COLLATION OF CASEINOLYTIC ENZYMES YIELD FROM *BACILLUS CEREUS* 13BN USING ABUNDANT TYPES OF MEDIA POTENTIALLY AS BLOOD ANTICOAGULANTS

Mazlina Mohd Ariffin [1,2*] , Mohamad Izzuan Mohd Ishar [1] , Zarita Zakaria [2]

[1] School of Education, Faculty of Science Social and Humanity, Universiti Teknologi Malaysia, 81310 Skudai, Johor Bahru, Johor.
[2] Department of Biosciences, Faculty of Science, Universiti Teknologi Malaysia, 81310 Skudai, Johor Bahru, Johor.
Email: mazlina.mohdariffin@gmail.com*

ABSTRACT
Previously, a proteolytic enzyme producer from one of Malaysian fermented food - *belacan* was successfully isolated and named *Bacillus cereus* 13BN. This caseinolytic enzyme is a systemic enzyme, operationally needed to break down the extra amount of fibrin of the blood clots that belongs to the family of serine protease. Considering the potential of this enzyme as an alternative to the commercially available anticoagulant, the best condition for the yield of the enzyme of ***"B. Cereus 13BN"*** was optimised by slightly modifying the arrangement of broth of nutrient, using five types of carbon source, which are lactose, galactose, maltose, glucose, and glycerol. Five types of nitrogen sources which include casein, gelatine, and peptone of proteose, ammonium nitrate, and ammonium were also used in modifying the composition of nutrient broth, as well as the influence of different incubation periods. It showed that maltose and casein had constantly increased caseinolytic activity during incubation. Both sources had the highest caseinolytic and activity at 36 hours of incubation, which was 5.824 ± 0.065 U/mL for maltose, and during 72 hours of incubation, which was 3.948 ± 0.772 U/mL for casein, respectively. As for the control, the highest caseinolytic activity was at 36 hours of incubation, which was 1.390 ± 0.074 U/mL respectively. After combining these two sources, the analysis using statistical software showed that the highest caseinolytic enzymes can be optimised during 72 hours of incubation with desirability of 0.540.

Keywords: *Bacillus cereus* 13BN; protease; caseinolytic activity; carbon and nitrogen source, incubation period

1. INTRODUCTION

Nowadays, various types of cardiovascular disease had already become one of the main reasons that lead to a huge amount of death cases worldwide[6]. Symptoms that lead to cardiovascular diseases, known as CVD such as hypertension, high blood pressure and an unbalanced diet can be seen commonly occur to humankind worldwide. When a person has critical CVD, those symptoms can cause blood clotting in blood vessels, thus leading to thrombosis, and highly fatal to patients if not treated[6].To reduce the deposition of

blood clotting or thrombosis, a microbial enzyme known as natto kinase has been introduced to the pharmaceutical and medicinal world[4]. This is because, these enzymes are systemic enzymes, come from the serine protease family, and are also critically involved in breaking down the excess fibrin of blood clots in the blood vessels [4].Other commercialised natural thrombolytic enzymes such as urokinase, reptilase and also brinase have been utilised widely for clinical purposes [1]. Yet, these commercialised enzymes are still expensive [1]. Hence, *Bacillus cereus* 13BN [7] was used throughout this study. This is because the enzyme secreted by the bacteria was proven to have an anticoagulant agent, known as t-PA. It can hydrolyse fibrin along with fibrinogen totally without any support from proteolytic enzymes and it is easy and gives high chance to be an affordable medicinal anticoagulant product[7]. Thus, this research is conducted to compare the production of caseinolytic enzymes from *B. cereus* 13BN by slightly modifying the carbon source and nitrogen source of the nutrient broth compositions, and the general nutrient broth, at different incubation periods.

2. METHODS AND MEDIUMS

2.1 Bacterial strain

In this research, the *Bacillus cereus* 13BN strain was used, which was obtained from the previous research, from the paste of shrimp or known as *belacan*[7].

