of alginate beads collapsed. This was due to the insufficient amount of alginate polymer to hold the cells. High amount of mycelia in the gel resulted in a lower mechanical stability of the matrix. Therefore, when the sodium alginate came into contact with calcium chloride, the beads were dispersed instead of forming standard diameter spherical beads of 3 to 4 mm. Therefore, 6% (w/v) mycelia were considered as the optimum cell biomass and used in subsequent experiments.

Measurement of cells performance towards boric acid

Figure 3 represents the reaction time of the immobilized cells in responding to 0.2% (w/v) boric acid. The graph clearly shows that the cells started to give reaction to boric acid significantly at 3 h. Therefore, in this study, the reaction time was set at 3 h to enable the determination of boric acid as the color changes of the reaction from colorless to pale yellow could be observed when enzyme assay was performed. The short reaction time (less than three hours) of cell to boric acid is preferred characteristic in developing a bioassay system.

The response range of immobilized cells in various concentrations of boric acid is revealed in Figure 4. Setting the reaction time to three hours enables the determination of boric acid in the range of 0 to 1.2% (w/v). A linear relationship between the absorbance and the concentration of boric acid within the range of 0 to 0.215% (w/v) was observed. A plateau region was achieved when the concentration of boric acid solution was above 0.25% (w/v). This point represents the state where the immobilized cell was inhibited by boric acid. Moreover, as displayed in Figure 4, the straight line was obtained with the equation $y = -0.357x + 0.129$, giving the calculated correlation coefficient, $r = 0.996$. The limit of detection defined here as the three times the standard deviation of the response obtained for a blank (Alpat et al., 2008) was calculated to be 0.037% (w/v).
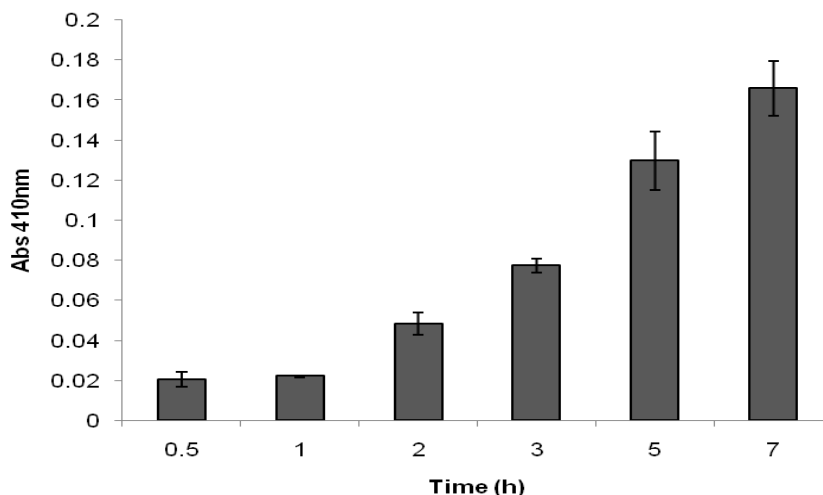
Figure 3. Response time of the immobilized cells within 7 h at boric acid concentrations of 0.2% (w/v). Operating conditions were Tris - HCl buffer pH 7 at temperature 45°C with 6% (w/v) cell concentration, 2% (w/v) sodium alginate and 0.25 M calcium alginate. Each bar represents mean value with the standard deviation of three measurements.
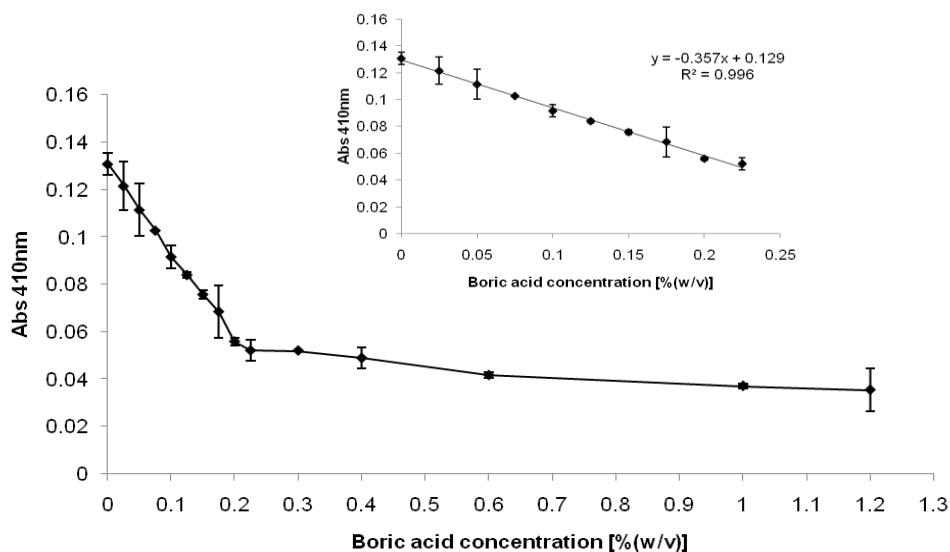

