Food Research 1 (5) : 176 - 182 (October 2017)
Journal homepage: http://www.myfoodresearch.com

FOOD RESEARCH

FULL PAPER

Production of sorbitol by repeated batch fermentation using immobilized of *Lactobacillus plantarum* strain (BAA-793) via solid state fermentation

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Article history:
Received: 10 June 2017
Received in revised form: 18 June 2017
Accepted: 17 July 2017
Available Online: 18 July 2017

Keywords:
Repeated batch fermentation,
Solid state fermentation,
Immobilization,
Sodium alginate

DOI:
http://doi.org/10.26656/fr.2017.5.064

Abstract

Sorbitol production from *Meranti* wood sawdust (MWS) using immobilized cells of *Lactobacillus plantarum* (BAA-793) with repeated batch fermentation via solid state fermentation (SSF) was studied. The storage stability of the immobilized cells, entrapped in sodium alginate beads was also investigated. This study started with the pretreatment of the MWS to recover the cellulose, followed by the entrapment of the bacterial cells in sodium alginate, then the fermentation process via SSF. The results showed that the storage stability of the immobilized cells in gel beads at 4°C were more stable when compared to stability at 35°C, even with high cell concentration. The cell concentration, when stored at 4°C showed great stability up to 2 days but started to decrease slightly as the storage time was extended to 5 days. The concentration of the cells was about log 10.23 CFU/mL (1.70 x 10^{10} CFU/mL) after 2 days, and log 9.48 CFU/mL (3.00 x 10^{9} CFU/mL), log 9.47 CFU/mL (2.98 x 10^{9} CFU/mL) and log 9.44 CFU/mL (2.80 x 10^{9} CFU/mL) after 3 to 5 days, respectively. The decrease in the concentration was not significant within the studied period of storage at 4°C. The repeated batch fermentation for sorbitol production through the reuse of the entrapped cells showed that the same bead cells can be reused for the fermentation process up to 5 cycles. The production of sorbitol using the immobilized cells of *Lactobacillus plantarum* (BAA-793) through solid state fermentation process showed a good result due to the high concentration of cells entrapped in the beads, giving rise to a high sorbitol production.

1. Introduction

Generally, sorbitol belongs to the sugar alcohol group of polyols. Sorbitol is also the most important polyol bulk sweetener and has been in use as a sweetener for diabetics since the early 1930's (Nezzal *et al*., 2009). Sorbitol and other polyols have been used to reduce intracranial pressure (ICP) since the early 1960s (Barros *et al*., 2006; Saha, 2006). The real breakthrough came when the first sugar-free confectionery products were launched in the late 1970's. Those products focused on health aspects such as reducing the calorie content in many types of foods, reducing tooth decay, and as a sugar substitute, but were not specifically designed nor were they suitable for diabetics. Furthermore, novel dairy products enriched in polyols were developed in order to reduce diabetes (Ladero *et al*., 2007; Kumar and Murugalatha, 2012).

Solid state fermentation (SSF) can be described as the cultivation of microorganisms in the absence of free water under controlled conditions (Pandey *et al*., 2001). Generally, SSF has been utilized to convert moist agricultural polymeric substrates such as soy, rice, sawdust, wheat and other substrates into fermented food products including industrial enzymes, fuel, and nutrient enriched animal feeds (Pandey, Palni and Bisht 2001*;* Pandey *et al*., 2004). In addition, solid state fermentation was preferred to liquid state fermentation because of being a simple technique, having low wastewater output (liquid waste is not produced), less chances of contamination, low capital investment (cheaper costs), lower levels of catabolite repression, better product recovery, less time-consuming and higher quality of production (Manpreet *et al*., 2005).

