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Calcium alginate entrapped cells of *Penicillium digitatum* FETL DS1 for the improvement of tannase production

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ABSTRACT

The ability of immobilized cell cultures of *Penicillium digitatum* FETL DS1 to produce extracellular tannase was investigated. The production of enzyme was increased by entrapping the fungus in calcium alginate beads compared to the free suspended cells. Using the optimized parameters of 40 calcium alginate beads, 1.5% (w/v) of sodium alginate and inoculum size of 1 x 10^6 spores/mL in 50 mL cultivation medium in a 250 mL shake flask system, 6.06 U/mL tannase was obtained from the immobilized cells compared to the free suspended cells (4.34 U/mL). It was approximately a 39.63% increment.

KEYWORDS

Immobilized cells;
Penicillium digitatum FETL DS1;
Tannase;
Calcium alginate beads.

INTRODUCTION

Tannin acyl hydrolase (EC 3.1.1.20) also known as tannase, catalyzes the hydrolysis of ester bonds present in gallotannin, complex tannin and gallic acid esters[20]. Tannase has a great amount of applications and the main commercial application at present is in the preparation of instantaneous tea, action on tea polyphenols[15, 27], beer chill-proofing and wine making, besides reducing tannin levels in fruit juices[25]. Tannase also can be used as clarifying agent in coffee flavoured soft drinks[2] and to improve the nutritional properties of tannin-rich forage[4]. It is an inducible enzyme that can catalyse the breakdown of ester linkages in hydrolysable tannins such as tannic acid resulting in the production of gallic acid and glucose[5].

Despite the many important applications of tannase, high scale use of tannase is restricted due to the high cost of its production. Even though attempts to produce high tannase activity in submerged fermentation culture using high agitation speeds have been made, it was not successful due to damage to the fungal mycelia caused by the shear forces that occurred during high agitation speed. Therefore, it is speculated that the protection of cells is inevitable, at the same time maintaining adequate amounts of oxygen in the culture. The protection of the cells can be done through immobilization techniques and the damage of fungal mycelia can be reduced through protection by entrapping the cells within the carrier matrix[10]. One of the most interesting techniques to increase the amount of tannase production is the use of immobilized cells in the production. Immobilization offers many advantages

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which are direct consequences of the simple fact that immobilized cell particles are about 1,000 times larger than free cells, making them easy to handle and capable of being conveniently packed in a fermenter system for industrial processes[10]. The confinement of the cell to support materials implies a number of desired properties such as protecting the cell from detrimental environment perturbation. Besides that, the support materials provided a stabilization effect on the cellular activities that enhanced operational stability and immobilized preparation can be stored for a longer period of time. In an immobilized system, the enzymes secreted are largely free of cells and cell debris which substantially facilitated downstream processes[8, 10].

Even though there are several methods in immobilization, calcium alginate gel entrapment is the most common method[23]. Alginate entrapment cells is one of the simplest, non-toxic and most importantly is one of the cheapest methods of immobilization[1]. Alginates are commercially available as water soluble sodium alginates and they have been used for long time in the pharmaceutical and food industries as thickening, film forming and emulsifying agents[3]. Sodium alginate is water soluble, whereas the polyvalent cations of calcium are water insoluble, and finally these two form calcium alginate beads which are preferred to be one of the carriers for immobilization.

Bacteria[19], yeast[28] and filamentous fungi[6, 17, 24] have been reported to be able to produce extracellular tannase. However, this is the first report on *Penicillium digitatum* that can produce extracellular tannase. *Penicillium digitatum* FETL DS1 which was isolated from the dumping sites of tannin-rich barks of *Rhizophora apiculata* in the mangrove area in Perak, Malaysia is an aerobic fungus which is sensitive to oxygen limitation in the cultivation process. Therefore, the aim of the present investigation was to study the immobilization of the *Penicillium digitatum* FETL DS1 cells for higher tannase production using the calcium alginate entrapment method.