Figure 4. The response range of the immobilized cells towards different concentrations of boric acid. Inset is the linearity range of the cells towards boric acid [0 to 0.215% (w/v)]. Operating conditions were Tris - HCl buffer pH 7 at temperature 45°C with 6% (w/v) cell concentration, 2% (w/v) sodium alginate and 0.25 M calcium alginate. Each point represents mean value with the standard deviation of three measurements.

The reproducibility test of the immobilized cells for the production of β-glucosidase is presented in Figure 5. Ten different sets of cell beads were tested at 0 and 0.2% (w/v) boric acid. The result indicates that the immobilized cell beads was reproducible, giving the relative standard deviation (RSD) of 4.96% (n = 10, mean = 0.057) and 4.81% (n = 10, mean = 0.099) when in the presence (0.2% w/v) and absence of boric acid, respectively. The low relative standard deviation of both treatments demonstrates the high precision and uniformity of analysis. In addition, a low % RSD from ten different sets of immobilized cells prepared at different times represents a good reproducibility from beads preparation to the next beads.

The immobilized cell beads were tested for its selectivity to boric acid in comparison with two chemicals,
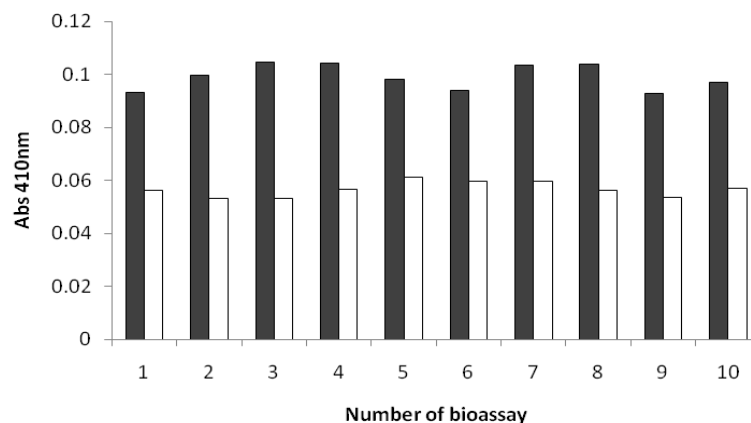
Figure 5. Reproducibility test of immobilized cells in the production of β-glucosidase at boric acid concentrations of 0% (black histogram) and 0.2% (w/v) (white histogram). The number of assay represents ten different sets of immobilized cells. Operating conditions were Tris - HCl buffer pH 7 at temperature 45°C with 6% (w/v) cell concentration, 2% (w/v) sodium alginate and 0.25 M calcium alginate.
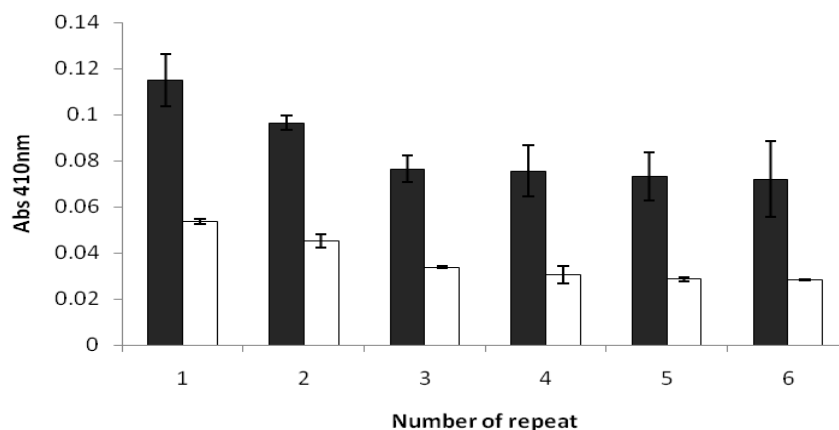

