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Growth kinetic of *Rhizopus* sp. immobilised on loofah sponge for whole-cell biocatalyst in different cultivation media

Azlin Suhaida Azmi *, Nur Atikah Naqiah Mohd Bakari, Fathilah Ali, Noor Illi Mohamad Puad, Farah Ahmad

Department of Chemical Engineering and Sustainability, Kulliyyah of Engineering, International Islamic University Malaysia, P.O. Box 10, 50728 Kuala Lumpur, Malaysia

*Corresponding email: azlinsu76@iium.edu.my

Abstract

Immobilisation of filamentous fungi such as *Rhizopus* sp. onto biomass support particles (BSP) surface was studied as whole-cell biocatalyst. The function was to aid fermentation process. By using the microbial immobilisation process, the complex procedures of isolation, purification and immobilisation of extracellular enzyme can be avoided. Loofa sponge was selected as the BSP to aid in the immobilisation of the cells. In this study, the growth of immobilised *Rhizopus* sp. on loofa sponge was compared in four different cultivation media and the attachment of *Rhizopus* sp. on loofa sponge was investigated using scanning electron microscopy (SEM). The media used in this study were hydrolysed cassava starch, cassava dextrose, potato dextrose, and soy dextrose. The process condition and other parameters which were temperature, pH, inoculum dilution, and weight of loofa sponge, fixed at 30 °C, 7, 20 mL, and 2 g, respectively. The highest and lowest of maximum growth (y_{max}) of the immobilised cells were determined from potato dextrose and soy powder mixed with dextrose media, respectively at 1.5281 g/g and 1.0370 g/g. Whilst, the highest and lowest observed rate constant (k) were obtained from cassava starch mixed with dextrose and soy powder mixed with dextrose, which respectively at 2.9403 day^{-1} and −0.8763 day^{-1}. SEM images showed the presence of mycelia attached to the loofah sponge after immobilisation process. In conclusion, *Rhizopus* sp. has been successfully immobilised on loofah sponge as a whole-cell biocatalyst.

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1.0 Introduction

Fermentation is used as means to produce a variety of fermentation products such as organic acids and alcohols. These products are in a high demand as feedstock in many other industrial products (Huang et al., 2021; Miller et al., 2019). *Rhizopus* sp. is one of the fungi used as an efficient biocatalyst in such fermentation process due to its robustness which requires minimal nutrients for its growth (Azmi et al., 2016; Maslova et al., 2019). The fungi being able to produce several industrial enzymes such as protease, amylase, and lipase (Benabda et al., 2019; López-Fernández et al., 2020), assist fermentation. Using the intracellular *Rhizopus* enzyme, the complex separation procedures involving the extracellular enzyme can be avoided. The isolation, purification and immobilisation of extracellular enzyme are the main obstacles in achieving low-cost production process.

Immobilisation of whole-cell biocatalysts can enhance the performance of the biocatalysts by elongating their lifetime and having high stability of immobilised cells in contrast with the free cell as shown by more efficient performance when reusing the enzyme. This immobilisation technique also gives benefits in increasing specific biocatalyst loading and the simplicity of the recycling and the downstream processes (Sattari et al., 2015). Immobilisation also generates higher cell density, increased specific productivity, easy separation of catalyst and products, increase the possibility of continuous bioreactors arrangement without cell wash-out, recycling of biocatalyst and reducing the cost (Polakovič et al., 2017).

The particles that are selected as cell support for the immobilisation processes are known as biomass support particles (BSPs). The cell support must be mechanically strong for long term usage, nonreactive and nontoxic and the procedure for the immobilisation process also must be easy to perform (Maslova et al., 2019). Biopolymers are an effective support for the immobilisation of enzyme respective to their characteristics which are biodegradability, biocompatibility, and high affinity to peptides including enzymes. Furthermore, natural biopolymers

support larger quantity of peptides binding and smooth the control of catalysed processes.