MATERIALS AND METHODS

Microorganism, culture maintenance and inoculum preparation

Penicillium digitatum FETL DS1, isolated from the dumping area of tannin-rich barks of *Rhizophora apiculata* in mangrove areas in Perak, Malaysia, was used throughout the study. The fungal culture was maintained on 2% (w/v) malt extract agar slant supplemented with 0.01% tannic acid at 30°C for 5 days aerobically until sporulate, before storing them at 4°C for further use. The subculturing was performed every 3 weeks to ensure its survival.

The inoculum was prepared by adding 5.0 mL of sterile distilled water to the agar slants and shaking it vigorously. The spore suspension that obtained was adjusted to 1 x 10^7 spores/mL using a haemocytometer slide chamber (Neubauer, Germany) and used as inoculums.

Cultivation method for free cells of Penicillium digitatum FETL DS1

Cultures were grown in 250-mL Erlenmeyer flasks containing 50 mL of modified Czapek-Dox liquid medium (%; w/v): 0.25% $NHCl_4$, 0.1% KH_2PO_4, 0.05% $MgSO_4.7H_2O$, 0.05% KCl and 4% tannic acid (as a sole carbon source). The pH of the medium was adjusted to 6.5 prior to autoclaving. The 1% (v/v; 1 x 10^7 spores/mL) of inoculum was added into the medium and the cultivation was carried-out at 30°C for 10 days with agitation speed of 200 rpm. Tannase activity and biomass determinations were done every 24 hour intervals. The experiments were carried-out in triplicate and the values were reported as standard deviations.

Immobilization of Penicillium digitatum FETL DS1 using the entrapment method

Sodium alginate (Nacalai Tesque, Japan) with a final concentration of 1.5% (w/v) was added to a 5% (v/v) spore suspension of 1.0 x 10^7 spores/mL and mixed using a magnetic stirrer at room temperature. The spore-alginate slurry was sucked into a syringe (Terumo, Japan). Calcium alginate beads were formed by dropping the mixture of spore-alginate slurry into 0.2 M calcium chloride solution following a brief shaking for every drop. The beads were then incubated in the calcium chloride solution for another 30 minutes to complete the gel formation before storing them at 4°C for further use.

Fifty beads of the calcium alginate that contained spores each with a diameter of about 4.0 mm were

inoculated into 50 mL of modified Czapek-Dox liquid medium (%; w/v): 0.25% $NHCl_4$, 0.1% KH_2PO_4, 0.05% $MgSO_4.7H_2O$, 0.05% KCl and 4% tannic acid (as a sole carbon source). The pH of the medium was adjusted to 6.5 and the cultivation was carried-out at 30°C and at 200 rpm of agitation speed for 10 days. Tannase activity and biomass determinations were done every 24 hours. The experiments were carried out in triplicate and the values were reported as standard deviations. This experiment was conducted to determine the optimal cultivation time that produced the highest tannase production through the calcium alginate immobilization.

Improvement of culture conditions by calcium alginate immobilization

Immobilization was carried out by placing different numbers of sodium alginate beads (10, 20, 30, 40 and 50) into 250-mL Erlenmeyer flasks containing 50 mL of optimized modified Chapex-Dox medium [(%; w/v): 0.25% $NHCl_4$, 0.1% KH_2PO_4, 0.05% $MgSO_4.7H_2O$, 0.05% KCl and 4% tannic acid] with initial medium pH of 6.5, which was sterilized prior to placing in the calcium-alginate beads containing the fungal spores. The preparation was incubated at 30°C and at 200 rpm agitation speed for 10 days. Tannase activity and biomass determinations were done at 24 hour intervals. The experiments were conducted in triplicate and the values were reported as standard deviations.

Besides the number of calcium alginate beads, the inoculum sizes were also studied in order to get the best spore concentration to be entrapped in calcium alginate beads. Various concentrations of fungal spores were studied (10^1, 10^2, 10^3, 10^4, 10^5, 10^6, 10^7 and 10^8 spores/mL). The effects of sodium alginate concentrations on the size of inoculum were also determined and the concentrations of sodium alginate used were 1.0%, 1.5%, 2.0% and 2.5%. The cultivations were done for 4 days (optimum cultivation period) at 30°C and at 200 rpm agitation speed.

