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Immobilization of *Providencia stuartii* Cells in Pumice Stone and Its Application for *N*-Acetylglucosamine Production

Immobilization of *Providencia stuartii* Cells Using Pumice Stone

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SUMMARY

Research background. Shrimp shells contain chitin that can further be processed into *N*-acetylglucosamine which has been extensively used to treat joint damage. *Providencia stuartii* isolated form previous research has strong chitinolytic activity and may be utilized in the form of immobilized cells to be used in repeated fermentation. Pumice is a porous and rigid stone that offers superior mechanical strength, making it suitable to be used for immobilization process.

Experimental approach. The research used experimental method to conduct the submerged fermentation process with different pumice stone size and pumice stone:growth medium ratio (*m*/*V*). The fermentation was carried out for 4 days at 37 °C and pH of 7.0. The optimum pumice stone size and pumice stone:growth medium ratio (*m*/*V*) were used to determine the optimum fermentation cycle to produce *N*-acetylglucosamine.

Results and conclusions. Pumice stones of 1.0 × 1.0 × 1.0 cm and pumice stone:growth medium ratio (*m*/*V*) of 1:5 were found to be the optimum conditions which successfully

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immobilized (89.99±1.65) % cells and produced (331.37±7.34) g/L *N*-acetylglucosamine. The highest *N*-acetylglucosamine concentration of (322.97±2.46) g/L was obtained in the first fermentation cycle which then decreased and remained stable throughout the last three cycles of fermentation.

Novelty and scientific contribution. P. stuartii was a strong chitinolytic bacteria previously isolated from rotten shrimp shells and was used for the first time in immobilized form to produce *N*-acetylglucosamine. The findings in this research showed potential use of *P. stuartii* cells immobilized in pumice stone for continuous production of *N*-acetylglucosamine using fermentation method.

Key words: cell immobilization; chitin; *N*-acetylglucosamine; *Providencia stuartii*; pumice stone; repeated fermentation

INTRODUCTION

Shrimp shells comprise of 30-40 % protein, 30-50 % calcium carbonate and 20-30 % chitin, depend on the type of the shrimp (*1*). The monomeric unit of chitin includes *N*-acetylglucosamine, an amino sugar that plays role in stimulating joint functions and forming the structure of cartilage (*2*). The lack of glucosamine might lead to the symptoms of osteoarthritis, which is often developed by 90 % of people above 40 years old (*3*). *N*-acetylglucosamine can be produced through chemical synthesis, enzymatic process, or microbial fermentation method (*2*). The chemical synthesis is not necessarily preferred due to its lower yield and environmental issues because strong acids are used (*4*). Meanwhile, enzymatic process poses a great challenge with its great cost for enzyme purification, lower yield, and enzyme stability issues (*2*). Hence, a microbial fermentation method is more preferred to produce *N*-acetylglucosamine (*5*).

The microorganisms which can be used to produce *N*-acetylglucosamine are those that can produce chitinolytic enzyme to break down chitin into glucosamine (*6*). Previous research has successfully isolated 17 microorganisms that possess chitinolytic activity from rotten Tiger shrimp shells. About 8 of them possessed strong chitinolytic activity, however, *Providencia stuartii* was the strongest chitinolytic bacterium isolated (*7*). Chitinolytic index of *P. stuartii* in the previous research was about 4.46 after incubation for 48 h at 37 °C (7) and it is higher compared to other chitinolytic bacteria isolated from similar sources (solid and liquid waste of shrimp shells), *i.e.*, *Acinetobacter johnsonii* (chitinolytic index of 2.069) and *Bacillus amyloliquefaciens* (chitinolytic

index of 2.084) (*8*). Although *P. stuartii* is pathogenic, mild heat treatment will be sufficient to inactivate it because of its mesophilic nature (*9*). Therefore, *P. stuartii* was considered potential to produce high amount of *N*-acetylglucosamine from shrimp shells.