Immobilisation of *Rhizopus* sp. on loofa sponge as a whole-cell biocatalyst was investigated in this current study. In this study, the growth of immobilised *Rhizopus* sp. on loofah sponge was compared in different mixtures of cultivation media which were cassava starch, potato dextrose broth, soy powder with dextrose, and cassava starch with dextrose. The immobilisation was conducted at fixed process parameters of temperature, pH, inoculum dilution, and weight of loofah sponge at 30 °C, 7, 20 mL and 2 g, respectively. The cell dry weights (g cell/ g loofah sponge) were obtained, and the growth kinetics was fitted in a regression equation model. Then, the presence of the *Rhizopus* sp. on a loofah sponge was investigated using scanning electron microscopy (SEM).

2.0 Methodology

2.1 Materials and medium preparation

Cassava tuber and loofah sponge were purchased from the local market (Selangor). Potato dextrose broth and dextrose powder were purchased from Difco Laboratories and Global Sdn. Bhd., respectively. *Rhizopus* sp. was isolated from bread and grown on potato dextrose agar (PDA) at an incubation temperature of 37 °C for 4 days. The spores were collected by shaving using the L-loop and extracted with 25 mL of sterilized distilled water. Then, the spores were filtered through sterilized filter paper. The inoculum size was 10^5 spores/mL.

Cassava tubers were peeled and washed with water. Then, it was dried in an oven at 80 °C for 24 hours. The dried pieces were ground to powder and stored in a microwavable container until further use. Four media (Table 1) were prepared in the 200 mL of distilled water in 500 mL beakers. The media were heated on a hot plate at 50 °C until dissolved and strained through a sieve. Each medium was transferred in a 500-mL Schott bottle and autoclaved at 121 °C for 30 minutes and left to be cooled at room temperature.

2.2 Rhizopus sp. immobilisation on loofa sponge

A modified method based on Ranjit & Srividya (2016) was adopted. Loofa sponge was submerged in distilled water and autoclaved three times at the temperature of 121 °C for 15 minutes. The distilled water was replaced few times to remove any chemical that may contaminate the culture medium. Approximately 2 g of loofa sponge (about 5 to 7 mm cubic size each) was put in each flask. A 50 mL of the cultivation medium was added to the spores. After sterilisation, each flask was put in the water bath at 30 °C for seven days. The pH was adjusted to 7 using hydrochloric acid and sodium hydroxide.

Table 1: Cultivation medium and its composition

Medium	Composition
Cassava starch	5 g of cassava powder
Potato dextrose	5 g of potato dextrose broth powder
Soy dextrose	4 g of soy powder and 1 g of dextrose
Cassava dextrose	4 g of cassava and 1 g of dextrose

2.3 Cell dry weight and growth model equation

A piece of loofah sponge immobilised with *Rhizopus* sp. was taken from each of the medium (Table 1) every day for seven days. Each piece of the sponges was put on a small piece of aluminium foil and dried in the oven at 50 °C for one day. Then, the sponge was placed in a desiccator for about 10 minutes. The sponges were weighted on a weight balance. The immobilised cells' dry weight (ICDW) was calculated using Eq. (1).

$$ICDW = \frac{A-B-C}{C} \quad (1)$$

where *A, B* and *C* are the weight of immobilised cell attached to a piece of loofa sponge on an aluminium foil, weight of aluminium foil, and weight of a piece of loofa sponge, respectively in gram (g). The growth model equation is shown in Eq. (2).

$$y = y_{max}(1 - e^{-kt}) \quad (2)$$

where y, y_{max}, k and t are growth (g/g), maximum growth (g/g), observed rate constant (day^{-1}) and time (day), respectively.

2.4 Analytical methods and equipment cell

The morphology of *Rhizopus* sp. immobilised on loofa sponge was investigated using scanning electron microscopy (JSM-5600, JEOL, Tokyo, Japan). The concentration of glucose, lactic acid and ethanol were determined from the supernatant after the immobilisation process. HPLC was used to analyse the concentration of the products. The samples were centrifuged at 5000 rpm for 15 minutes and filtered using syringe 0.45 μm syringe filter (Azmi et al., 2016).