Crude enzyme extraction

The culture broth was filtered through a Buchner funnel containing a pre-weighed filter paper (Whatman No.1 paper) to separate the mycelial biomass. The cell-free culture filtrate containing the crude enzyme was then assayed for extracellular tannase activity.

Fungal growth determination

The growth of free cells in the medium was estimated by filtering the culture broth through a Buchner funnel containing a pre-weighed Whatman No. 1 filter paper. The retained biomass was then washed with distilled water to remove any remaining substrate and later both the mycelial mass and the filter paper were dried to a constant weight at 65°C. Subsequently, they were re-weighed and the weight of the dry mycelial mass was calculated by subtracting the weight of the filter paper.

The dry weight of the immobilized mycelia in calcium alginate beads was determined by dissolving known amounts of beads in a 10 mL sodium hexametaphosphate solution (23.4%, w/v) as previously described by[9]. The mycelium was filtered using Whatman No. 1 filter paper as described above.

Scanning electron microscopy

Morphological observation of the free and calcium alginate immobilized cell preparations were performed using a scanning electron microscope (Leica, Cambridge S-360). The 4-day-old fungal mycelia were harvested, washed three times with sterile distilled water and then blotted dry in Whatman filter paper. The samples for SEM were prepared as described previously by[21].

Tannase activity assay

Extracellular tannase activity was determined according to the method described previously by[12]. One millilitre of the culture filtrate was incubated with 1 mL of tannic acid solution (1% w/v tannic acid in 0.2 M acetate buffer, pH 5.0) at 40°C for 30 min in a water bath. The enzymatic reaction was then stopped by precipitation of the remaining tannic acid with the addition of 4 mL of standard protein solution (1.0 mg/mL bovine serum albumin) at room temperature (30±2°C). The gallic acid produced was not precipitated by the protein solution in this method due to its low molecule weight. A reference tube with the heat inactivated enzyme was treated in the same manner except that the tannic acid was precipitated immediately without incubation. The tube was then centrifuged at 3,000 rpm (Chermle Z 320) for 20 min and the absorbance of 1:80-fold diluted supernatant (con-

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taining liberated gallic acid by action of tannase on tannic acid) against distilled water as blank in a UV spectrophotometer (UV-120-01 Shimadzu, Japan). The amount of gallic acid produced in the reaction mixture was then estimated from a reference curve standardized with commercial gallic acid (Fluka AG) with concentration ranging from 0 to 30 µmol/mL. One Unit of tannase was defined as the amount of enzyme required to release 1.0 µmol of gallic acid per min per mL of fermentation broth under standard assay conditions.

RESULTS

Tannase production by free cells

The results obtained for the production of tannase by free suspended cells in a shake flask system is shown in Figure 1, where the maximal cultivation period for the highest enzyme production was at the 4 days of cultivation with about 4.34 U/mL tannase activity. The tannase production was found to be expressed during the secondary metabolic phase of growth, the onset of which was triggered by the limitation of carbon and nitrogen sources. The enzyme activity dropped gradually after achieving its maximum activity. However, the fungal growth was increased slowly after achieving the maximal tannase production.

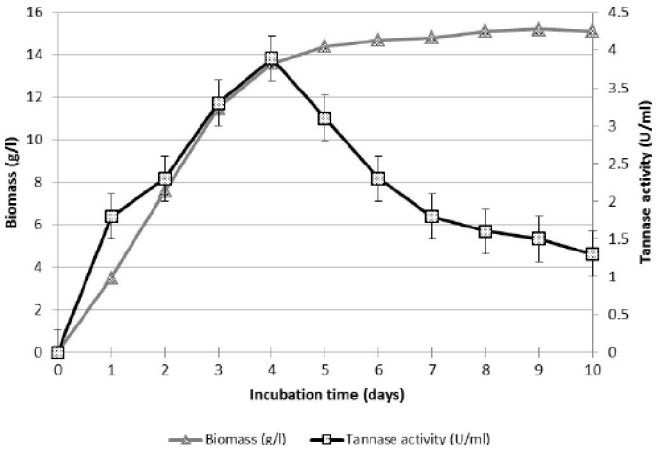

Figure 1 : Tannase production by free suspended cells of *P. digitatum* FETL DS1 in a shake flask system.