2.2 Preparation of Nutrient Broth (NB) medium

The *Bacillus cereus* ***13BN*** strain was incubated in 3 types of ***"nutrient broth"*** (NB), which were modified carbon source NB (MCNB), modified nitrogen source NB (MNNB), modified carbon-nitrogen source NB and the general NB. These NB media were freshly prepared from the beginning. **Table 2.1 and 2.2**showthe different compositions of NB used for culturing the bacteria;

Table 2.1 Composition of modified ***"nutrient broth"*** (NB) using different types of carbon source

Composition	Amount needed for 100mL (g)
Modified carbon source (lactose, galactose, maltose, glucose, and glycerol)	0.2
Peptone	0.5
Sodium chloride (NaCl)	0.5
Distilled water	100 mL

Table 2.2 Composition of modified ***"nutrient broth"*** (NB) using different types of nitrogen source

Composition	Amount needed for 100mL (g)
Carbon source that has the highest proteolytic activity	0.2
Modified nitrogen source (gelatin, casein, proteose peptone, ammonium sulphate, and ammonium nitrate)	0.5
Sodium chloride (NaCl)	0.5
Distilled water	100 mL

To homogenise the media were heated a little and were adjusted to the pH level of 7.0. During this study general ***"nutrient broth" or, (NB)*** was used as for control.

2.3 Optimisation of incubation period and media for the production of the enzyme

Various sources had been analysed at 37℃ using 200 rpm influencing the period of incubation with the period of 72 hours. The assay of caseinolytic activity regarding utilizing standard procedures determines the ideal conditions for the production of the enzyme that has been described in subsection 2.4. ***"The caseinolytic activity"*** was accepted as 100% at the ideal time frame regarding the production of the enzyme

2.4 The effects and incubation time using different carbon and nitrogen sources

The source of carbon that has been used in the general component of ***"nutrient broth" or, (NB)*** that refers to the extract of yeast was exchanged with carbon source of five types of carbon at ***"1% w/v"***, which were galactose, lactose, glucose, maltose, and glycerol. For nitrogen source, five types of nitrogen source replaced peptone at ***"1 % w/v"*** (casein, gelatin, proteose peptone, ammonium sulphate, and ammonium nitrate). These modified media of ***"nutrient broth"*** incubated the bacteria at 37℃ using 200 rpm for 72 hours.

2.5 Enzyme Assays

Generally, ***"B. cereus 13BN"*** was cultured in 3 different types of ***"nutrient broth" (NB)*** previously at a temperature of 37℃ with an incubation time range of 0 to 72 hours using 200 rpm. The collection of supernatants from every medium was collected within incubation time for the assay of caseinolytic activity along with the estimation of the concentration of protein. Lastly, the supernatants were collected for 15 minutes using 4000 rpm at 25℃.

2.6 Determination of caseinolytic activity

The caseinolytic activity for ***"B. cereus 13BN"*** was measured utilizing casein as substrate while tyrosine as standard with concentrations of tyrosine including ***"0.111, 0.055, 0.442, 0.553, and 0.221"*** µmoles. This assay was carried out for 10 minutes at 37℃.

2.7 Statistical Analysis

The data analysis of samples obtained from both the enzymatic assay and the Lowry assay will be calculated and obtained using ***"statistical computer software"*** such as Design Expert Software and Microsoft Excel.

3. DISCUSSION AND RESULTS

3.1 The results for the general composition of nutrient broth (control)

During this study, the common nutrient broth (NB) was used as a control. Within 0-72 hours of incubation, every 12 hours, 6 mL of culture was pipette and transferred into a 15 mL Falcon tube. 2 mL of 6 mL culture was used for OD reading, using 600 nm of absorbance, while the other 4 mL was centrifuged for 15 minutes, at 25℃ and 4000 rpm. Then, the supernatant was collected for determining the protein concentration and also the specific caseinolytic activity using the assays stated before.