Cell immobilization refers to a technique to fix the cells onto a solid support system, into a solid support matrix or retained by a membrane to keep the cell stability and enables repeated or continued utilization (*10*). Immobilization also results in high concentration of cells. Immobilization of bacteria cells can be done on a solid, porous matrix through entrapment method, which is relatively rapid and simple yet offers high stability of cells (*11*). Immobilization of *P. stuartii* allows the cells to be repeatedly used for the *N*-acetylglucosamine production.

Pumice is a porous and rigid stone which offers high mechanical strength. The high porosity of about 90 % makes it preferable to be used in immobilization process as it may lead to a large surface area with low commercial cost (*11*). Moreover, pumice, which is primarily composed of silica, is also an ideal immobilization matrix. It is because of its inertness and stability that make it reusable (*12*). It was also reported from a previous research that the productivity of immobilized cells in pumice stones is two-folds higher than the suspended cell system (*13*).

Pumice stone has been used to immobilize several microorganisms, such as *S. cerevisiae* (*14*), *Penicillium digitatum* (*15*), *Clostridium beijerinckii* NRRL B-593 (*16*), and *Aspergillus niger* (*17*). Furthermore, pumice stones which have been used to immobilized microorganisms can be used for protease production (*18*), fructooligosaccharides production (*19*), lactic acid production (*20*), and for improving β-glucanase productivity (*21*).

In this research, *P. stuartii* cells were immobilized using pumice stone by entrapment method for further use in *N*-acetylglucosamine production from shrimp shells. The objectives of this research were to determine the optimum size of pumice stone, optimum ratio of pumice stone and growth medium (*m*/*V*), as well as the optimum fermentation cycle of immobilized cells used in the fermentation-based production of *N*-acetylglucosamine.

MATERIALS AND METHODS

Materials

The main materials used in this research were the shells of whiteleg shrimp (*Penaeus vannamei*) obtained from PT. First Marine Seafood, Muara Baru, Jakarta Utara, the culture of *Providencia stuartii* which is obtained from previous research (*7*) and the pumice stones from Aquadratic Aquarium Supplier, Bandung, Indonesia as the immobilization matrix. The chemicals

and media used in this research were the aquadest, standard *N*-acetylglucosamine (Sigma-Aldrich, St. Louis, MO, USA), Nutrient Agar (NA) , Nutrient Broth (NB), Bovine Serum Albumen (BSA), Coomassie Brilliant Blue G-250, dipotassium phosphate, potassium dihydrogen phosphate, ammonium sulphate, magnesium sulphate heptahydrate, ninhydrin, and pH buffer 7 (all from Merck, Darmstadt, Germany).

Shrimp shells powder preparation

Shells of *Penaeus vannamei* were separated from the leftover meat, washed and sun-dried for two days. The dried shrimp shells were then crushed into shrimp shells powder by using mill (Fomac FCT-Z500, Jakarta, Indonesia) and sieved with 60-mesh sieve, yielding in a smooth shrimp shells powder (*22*).

Immobilization of P. stuartii cells using pumice stone

The immobilization of *P. stuartii* cells began by preparation of pumice stone as the immobilization support. The pumice stones were cut into different sizes of 1.0 × 1.0 × 1.0 cm, 1.5 × 1.5 × 1.5 cm, and 2.0 × 2.0 × 2.0 cm, boiled for 10 min, washed three times, and dried overnight at 60 °C using oven (Memmert UNE 800, Schwabach, Germany). Pumice stones were then sterilized using autoclave (Hirayama Hiclave HVE 50, Saitama, Japan) at 121 °C for 15 min before use. Meanwhile, 1 mL of *P. stuartii* culture was inoculated into 300 mL growth medium. Growth medium used in this research consisted of 2.4 g Nutrient Broth (NB), 0.09 g KH_2PO_4, 0.21 g K_2HPO_4, 0.03 g $MgSO_4.7H_2O$, and 2.1 g $(NH_4)_2SO_4$ in 300 mL aquadest (*23*).