Generally, the physical confinement or localization of the intact cells to a certain region of space without

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eISSN: 2550-2166

loss of desired biological activity is called immobilization of whole cells (Pilkingtonn *et al*., 1998; Ibrahim *et al*., 2013; Amenaghawon and Oiwoh, 2015). The immobilization of whole cells plays an important role in microbial ecology and is widespread in nature. Whole cell immobilization occurs to some extent in all microbe-based industrial processes as well, including water and wastewater treatment (Pilkingtonn *et al*., 1998; Ibrahim *et al*., 2013; Amenaghawon and Oiwoh, 2015). Before this, the immobilization of whole cells process and its application in the bioprocessing has been interesting for 30 years and it also has introduced in industrials (Behera *et al*., 2011). Besides that, immobilization also consists immobilizing cells of microorganism inside or on the surface of a carrier in a way that preserves their catalytic activity (Jack and Zajic, 2006). Based on previous research, immobilization cells systems are classified according to the mechanism of immobilization. Cells Immobilization has many types of methods such as physical entrapment within the porous matrix, adsorption, encapsulation, covalent binding to a carrier and cross-linking of cells. Currently, 50% of the world's supply of biochemicals such as lactic acid, sorbitol, bioethanol and other biochemical products are provided through free cell fermentation, but the productivity of the fermentation process is very low. However, employing the cell immobilization technique that provides high density can increase the productivity of generating biochemical products. Besides that, the immobilization process is one of the most attractive methods for maintaining high cell concentration in a bioreactor for processing biochemical products (Gündüz, 2005; Cláudia *et al*., 2013). The cell immobilization system offers the greatest advantages for several industrial process, such as having an easy to handle biocatalyst, easy separation of biological materials from the reaction medium, employing high cell loading capacity, improving the production rate of products and other advantages (Bayat *et al*., 2015; Tallur *et al*., 2015).

The present work has a focus on storage stability of cells immobilization and repeated batch fermentation using immobilized cells employing entrapment technique due to sorbitol production.

2. Materials and methods

2.1 Materials and culture

The raw material used was *Meranti* wood sawdust, which was obtained from Kilang Kayu Aman Sdn Bhd, Gambang Kuantan, Pahang. *Meranti* wood sawdust is of the hardwood type that contains a high cellulose content compared to other types. *Meranti* wood sawdust was taken by bulk from the sawmill plant in order to make sure the quality of the materials was the same throughout the research. The bacterial strain that was used in this study was *Lactobacillus plantarum* (BAA-793). The bacterial strain was purchased from the America Type Culture Collection (ATCC) type.

2.2 Preparation of cellulose extraction

Meranti wood sawdust (MWS) must first be treated using physical pre-treatment (cutting, milling, and drying) and chemical pre-treatment. Chemical pre-treatment involved a pre de-lignification process using sodium hydroxide (NaOH), with the first stage of the pre -treatment process using peracetic acid (CH_3COOH) and the second stage using sulfuric acid (H_2SO_4) (Zhang *et al*., 2006; Zhao *et al*., 2008; Fang *et al*., 2010; Rusmawarni, 2011).

The type of bacteria used in this study was *Lactobacillus plantarum* (BAA- 793), which was purchased from America Type Culture Collection (ATCC). This strain is an anaerobic bacteria type. To handle this bacterium, the anaerobic condition must be applied in order to get a good growth of the bacterium.

2.3 Culture preparation

The bacterium was cultivated under anaerobic condition in the MRS medium for 24 hours at 30^oC inside incubators (Ladero *et al*., 2007). The liquid medium of MRS was used for inoculum preparation. 100ml of MRS medium was transferred into 250 ml Schott bottles and one loop of bacteria (*Lactobacillus plantarum*) from the agar plate was transferred into 100 ml of MRS medium. Then, nitrogen gas (N_2) was used to purge the Schott bottles that contained the MRS medium and bacteria in order to remove the oxygen gas (O_2) inside the Schott bottles and to maintain the anaerobic condition during the cultivation process. Then, it was kept in an incubator at 30^oC. After 24 hours of cultivation, the optical density of the inoculums was checked using UV-Vis Spectrophotometer. The optical density was taken at a wavelength equal to 600nm (OD_{600}). The values of OD_{600} should be less than 0.4 or 0.1 to 0.2 for successful cultivation (Sabu *et al*., 2008).