3.0 Results and discussion

3.1 Growth kinetics of immobilised Rhizopus sp.

Fig. 1 shows the graph of growth kinetics of *Rhizopus* sp. attached to loofah sponge during the immobilisation process in seven days of growth duration. The immobilised cells were grown in various media which were cassava starch, potato dextrose, cassava dextrose, and soy dextrose broths. The process condition and other parameters which

were temperature, pH, inoculum dilution, and weight of loofah sponge were respectively fixed at 30 °C, 7, 20 mL, and 2 g. The growth was based on the dry weight of cell per weight of loofah sponge (g/g) as calculated based on Eq. (1).

The growth data were fitted to the exponential association growth (y) model of Eq. (2) and plotted in Fig. 1. The values for the maximum growth (y_{max}), observed rate constants (k), and coefficient of determination (R^2) are shown in Table 2. The R^2 are all above 0.80 except for soy dextrose broth which indicates that more than 80 % of the data fit the regression model.

The highest y_{max} of the immobilised cells was obtained from the potato dextrose broth with 1.5281 g/g followed by immobilised cells in cassava dextrose broth with 1.4170 g/g as shown in Table 2. Whilst the lowest y_{max} was obtained when immobilised in soy dextrose broth with 1.0370 g/g. This study showed higher cell mass attachment as compared to the study done by Alasali et al. (2022), with the observation after 2 days compared to current study after 7 days. However, quite similar cell density was obtained as studied by He et al. (2016) where the observation made after 3 days. Cassava dextrose and potato dextrose broths had higher content of starch and simple sugar compared to soy dextrose medium. Cassava contained more than 80% of starch (Karlström et al., 2016) with the addition of simple sugar like dextrose. On the other hand, Robertson et al. (2018) and Stevenson et al. (2006) reported that the concentration of starch in potato and soybean were 16 % and between 0.19 to 0.91 %, respectively. Thus, immobilised cells in soy dextrose broth recorded lower growth than the other media. *Rhizopus* sp. has amylolytic characteristics where it converted starch to glucose. When there was low starch concentration, the glucose concentration also became low, hence lower production of metabolites and the growth of cell were achieved.