The pre-treated pumice was then submerged in the growth medium with pumice stone:growth medium ratio (*m*/*V*) of 1:5, 1:10 and 1:15. The pumice was left in the growth medium for 2 h at 37 °C using incubator (Memmert BE600, Schwabach, Germany). The number of immobilized cells was also counted, by subtracting the number of unimmobilized cells from the initial number of cells. The number of cells were counted using haemocytometer (*24*).

Submerged fermentation was done for 4 days at 37 °C and pH media of 7.0 with periodic shaking. These temperature and pH were reported to be the optimum conditions for the growth of *P. stuartii* (*25-26*). Fermentation process was carried out by putting the immobilized cells from different treatments into the 300 mL fermentation media, consisted of 30 g shrimp shells powder,

0.09 g KH_2PO_4, 0.21 g K_2HPO_4, 0.03 g $MgSO_4.7H_2O$, and 2.1 g $(NH_4)_2SO_4$ in 300 mL aquadest (*23*).

To stop the fermentation, media were then heated at 70 °C for 45 min using waterbath (Memmert WNB-14, Schwabach, Germany), followed by centrifugation at 4000 rpm for 15 min using centrifuge (MPW e-223, Westertimke, Germany) and filtration with Whatmann No. 1 filter paper. The filtrate obtained was then analysed for its *N*-acetylglucosamine content (*27*). Optimum size of pumice stone and optimum pumice stone:growth medium ratio (*m*/*V*) were then determined based on *N*-acetylglucosamine concentration obtained from the fermentation.

Determination of optimum fermentation cycle

The optimum size of pumice stone and optimum pumice stone:growth medium ratio (*m*/*V*) were then used to determine the optimum fermentation cycles. The fermentation process was done repeatedly up to four cycles (*28)*. Each cycle of fermentation was done at 37 °C and pH of 7.0 for 4 days, shaken periodically. After each cycle, the fermentation process was terminated through heating and the concentration of *N*-acetylglucosamine was quantified. *N*-acetylglucosamine concentration was quantified using UV-Visible spectrophotometer (Thermo Scientific Genesys 10s, Waltham, MA, USA) at 324 nm wavelength (*27*).

Scanning electron microscopy (SEM) analysis on immobilization support

Pumice stones of 1.0 ×1.0 ×1.0 cm which had been immobilized with the *P. stuartii* cells were collected from the growth medium and dried overnight in the incubator (Memmert BE600, Schwabach, Germany) at 37 °C. The prepared pumice samples were then sent to PT. Qantaz W. K (Labq.id) for the SEM analysis. The immobilized pumice stone was observed with a Thermo Scientific™ Quanta FEG 650 Scanning Electron Microscope (Thermo Fisher Scientific, Waltham, MA, USA).

Data analysis

To determine the optimum fermentation cycle, experimental design used was Completely Randomized Factorial Design with five replications. Data obtained in this research were analyzed statistically with Analysis of Variance (ANOVA) using SPSS Software, version 22.0. Further analysis was done using Post hoc Duncan test.

RESULTS AND DISCUSSION

Cell immobilization is a technique of fixing cells into a support to keep the cell stability, allowing the possibility of repeated or continued use (*10*). Therefore, immobilized cells counting was conducted to assure that *P. stuartii* cells had been immobilized into the pumice stones. Table 1. shows the percentage of immobilized *P. stuartii* cells from different treatments. The initial number of *P. stuarti* cells prior to immobilization was 10^7 CFU/mL, as for fermentation process, bacterial cell count required is about 10^6-10^7 CFU/mL (*29*).