2.4 Cells immobilization

The bacteria (*Lactobacillus plantarum*) cells that were grown in MRS broth were mixed with an equal medium volume of 2% Na-alginate solution (1:1, v/v). Then, after being mixed, the alginate-cell solution was dropped slowly into 0.2% of calcium chloride ($CaCl_2$)

solution using a syringe needle. After that, the alginate solidified when it made contact with the $CaCl_2$ solution and formed gels beads, thus entrapping the bacteria cells. The beads were allowed to harden in $CaCl_2$ solution for 30 minutes. Then the beads were washed with 0.85% of NaCl solution and distilled water to remove excess calcium ion and cells. The beads were stored at 4°C for further experiments. To improve the immobilization results, the ratio of $CaCl_2$ (0.2%) and NaCl (0.85%) that were used in solution preparation was 1:1, v/v (Yan *et al*., 2001; Bangrak *et al*., 2011; El-Borai *et al*., 2013).

2.5 Storage stability

The storage stability of immobilized cells in beads (entrapped in Na-alginate) was also investigated in order to measure cell concentration and population. The gel beads were stored at 4°C in the freezer and 35°C in an incubator. The storage time varied from 1 to 7 days for both conditions. Then, the cell numbers were counted using plate counting.

2.6 Immobilization cells storage

The gel beads that were stored at 4°C for 2 to 5 days were used for repeated batch fermentation and no significant reduction of cell concentration occurred during this time period.

2.7 Colony counting

Ten dried gel beads containing bacteria cells were depolymerized in 10 mL of 1% (w/v) sodium citrate solution using a 100ml beaker with gentle shaking for 20 minutes at room temperature in order to produce a cell suspension. After that, the cells suspension was serially diluted using pour plate method and incubated at 35°C for 3 days. The plates with 30 to 300 colonies were valid for use, while the plates with more than 300 colonies could not be counted and were referred to as too many to count (TMTC). Meanwhile, plates with fewer than 30 colonies were also not valid for colony counting and were referred to as too few to count (TFTC). The equation for colony counting is shown below

$$\text{CFU/mL} = \frac{\text{Number of Colonies (CFUs)}}{\text{Dilution} \times \text{Amount Plates}} \quad (1)$$

2.8 Fermentation of cellulose using SSF process

Two grams of the sample were put into a 100ml Erlenmeyer conical flask, then it was moistened with 50% of distilled water in order to achieve the desired moisture content (Sabu *et al*., 2008; Natarajan and Rajendran, 2012). All the apparatus and materials were sterilized at 121°C for 15 minutes to avoid contamination (Natarajan and Rajendran, 2012). This experiment was conducted in a laminar flow to avoid contamination and loss of viability. After the sterilizing process, the samples were cooled. Then, 10% of beads of immobilized cells were put into the samples (Yan *et al*., 2001; Hsieh and Yang, 2004; Bangrak *et al*., 2011; El-Borai *et al*., 2013). The samples were purged with nitrogen gas in order to remove oxygen gas to maintain the anaerobic condition and then they were incubated at 35°C. All the experiments were carried out in 3 sets to get their average values.

2.9 Repeated batch fermentation

The recycling of immobilized bacteria cells (gel beads) was studied in order to calculate how many cycles the gel beads could be used for sorbitol production. For this step, the condition parameters for fermentation followed the optimization values, which were fermentation time: 4 hours, substrate amount: 1.0g and moisture content: 50%. After finishing one cycle of fermentation, the beads inside the samples were collected and washed using sterilized distilled water for reuse in the next cycle of fermentation.

2.10 Analysis methods

The sorbitol production was determined using High-Performance Liquid Chromatography, (HPLC Agilent, 1200 series). The column for the quantification of sorbitol was Rezex Chromatographic Method, RCM Monosaccharides 300 X 7.8mm with water as a mobile phase. The sugar was eluted with deionized water at a flow rate 0.6mL/min and the column, maintained at 75°C with the retention time is 30 min. This method used Refractive Index, RI as a detector (Saha and Nakamura, 2003).