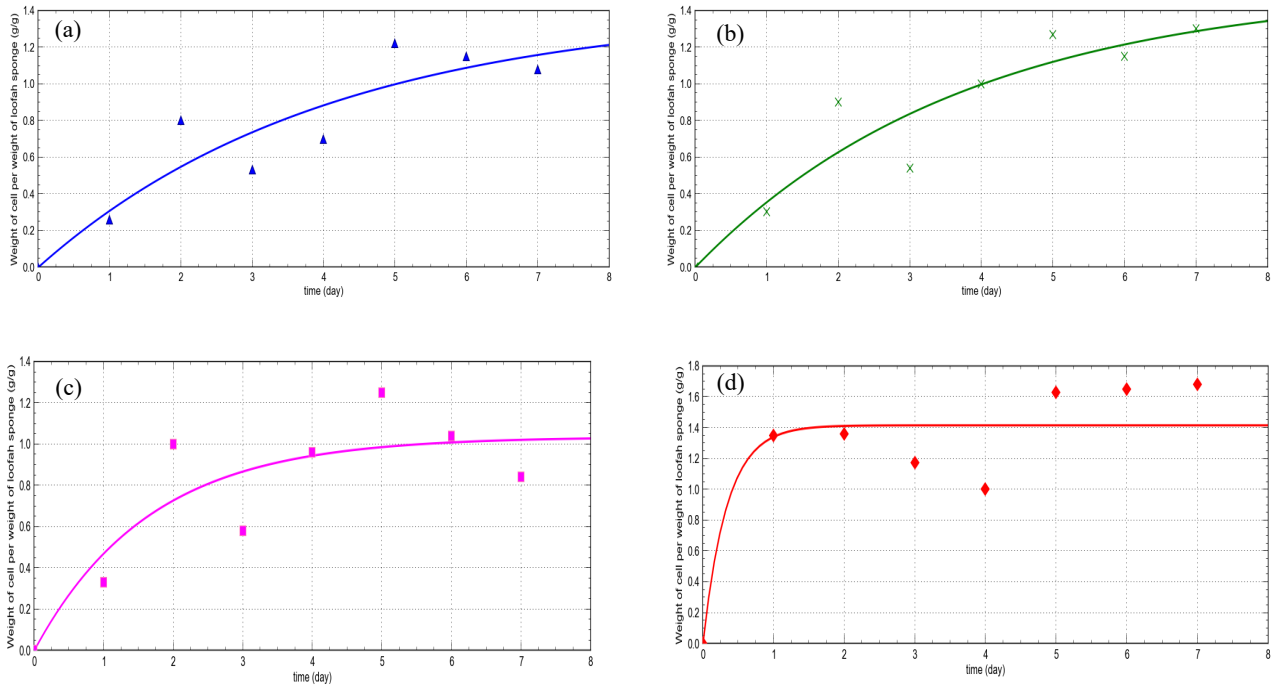

Fig 1: Growth kinetics of *Rhizopus* sp. on loofah sponge in (a) hydrolysed cassava, (b) potato dextrose, (c) soy dextrose, and (d) cassava dextrose

Table 2: Values of constants y_{max} and k and coefficient of determination (R^2)

	y_{max} (g/g)	k (day^{-1})	R^2
Cassava starch	1.4104	0.2467	0.8497
Potato Dextrose	1.5281	0.2652	0.8798
Soy Dextrose	1.0370	−0.8763	0.7694
Cassava Dextrose	1.4170	2.9403	0.8100

3.2 Rhizopus sp. Morphology and Cell Attachment

Fig. 2(a) shows the neat loofah sponge before immobilization, while Fig. 2(b) shows the cell mass attached to the loofah sponge which was clearly shown by the presence of black attachment on the sponge. While Fig. 2(c) shows the morphology of *Rhizopus* sp. under microscope. The species was isolated and grown on PDA. *Rhizopus* was characterized by its black sporangiospores as can be seen in the figure where some has been released in the liquid medium and some were still intact inside the

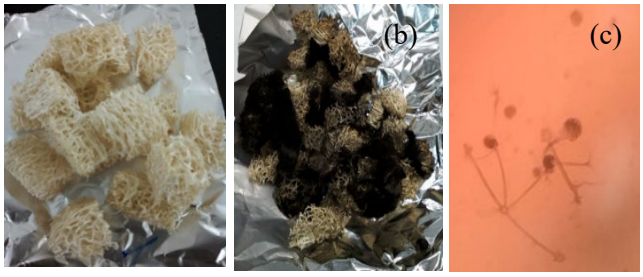

Fig. 2:(a) Neat loofah sponge, (b) dried immobilised cell on loofah sponge grown in potato dextrose, and (c) morphology of the *Rhizopus* sp. under microscope (250× of magnification).

spherical structure of sporangium on the tips of sporangiophores. It was also being characterized by a body of branching mycelia composed of three types of hyphae: stolons, rhizoids, and unbranching sporangiophores (Melissa Petruzzello, 2016) .

Fig. 3 shows SEM images of loofah sponge without (Fig. 3(a)) and with cell attachment on the sponge after 7 days of immobilisation process. Fig. 3(b) to 3(c) clearly show the presence of *Rhizopus* sp. that are firmly attached and covered the loofah sponge. These resulted from immobilisation process using different medium. These validate the presence and attachment of *Rhizopus* sp. All figures, except Figure 3(a), clearly shown mycelia of *Rhizopus* which were firmly grown and covered on the sponge fibres. The cells immobilised during the cultivation after adhesion of the cell to the porous matrix surface, loofa sponge. Then, the immobilization generated higher cell density and increased the cell mass as also observed by Cabulis et al. (2012).