Figure 6. Repeatability test of immobilized cells in the production of β-glucosidase at boric acid concentrations of 0% (black histogram) and 0.2% (white histogram) (w/v) boric acid. Operating conditions were Tris - HCl buffer pH 7 at temperature 45°C with 6% (w/v) cell concentration, 2% (w/v) sodium alginate and 0.25 M calcium alginate. Each bar represents mean value with the standard deviation of three measurements.

namely benzoic acid and sorbic acid. These chemicals were normally used as food preservative to avoid degradation and alteration by microorganism during storage (Han et al., 2008). Figure 6 shows that benzoic acid gave no response to the system because its response has no significant difference (p < 0.05) as compared to the control. In contrast, sorbic acid shows positive response to the system as the signal was higher than the control which leads to considerable positive interference (the signal up to 44%). The positive interference was probably due to the resistance of the microorganism towards boric acid (Marzec et al., 1993). Apparently, benzoic acid and sorbic acid did not show

inhibition effect to the system as boric acid did, therefore, the presence of benzoic acid and sorbic acid would not interfere with boric acid determination (Figure 7).

Conclusion

The optimum conditions of β-glucosidase production by immobilized *Paecilomyces variotii* were performed and its behavior towards boric acid was studied. The cells not only exhibited good reproducibility with an acceptable RSD values and gave a linear response range towards boric acid [0 to 0.215% (w/v)], but also provided a short
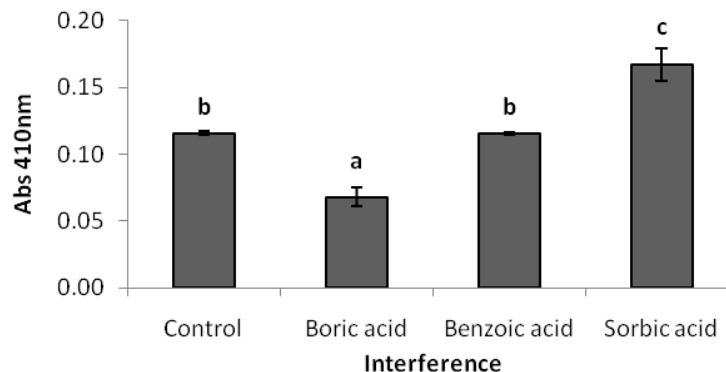
Figure 7. The production of β-glucosidase by immobilized cells in the presence of benzoic acid and sorbic acid. Each bar represents mean value with the standard deviation of three measurements. Operating conditions were Tris - HCl buffer pH 7 at temperature 45°C with 6% (w/v) cell concentration, 2% (w/v) sodium alginate and 0.25 M calcium alginate. The bars marked with different letters are significantly different in Duncan test ($p < 0.05$).

response time in the presence of boric acid. This suggested that such boric acid sensitive *P. variotii* is a potent biological element that could be further used in developing a microbial bioassay system for analysis of boric acid. However, additional investigation is inevitable in order to determine the efficiency of the system in sample analysis. Therefore, our next attempt is to focus on the applications of the system into food and environmental analysis so that the concept may provide significant contribution towards the development of a reliable biosensor to detect boric acid.

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