Tannase production by immobilized cell preparation

The profile of tannase production by calcium alginate immobilized cells is shown in Figure 2. The enzyme production is markedly enhanced by immobilization (50 beads per 50 mL medium) with about 5.65 U/mL which also achieved on the day 4 of cultivation. The fungal growth is also higher in the calcium alginate beads than the free suspended cells.

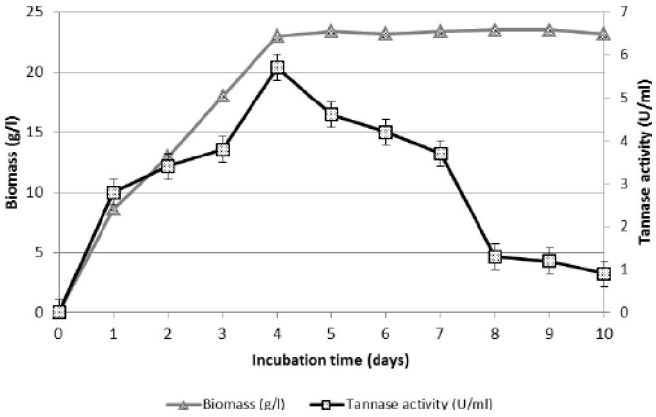

Figure 2 : Tannase production by immobilized preparation of *P. digitatum* FETL DS1 in calcium alginate entrapped cells in a shake system (non-optimum condition)

The optimization of fungal cell entrapment in calcium alginate beads was carried out in order to get maximum enzyme production. Two parameters including the inoculum sizes and concentration of sodium alginate and beads were investigated.

Effect of inoculum size

Optimization of spore concentration on the production of the tannase by immobilized cells of *P. digitatum* FETL DS1 in calcium alginate beads was carried out using 1% (v/v) of spore suspensions of varying spore counts from 1 x 10^1 – 1 x 10^8 spores/mL (Figure 3). The

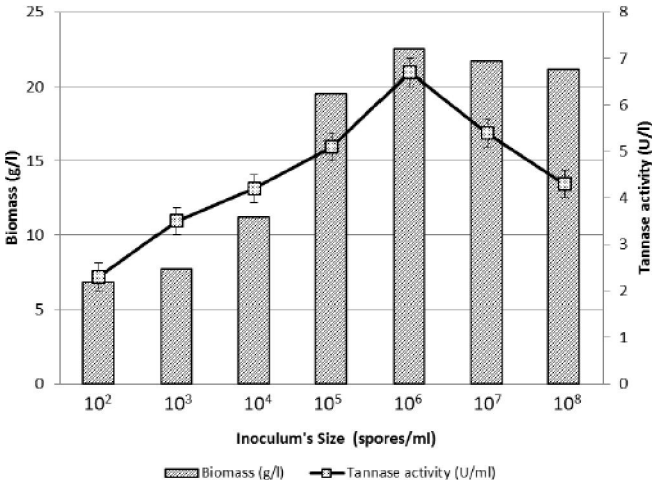

Figure 3 : Effect of inoculum size on tannase production by immobilized cells of *P. digitatum* FETL DS1 in calcium alginate entrapped cells in a shake flask system

result revealed that the higher inoculum size produced higher tannase production and the optimized inoculum size of 1 x 10^6 spore/mL produced the highest tannase of about 7.50 U/mL. The highest growth also achieved when the same inoculum size was used. Higher or lower inoculum sizes than the optimum one produced lower tannase activity.