Table 1 shows that pumice stones can be effectively used as a matrix for cell immobilization process with the highest immobilized cells percentage of (89.99±1.65) %. This outcome indicates that most of the bacterial cells had been entrapped in the pumice stones pores. Larger size of pumice stone tends to lead to the lower percentage of immobilized cells. This result correlates with previous research on immobilization of *T. turnirae* cells for protease production, which stated that pumice stones that have smaller size and rougher surface offers superior microenvironmental conditions for cell immobilization (*18*), such as larger contact area and more favourable binding sites for the cell surface structures to interact (*30*). Thus, leads to higher immobilized cells percentage. In addition, carrier with a large surface area to volume ratio may result in an efficient immobilization, as the cells should firstly attach to the surface of the support before progressively be entrapped in the pores (*31*).

This result is higher compared to another research that immobilize *Pseudomonas putida* using pumice particles, in which immobilization efficiency was 67.83 % (*32*). It is also higher than other researches using alginate beads to immobilize chitosan, *i.e.,* about 74 % (*33*), calcium agar beads and agar beads to immobilize α-amylase, *i.e.,* 80 % and 63.83 %, respectively (*34*). Therefore, it can also be inferred that the macroporous pumice have greater loading capacity compared to the natural gels.

Higher ratio of pumice stone to growth medium (*m*/*V*) contributes to higher immobilized cells percentage because it provides more amount of supports for the immobilization process. Higher amount of added supports naturally results in a higher loading into the support materials regardless the types of material (*35*). In this research, pumice stone:growth medium ratio (*m*/*V*) of 1:5 offered the most suitable amount of carriers compared to the other treatments.

To know the efficiency of pumice stone in immobilizing *P. stuartii* cells, *N*-acetylglucosamine produced after fermentation was also measured. Fermentation was conducted for 4 days at 37 °C with pH media of 7.0. This temperature and pH were required for optimum

growth of *Providencia stuartii* (*25-26*). Results shows that different pumice stone sizes and different pumice stone:growth medium ratios (*m*/*V*) affect the production of *N*-acetylglucosamine. Fig. 1 shows the effect of different pumice stone sizes and pumice stone:growth medium ratio (*m*/*V*) on *N*-acetylglucosamine production.

Fig. 1 depicts that the production of *N*-acetylglucosamine decreases along with an increase of pumice size. The highest *N*-acetylglucosamine concentration was obtained from cells immobilized in the pumice stones of 1.0 × 1.0 × 1.0 cm. Moreover, pumice stones of 1.0 × 1.0 × 1.0 cm, which has the highest percentage of immobilized cells, alsoproduce the highest concentration of *N*-acetylglucosamine. It is also because a large surface area of the supporting material's porous structure promotes an efficient immobilization, resulting in high yield of products (*36*).

On the contrary, another research found that protease production firstly increases before decreases as the pumice stone size gets larger (*18*). Such difference in result suggests that pore size also influences the performances of immobilized enzymes and cells (*37*). *P. stuartii* are facultative anaerobes, therefore they require oxygen for their growth. However, oxygen transfer, which supports the growth of immobilized *P. stuartii* cells (*21*), is also affected by pore size of immobilization support. Pumice stones have irregular pores and varied connectivity (*38*), which might contribute to different result from previous research.

Fig. 1 also shows that higher pumice stone:growth medium ratio increases the production of *N*-acetylglucosamine. The highest *N*-acetylglucosamine obtained from cells immobilized with pumice stone:growth medium ratio (*m*/*V*) of 1:5. This correlates with the result of percentage of immobilized cells (Table 1). Higher percentage of immobilized cells means more cells are available to ferment shrimp shells powder, producing the higher concentration of *N*-acetylglucosamine. Furthermore, optimum yield can be achieved with the proper carrier amount. Increasing amount of carrier may provide more space for the free cells to be immobilized, which further leads to higher yield, unless it has reached the optimum value (*35*).