2.11 Electron microscopic scanning

The external appearance and surface structure of the beads were observed using scanning electron micrograph (SEM). The beads of bacteria were dried in an oven for 1 day in order to remove the moisture content. The beads were observed and examined using scanning electron micrograph (SEM).

3. Result and discussion

3.1 Effect of storage stability on cell viability

Determining the storage stability of immobilized cells for either short-term storage or long-term storage is an important procedure in the immobilization process and is also an essential factor for practical application of

immobilized cell system (Ying *et al*., 2007). In this study, storage stability was investigated in order to determine the cell viability of *Lactobacillus plantarum*. Figure 1 shows a graph representing the storage stability of immobilized *Lactobacillus plantarum* (BAA-793) bacteria cells that were stored at 4°C and 35°C.

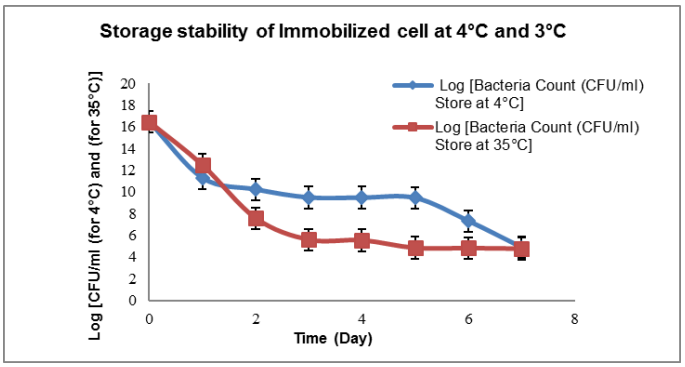

Figure 1. The storage stability of immobilized cells at 4°C and 35°C

Based on Figure 1, the initial concentration of viable cells before storage at 4°C and 35°C that was represented at (t_0) was about log 16.46 CFU/ml (2.90 x 10^{16} CFU/ml), in which the concentration of cells was very high because the cells were still fresh/concentrated after being immobilized using Na-alginate. Then, after storage at 4°C for 1 day, the concentration of cells decreased to log 11.29 CFU/ml (1.95 x 10^{11} CFU/ml). Otherwise, after storage at 35°C for 1 to 3 days, the cell concentration decreased to log 12.47CFU/ml (2.98 x 10^{12} CFU/ml), log 7.58 CFU/ml (3.70 x 10^{7} CFU/ml) and log 5.60 CFU/ml (4.00 x 10^{5} CFU/ml) on day 1, day 2 and day 3, respectively. The concentration of cells that were stored at 35°C was lower compared to storage at 4°C because the compatibility of temperature is important in order to maintain the cell concentration. Meanwhile, the cell concentration at 4°C was maintained and started to stabilize at 2 to 5 days, where the cell concentrations were about log 10.23 CFU/ml (1.70 x 10^{10} CFU/ml), log 9.48CFU/ml (3.00 x 10^{9} CFU/ml), log 9.47 CFU/ml (2.98 x 10^{9} CFU/ml) and log 9.44 CFU/ml (2.80 x 10^{9} CFU/ml) on day 2, day 3, day 4 and day 5, respectively, and no significant reduction of cell concentration occurred during this time period. Lakshmi and Hemalatha (2015) reported that the time required for stabilization of cell content in the gel bead is approximately 48 hours of cultivation. Then, after 5 days of storage at 4°C, the cell concentration decreased until day 7. Otherwise, at 35°C storage, cell concentration was maintained from day 3 until day 7, whereby the cells concentrations were about log 5.60 CFU/ml (4.00 x 10^{5}), log 5.55 CFU/ml (3.60 x 10^{5} CFU/ml), log 4.84 CFU/ml (7.00 x 10^{4} CFU/ml), log 4.82 CFU/ml (6.70 x 10^{4} CFU/ml) and log 4.77CFU/ml (6.00 x 10^{4} CFU/ml) on day 4, day 5, day 6, day 7 and day 8, respectively. Besides that, these values had no significant reduction of cell concentration. In addition, the above observations from Figure 1 suggest that the immobilized cells of *Lactobacillus plantarum* (BAA-793) were more stable at 4°C when compared to 35°C. This is also consistent with other researchers (Geethanjali and Subash., 2013) who reported that immobilized enzymes are more stable at 4°C when compared to 25°C, since 4°C is the suitable temperature for the storage of most enzymes. In addition, storage stability is also related to accelerated stability, which is the stability of products with a predicted shelf life that is used to compare the relative stability of alternative formulations (Bajaj *et al*., 2012). The concept of accelerated stability is based on the Arrhenius equation (2), which is shown below, and also a modified Arrhenius equation (3) (Anderson *et al*., 1991; Bajaj *et al*., 2012).