Effect of sodium alginate concentration and bead number

Optimization of sodium alginate concentration on the production of tannase by immobilized cells *P. digitatum* FETL DS1 in calcium alginate beads was carried out using 1 x 10^6 spores/mL entrapped in various concentration from 1.0 – 2.5% (w/v) in 4 days (Figure 4A). The production of tannase improved with the increasing sodium alginate concentration and reached the maximum tannase activity of 5.80 U/mL at 1.5% (w/v). The effect of initial cell loading was studied by varying the number of alginate beads from 10 – 50 beads per 50 mL of cultivation medium in a flask. It was found that the production of tannase increased as the number of calcium alginate entrapped cells increased until it achieved the maximum tannase production of 5.90 U/mL when 40 beads were used (Figure 4B). The fungal growth was also found to increase tremendously when entrapped in calcium alginate beads. Higher or lower the sodium alginate concentration and beads number than the optimum one reduced the enzyme activity.

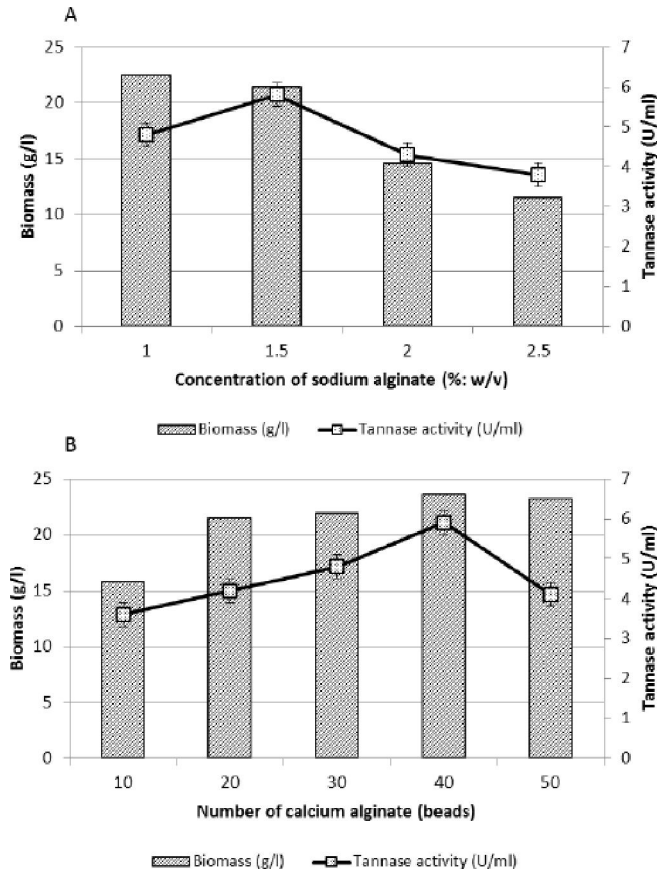

Figure 4 : (A) Effect of sodium alginate concentration and (B) Effect of bead numbers on tannase production by immobilized cells of *P. digitatum* FETL DS1 in calcium alginate entrapped cells in a shake flask system

Profile of tannase production by immobilized and free cells

Using an optimized number of calcium alginate beads (40 beads), 1.5% (w/v) of sodium alginate concentration and inoculum size 1 x 10^6 spores/mL, the time course for tannase production by the immobilized *P. digitatum* FETL DS1 was carried out (Figure 5). The maximum tannase production by calcium alginate beads