To ensure that *P. stuartii* cells were immobilized in pumice stone, SEM analysis was also done, and results can be seen on Fig. 2. Fig. 2 shows the electron micrographs of pumice sample after 2 h of *P. stuartii* cells immobilization. The results display the presence of cells which had been immobilized in the pores of pumice. Hence, these images also prove that pumice stone is suitable for immobilizing *P. stuartii* cells.

After the bacterial cells had been properly immobilized into the pumice stones, repeated fermentation was conducted to determine the stability of the immobilized cells. In this repeated fermentation, pumice stone with size of 1.0 × 1.0 × 1.0 cm and pumice stone:growth medium ratio of 1:5 were used. Statistical result using ANOVA shows that fermentation cycles have significant effect on the *N*-acetylglucosamine production ($p \leq 0.05$). The effect of fermentation cycles on *N*-acetylglucosamine production can be observed from Fig. 3.

Fig. 3 shows that the *N*-acetylglucosamine production significantly decreases from the first to the second cycle. The highest *N*-acetylglucosamine was obtained from the first fermentation cycle, *i.e*., (322.97±2.46) g/L. However, there was no significant difference between the *N*-acetylglucosamine concentration produced from the second to the fourth cycle with the lowest concentration of (239.63±16.69) g/L.

The decreasing result at the second cycle of fermentation was also found in a previous research that immobilize *Saccharomyces cerevisiae* in rice hulls for ethanol production. This might be caused by leaching of immobilized cells on the pumice stone's surface into the fermentation medium due to abrasion effect. This phenomenon leads to a reduction of the number of cells immobilized in the pumice stones, leaving mostly the cells which were entrapped within the pores (*39*). There is a possibility that some of the *N*-acetylglucosamine produced in the first cycle might possibly be contributed by the free cells which were leaked from the pumice stones (*19*).

However, the decreasing result was then followed by stable *N*-acetylglucosamine produced in the second, third, and fourth cycle. The same behaviour was also found from another research that immobilize *Aspergillus niger* mycelium using pumice stones for eight cycles of gluconic acid production (*17*). It was found that after having a decline in the production of gluconic acid from the first to the sixth cycle, the yield in the sixth up to eighth cycle did not continue to fall. This could be related to the fact that pumice stone has superior mechanical strength to protect the entrapped cells from the shear force (*11*), hence maintaining the number of remaining cells within the pores.

Moreover, the stable production of *N*-acetylglucosamine might also be contributed by the fact that cells within the supporting material had been properly adapted to and sufficiently maintained in the microenvironment of the pumice (*40*). High concentration of *N*-acetylglucosamine production obtained after 4 cycles of fermentation shows that immobilization

technique using pumice stones can be potentially applied for continuous fermentation using *P. stuarti* cells to produce *N*-acetylglucosamine.

CONCLUSIONS

Production of *N*-acetylglucosamine can be conducted through the repeated submerged fermentation from shrimp shells powder. In this research, cells of *P. stuartii,* a strong chitinolytic bacteria, were immobilized in pumice stone with size of 1.0 cm × 1.0 cm × 1.0 cm and could be used repeatedly for four cycles of fermentation. The highest concentration of *N*-acetylglucosamine, *i.e.*, (331.37±7.34) g/L was achieved in the first fermentation cycle, which then decreased in the second cycle and remained stable until the fourth cycle of fermentation. These research results showed potential application of immobilized *P. stuartii* cells for continuous production of *N*-acetylglucosamine from shrimp shells to treat joint damage or osteoarthritis. However, purity of *N*-acetylglucosamine obtained should be further analyzed.

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CONFLICT OF INTEREST

Authors declare there is no conflict of interest.

AUTHORS' CONTRIBUTION

Research concept and experimental design: Hardoko. Data collection: Devianita. Analysis and interpretation of data: Devianita, Yuniwaty Halim, Lucia C. Soedirga, Ratna Handayani. Manuscript preparation: Yuniwaty Halim, Devianita. Manuscript writing dan revision: Yuniwaty Halim. Critical input for revision of the manuscript: Hardoko. Overall, all authors have contributed equally based on their area of expertise.