$$lnK = \ln A \frac{\Delta E}{RT} \qquad (2)$$

From the equation (2), K is degradation rate/s, A is frequency factor/s, ΔE is activation energy (KJ/mol), R is universal gas constant (0.00831 kJ/mol), and T is absolute temperature (K).

$$\mathrm{Log}\left(\frac{k2}{k1}\right) - \left(\frac{-Ea}{2.309R}\right)\left(\frac{1}{T2}\ \frac{1}{T1}\right) \qquad (3)$$

From the equation (3), k1 and k2 are rate constants at temperature T1 and T2 in degree Kelvin, Ea is the activation energy and R is gas constant. From the both equations, it describes that the relationship between storage temperature and degradation rate.

3.2 Scanning electron micrograph (SEM) of sodium alginate bead immobilizing bacteria

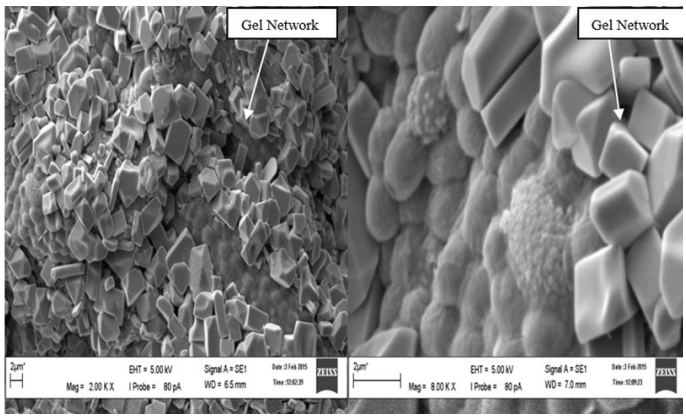

Figure 2. SEM image of sodium alginate bead immobilizing *Lactobacillus plantarum* sp. (BAA-793)

The external appearance and surface structure of the beads were observed using scanning electron microscope (SEM). Figure 2 shows the SEM image of calcium

alginate bead of immobilized *Lactobacillus plantarum* sp. (BAA-793). Based on Figure 2 below, the microscopic examination shows the appearance of cloudiness, which was caused by gelatinous material and also fragmented hyphae without cellular content. Besides that, the result also shows that the *Lactobacillus plantarum* bacteria remained confined to the subsurface.

The previous research related to immobilization process using entrapment method of mycelia that was described by Kuek and Armitage, (1985) and Kuek, (1991) also mentioned that after observing the beads using electron microscopy, it was confirmed that the mycelia remained confined to the subsurface (Kuek, 1991). Besides that, there were appearances of corrugation on the surface of the bead, as shown in Figure 2, which was similar to the previous observation of Chen *et al*. (2012), which used scanning electron microscopy of Ca-alginate and alginate-chitosan beads containing Acetobacter cells (Chen *et al*., 2012).