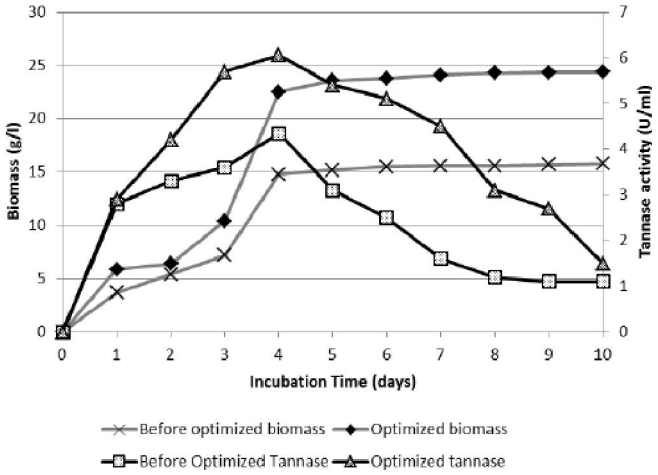

Figure 5 : Time course profile of tannase production by optimized and non-optimized immobilized preparation of *P. digitatum* FETL DS1 in calcium alginate entrapped cells in shake flask

TABLE 1 : Summary of tannase production by free suspended cells and calcium alginate (non-optimized and optimized) *P. digitatum* FETL DS1entrapped cells immobilization

Cell preparation	Tannase activity (U/mL)	Days of maximum activity
Free suspended cells	4.34	4
Calcium alginate entrapped cells immobilization (non-optimized)	5.65	4
Calcium alginate entrapped cells immobilization (optimized)	6.06	4

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was 6.06 U/mL. TABLE 1 shows the tannase production obtained from the free suspended cells, non-optimized and also optimized conditions of the calcium alginate entrapped cells. The results showed that there was an increment in tannase production of about 30.18% in the immobilization (non-optimized; 5.65 U/mL) compared to the free suspended cells (4.34 U/mL), and a slight increment of about 7.26% of tannase production when optimized conditions (6.06 U/mL) were used compared to the non-optimized (5.65 U/mL) conditions. Finally, there was an increment of 39.63% in the tannase production after the optimization conditions used compared to free suspended cells.

Scanning electron microscope observation of the fungal growth in a calcium alginate beads

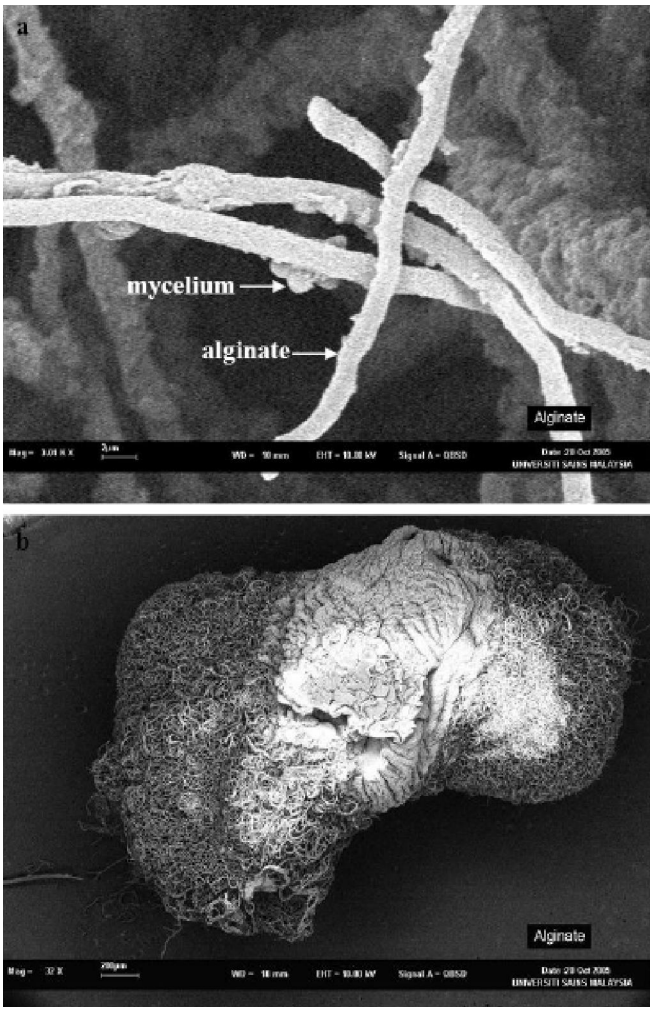

Figure 6 : SEM monograph of the entrapped *P. digitatum* FETL DS1 cells in calcium alginate beads. (A) Mycelia attached to the alginate polymer and (B) Dense and inter-wining fungal mycelia in a calcium alginate bead