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Table 1. Percentage of *P. stuartii* cells immobilized using different pumice stone size and pumice stone:growth medium ratio

Pumice stone size (cm × cm × cm)	*m*(pumice stone)/*V*(growth medium)	Immobilized cells/%
1.0 × 1.0 × 1.0	1:5	89.99±1.65
	1:10	85.19±3.21
	1:15	80.76±2.48
1.5 × 1.5 × 1.5	1:5	81.14±4.59
	1:10	80.48±1.14
	1:15	71.62±2.42
2.0 × 2.0 × 2.0	1:5	79.64±1.13
	1:10	71.28±1.77
	1:15	62.99±3.60

Note: Data are presented in mean value±SD.

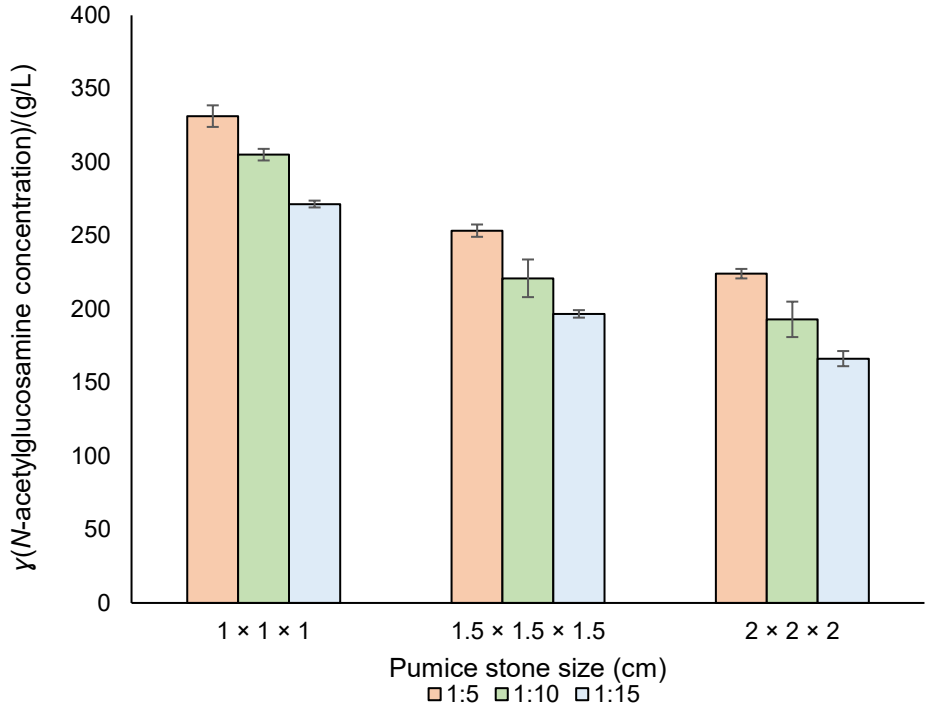
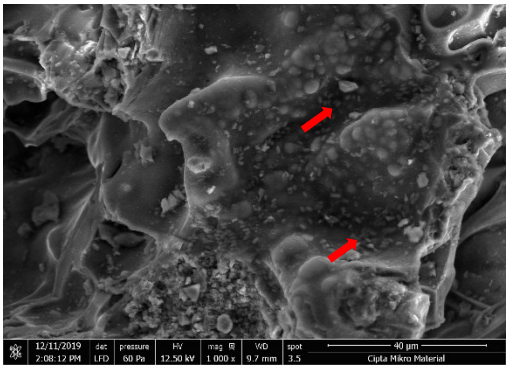
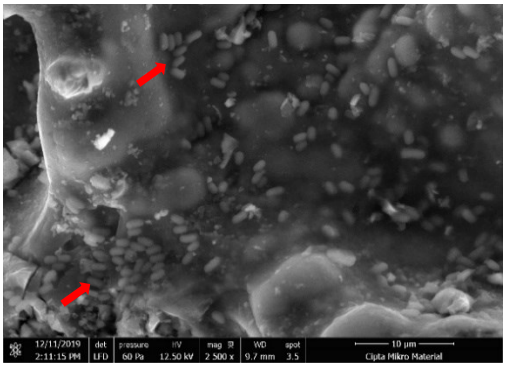