4.0 Conclusions

Rhizopus sp. has been successfully immobilised on loofah sponge as a whole-cell biocatalyst for starch fermentation. The growth kinetics of the immobilised *Rhizopus* sp. on the sponge was compared in four different cultivation media namely cassava starch, potato dextrose, soy dextrose and cassava dextrose broths. The highest to lowest growth of *Rhizopus* sp. that attached to the loofah sponge was in the following order of cultivation medium which were cassava dextrose, potato dextrose, cassava starch and soy dextrose broths as depicted in the exponential association growth model. In addition, the presence of the cell that attached to loofa sponge was also investigated using scanning electron microscopy (SEM). The fungi were observed and found attached to the loofah sponge by the presence of mycelia consisting of sporangiospores and black sporangia at the tip of sporangiospores. The mycelia of *Rhizopus* sp. were observed firmly attached and covered the surface of the loofah sponge.

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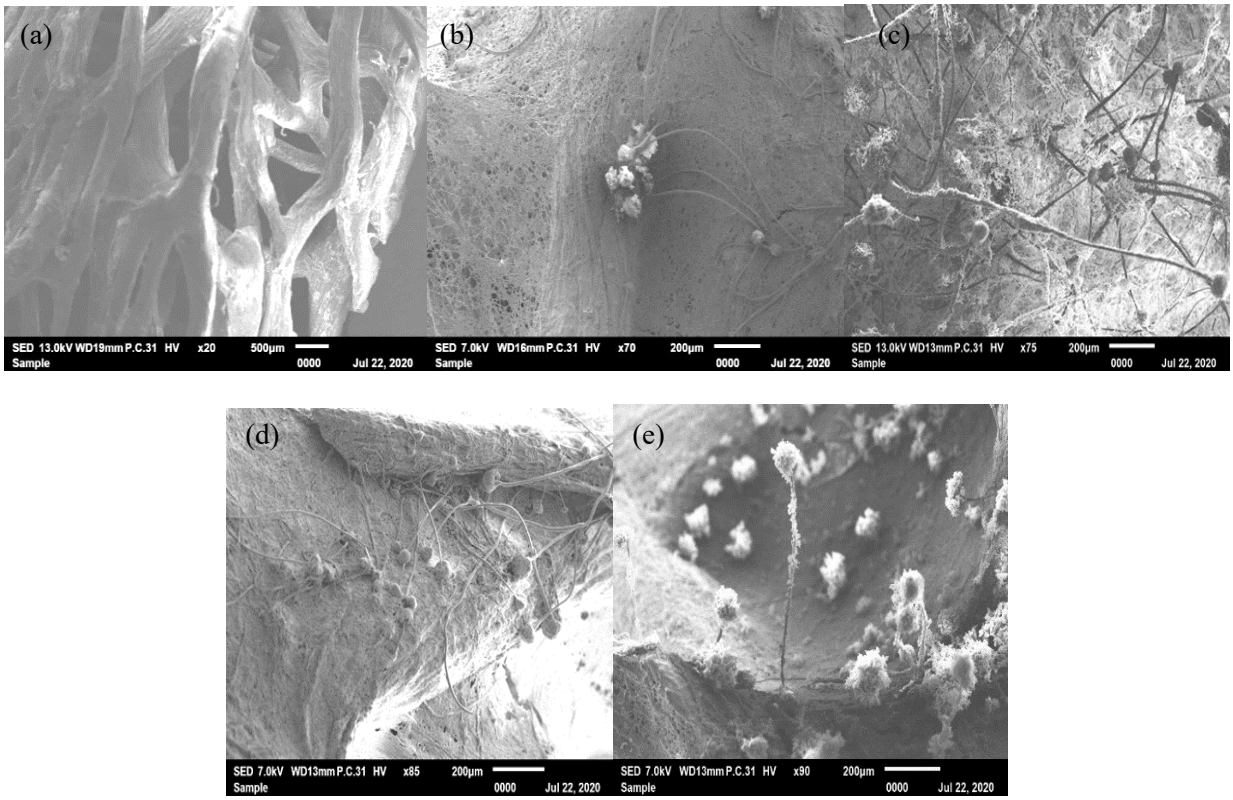

Fig. 3: Surface scanning electron microscopy (SEM) of loofah sponge (a) neat loofa sponge, and immobilised cell in (b) cassava starch, (c) soy dextrose, (d) cassava dextrose, and (e) potato dextrose broths

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