The fungal cells entrapped in the calcium alginate beads were examined using SEM. (Figure 6). Figure 6A shows a single calcium alginate bead exposing the mycelia within the beads. The clear distinct and dense mycelia ramified on the top surface layer of the bead compared to the mycelia in the inner part of the bead which is scarce (Figure 6B). This could be due to the diffusion limitation of the oxygen and nutrient.

DISCUSSION

Early work has shown that agitation resulted in the formation of mycelial pellets which sometimes suppressed the synthesis of enzymes[11]. Even though, agitation is necessary in the cultivation system to enhance the diffusion and mass transfer of gasses into, from and within the pellet, agitation can also bring major problems where it can make cells disintegrate or lysis as a result of the cell collision and shear forces during agitation or shaking[7]. Therefore, cell immobilization by the entrapment method is a good choice.

Immobilized of *P. digitatum* FETL DS1 cells in calcium alginate showed that the tannase production was comparatively higher than that by the free suspended cells. However, further observation revealed that although the matrix in this case functioned as a way to significantly increase the biomass concentration, increase in biomass did not result in an equivalent increase in enzyme synthesis, suggesting that the production of the enzyme by this method is not growth related.

Calcium alginate, which was used as support material allowed effective attachment of fungal mycelia. The SEM examination showed the attachment of fungal mycelia and the intensive colonization from within the alginate bead where the fungal spores were allowed to grow in situ. No suspended cells or mycelial pellets were detected in the culture broth and therefore it resulted in a clear liquid broth throughout the fermentation process. This condition suggested that the cells were protected and attached to the support matrix.

The mycelial growth is usually confined to the subsurface of the calcium alginate beads and therefore surface stability of the calcium alginate beads becomes an important factor to be considered. A membrane like coating is thought to be the result of contact between

sodium alginate and calcium chloride at the beginning of bead formation suggesting that the outer layer is mechanically weaker than the underlying calcium alginate. That was the reason for studying the effect of sodium alginate concentration on tannase production.

The increase of alginate concentration gradually reduced the leakage of cells into the cultivation medium followed by the limitation of growth and enzyme released[22]. However at the optimized alginate concentration, the entrapments of cells were maintained and the enzyme production was higher. This condition correlated to the optimized number of beads used (40 beads per 50 mL per flask). Many researchers have reported that the right concentrations of alginate were found to minimize the leakage of biomass out of the beads and as a formula of spherical and stable beads[13, 16, 26]. Besides, calcium alginate also maintained the porosity for the supply of nutrient and oxygen diffusion to the cells[14]. The structure of calcium alginate inside beads was gel-like. Despite the solid appearance, oxygen and nutrient diffusion through the calcium alginate used was clearly not so restrictive as to force mycelium to grow at the surface although further studies to be made on maximizing biomass by increasing the inoculums size may reveal otherwise.

Higher inoculum size than the optimized concentration resulted in reduced enzyme production and fungal growth. Klein[18] reported that high growth in the support material can cause diffusional limitation of oxygen and substrate in to the bead and thus reduced the enzyme production. Furthermore, the efficiency of enzyme production decreased with the increase of cell loading in immobilization due to inadequate substrate diffusion.

CONCLUSION

Immobilization of whole cells of *Penicillium digitatum* FETL DS1 for the production of extracellular tannase offers many advantages including the ability to separate cell biomass from cultivation broth and also enhancing productivity. However, proper selection of immobilization techniques and supporting materials was needed to minimize the disadvantages of immobilization. The cell entrapment in calcium alginate beads is one of the suitable method for cell immobilization because it is simpler, less expensive and non-toxic.