Fig. 1. Effect of pumice stone size (a) and pumice stone:growth medium ratio (*m/V*) (b) on *N*-acetylglucosamine concentration obtained after fermentation

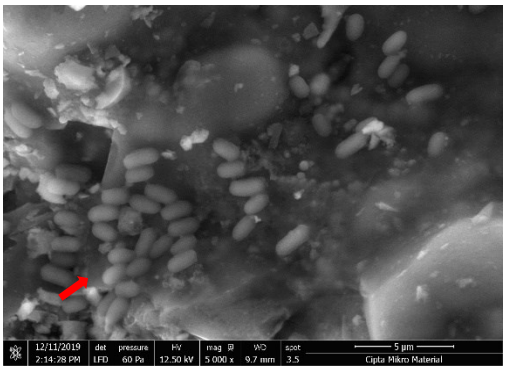
Note: Data are presented in mean value±SD.



(a)



(b)



(c)

Fig. 2. Observation results of *P. stuartii* cells immobilized in pumice stone observed using 1000x magnification (a), 2500x magnification (b), and 5000x magnification (c) under SEM

Notes: Arrows in the figures show some *P. stuartii* cells that have been entrapped in porous structure of pumice stones

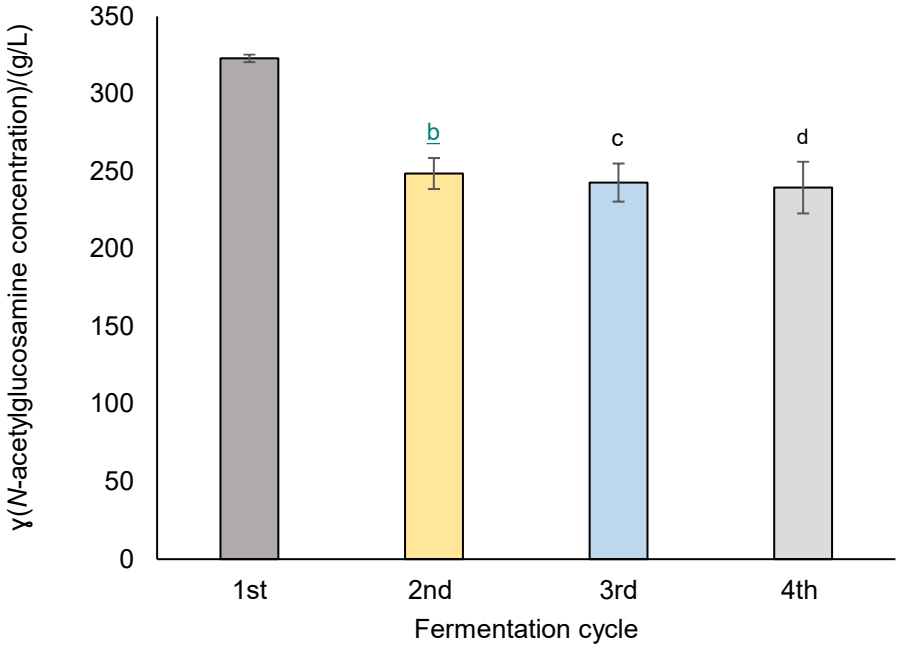


Fig. 3. *N*-acetylglucosamine concentration obtained from repeated fermentation cycle

Note: Data are presented in mean value±SD. The different letter notations indicate a significant difference ($p \leq 0.05$)