3.1.1 Determination of caseinolytic activity

After incubating the culture for 0-72 hours, the supernatants were collected and

analysed to determine the caseinolytic activity. The results were recorded in **Figure 3.1** below;

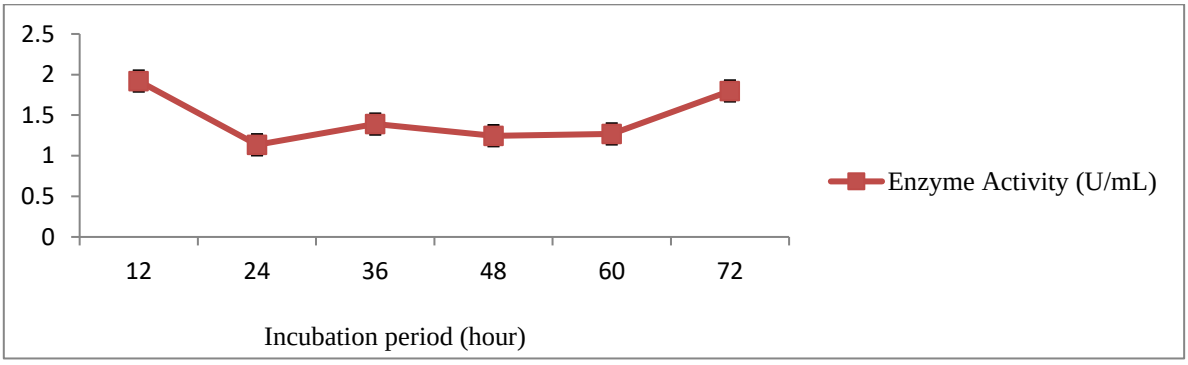

Figure 3.1 Readings of caseinolytic activity for each incubation period

Based on the results, showed that 12-hour incubation has the highest caseinolytic activity, which is1.920±0.172U/mL. Although the protein concentration recorded was 0.510±0.083 mg/mL, still has the highest caseinolytic activity reading, and also specific activity, compared to other types of carbon used. At 24-hour incubation, it showed that the caseinolytic activity result was the lowest, which is1.135±1.790 U/mL respectively. Both readings were shown in **Figure 3.1**.

3.2 Effects of modification of carbon and nitrogen source on nutrient broth composition

Throughout this study, the general composition of NB has been slightly altered, which is the carbon source in the NB, as stated in **Table 2.2** before. Hence, the processes in collecting the supernatant from each carbon source were exactly similar as stated in section **3.1**.

3.2.1 Determination of caseinolytic activity for both sources

The supernatant for each type of carbon source was collected at ***"12 hours, 24 hours, 36 hours, 48 hours, 60 hours, and 72 hours"*** incubation time. After collecting the supernatants, they were tested to determine the caseinolytic activity and specific activity for each incubation period. The results were recorded in **Figure 3.3** for the carbon source, while **Figure 3.4** for the nitrogen source as below;