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REFERENCES

[1] A.Adinarayana, K.V.V.S.N.Bapi Raju, P.Ellaiah; Process Biochem., **39**, 1331-1339 **(2004)**.
[2] C.N.Anguilar, G.Gutierrez-Sanches; Int.J.Food Sci.Tech., **7**, 373-382 **(2001)**.
[3] A.Anwar, S.A.Ul-Qader, A.Raiz, S.Iqbal, A.Azhar; World App.Sci.J., **7**, 1281-1286 **(2009)**.
[4] L.Ayed, M.Hamdi; Biotechnology Letters, **24**, 1763-1765 **(2002)**.
[5] A.Batra, R.K.Saxena; Process Biochem., **40**, 1553-1557 **(2005)**.
[6] V.Battestine, G.A.Macedo; Bioresour.Technol., **98**, 1832-1837 **(2007)**.
[7] I.Darah, M.L.Teo, C.O.Ibrahim; Holothuria Atra.Sains Malaysiana, **2(1)**, 87-97 **(1996)**.
[8] I.Darah, C.O.Ibrahim; Asia Pac.J.Mol.Biol.Biotechnol., **5(3)**, 143-154 **(1997)**.
[9] I.Darah, C.O.Ibrahim; Folia Microbiol., **43(2)**, 161-168 **(1998)**.
[10] I.Darah, G.Sumathi, K.Jain, S.H.Lim; Bioprocess.Biosyst.Eng., **34(7)**, 795-801 **(2011a)**.
[11] I.Darah, G.Sumathi, K.Jain, S.H.Lim; App.Biochem.Biotechnol., **165**, 1682-1690 **(2011b)**.
[12] A.M.Dechamps, G.Otuk, J.Lebeault; J.Ferment Technol., **61**, 55-59 **(1983)**.
[13] B.M.Doumèche, J.Heinemann, W.Büchs, Hartmeier, M.B.Ansorge-Schumacher; J.Mol.Catal.B., **18**, 19-27 **(2002)**.
[14] M.Elibol, A.R.Moreira; Process Biochem., **38**, 1445-1450 **(2003)**.
[15] A.B.El-Tanash, A.A.Sherief, A.Nour; Braz.J.Chem.Eng., **28**, 381-391 **(2011)**.
[16] A.M.Farag, M.A.Hassan, Enzyme.Microb.Tech., **34**, 85-93 **(2004)**.
[17] S.K.Hota, J.R.Dutta, R.Banerjee; Indian J.Biotechnol., **6**, 200-204 **(2007)**.
[18] J.Klein; Elsevier Applied Science Publisher Ltd., London, 1-8 **(1988)**.
[19] R.Kumar, A.Kumar, R.Nagpal, J.Sharma, A.Kunani; Ann.Microbiol., **60**, 177-179 **(2010)**.

FULL PAPER

[20] P.K.Lekha, B.K.Lonsane; Adv.and Appl.Microbiol., **44**, 215-260 **(1997)**.
[21] K.W.Lim, I.Darah; Mal.J.Pharm.Sci., **2(2)**, 9-17 **(2004)**.
[22] A.Martinsen, I.Storro, G.S.Braek; Biotechnol.Bioeng., **39**, 186-194 **(1992)**.
[23] J.N.Nigam; J.Biotechnol., **80**, 189-193 **(2000)**.
[24] R.Paranthaman, R.Vidyalakshmi, S.Murugesh, K.Singaravadivel; Adv.Biol.Res., **31**, 34-39 **(2009)**.
[25] S.Rout, R.Banerjee; Indian J.Biotechnol., **5**, 346-350 **(2006)**.
[26] A.A.Samia; J.Appl.Sci.Res., **4**, 1777-1781 **(2008)**.
[27] H.Vardin, H.Fenercioglu; Nahrung., **42**, 300-303 **(2003)**.
[28] X.Zhong, L.Peng, S.Zheng, Z.Sun, Y.Ren, M.Dong; A.Xu; Protein Expres.Purif., **36**, 165-169 **(2004)**.