3.3 Reuse of immobilized cells in a batch process for sorbitol production

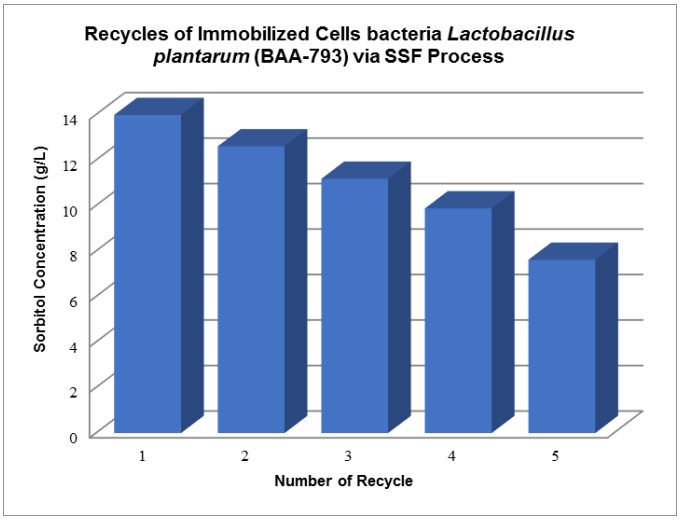

Figure 3. Recycles of immobilized cells bacteria *Lactobacillus plantarum* (BAA-793) via SSF process due to produce sorbitol

The cell immobilization process has many advantages and one is that the immobilized cells can be reused for repeated batch fermentation. In this research, the immobilized cells of *Lactobacillus plantarum* (BAA -793) via SSF process were reused after completing a cycle of the fermentation process. The immobilized cells of bacteria in gel beads that were used in the first fermentation batch of sorbitol were washed using sterilized distilled water in order to be reused for the next fermentation batch. The reuse of immobilized bacteria *Lactobacillus plantarum* (BAA-793) cells is very important from the point of view of reducing the cost of using the bacteria. Figure 3 shows the recycling of immobilized *Lactobacillus plantarum* (BAA-793) bacteria cells via SSF process in order to produce sorbitol.

From Figure 3, the data shows that sorbitol production was observed until the 5th cycle. The production of sorbitol was higher at cycle 1, where the production was about 13.516g/L. This is because the cell colonies on gel beads were at high concentrations when reacting with the fermentation medium and thus produced a high concentration of sorbitol. However, a gradual decrease was observed in the next cycle. The production of sorbitol for cycles 2, 3, 4 and 5 was 12.606g/L, 11.178g/L, 9.878g/L and 7.619g/L, respectively. The production of sorbitol decreased after cycle 1 because the cells which were immobilized in sodium alginate in gel beads were leaking into the fermentation medium of the first batch/first cycle, hence reducing the number of cells attached to gel beads and therefore reducing sorbitol production for the next cycles. Besides that, the number of cells attached to gel beads possibly leaked when the gel beads were washed with sterile distilled water in order to start the next cycle, and thus the sorbitol production decreased.

Besides that, the profiles of the beads are not uniform and the beads also changed shape during fermentation from big shape to small shape. The changing shape of the beads used for immobilizing bacteria cells during fermentation has also been proven by other researchers (Yan *et al*., 2001). In addition, the researcher that used the immobilization process where entrapped protease in calcium alginate beads was reused reported that the decrease in activity occurred on further reuse, which was due to the leakage of enzymes from beads during washing time (Anwar *et al*., 2009; Geethanjali and Subash, 2013).

4. Conclusion

In summary, the immobilization of the *Lactobacillus plantarum* (BAA-793) in sodium alginate beads ensured the survival of a high number of the cells during storage at 4°C. It also led to a high sorbitol yield using solid state fermentation. In addition, the immobilized cells in the gel beads can be used repeatedly up to 5 times with good sorbitol yield.

Acknowledgement

The authors thank the support of Faculty of Chemical Engineering and Natural Resources,

Universiti Malaysia Pahang (UMP) for providing the facilities.

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