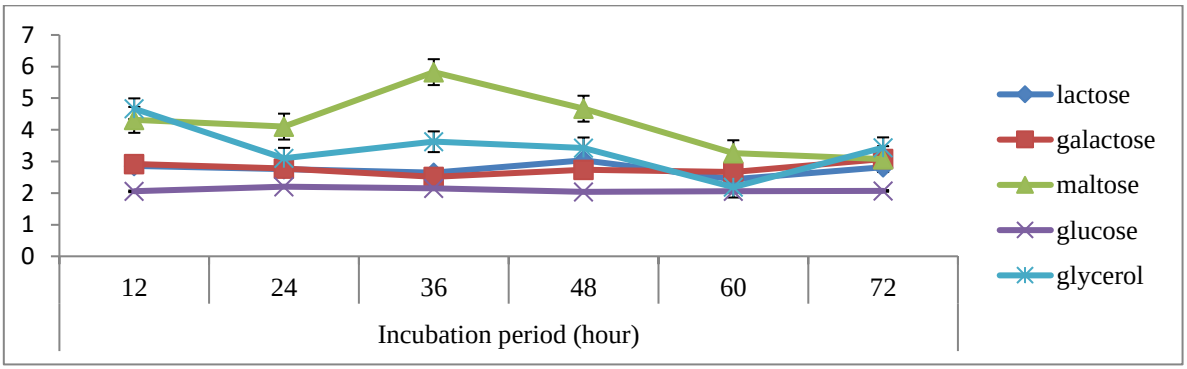

Figure 3.3 Caseinolytic activities of each carbon source during 12-72 hour

Based on the results shown in **Figure 3.3**, it was shown that maltose has the highest caseinolytic activity among other types of carbon, which was 5.824 ± 0.065 U/mL respectively. The reading was recorded as the highest during 36-hour incubation. While glucose has the lowest caseinolytic activity, which was 2.186 ± 0.615 U/mL respectively. The reason why the caseinolytic activity of this bacteria when using maltose as the carbon source, compared to glucose is because of the composition of these carbons. Maltose consists of two molecules of glucose bound together using a glycosidic bond [2]. This compound can be found as an intermediate and also as the final product in starch and glycogen production [2][3]. Compared to other types of disaccharides, maltose can be digested easier due to its compounds and gives a higher carbon source to the microbes, so that the cells can be more productive. On the other hand, glucose does not give the maximum and the highest amount of nutrients for the microbes to obtain energy. The metabolism of the microbes also is decreasing due to the inadequacy of carbon sources (glucose) in the media. That is why the caseinolytic activities for *B. cereus* 13BN using glucose as its carbon source have the lowest reading compared to others.

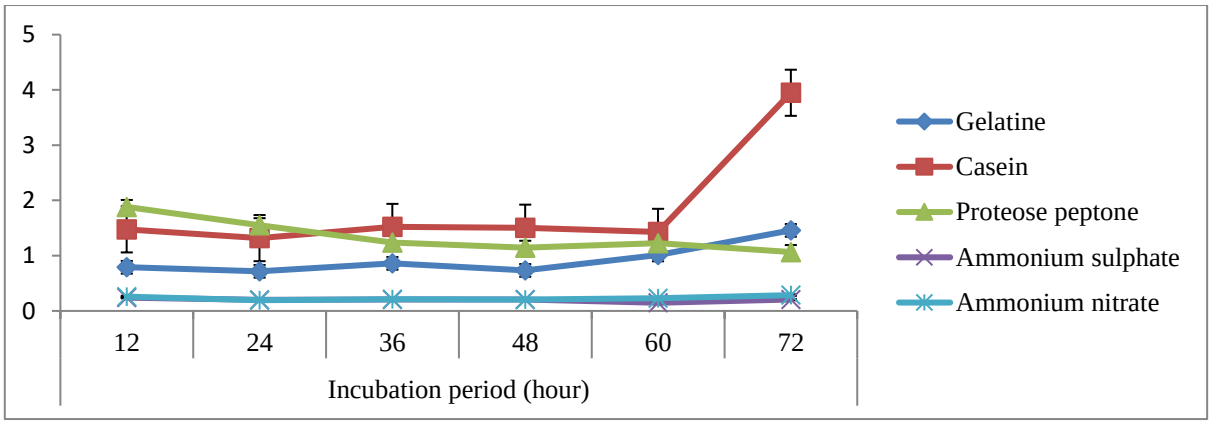

Figure 3.4 Caseinolytic activities of each nitrogen source during 12-72 hour

Based on **Figure 3.4**, showed that casein has the highest caseinolytic activity, which was 3.948±0.772 U/mL, during 72-hour incubation. Ammonium sulphate has the lowest caseinolytic activity, which was 0.148±0.453 U/mL. Casein has two properties that make it different than other milk proteins. The first one is easily dissolved in an amorphous calcium phosphate solution quicker due to phosphoserine [5]. Secondly, caseins are easily bound and coagulate with each other, forming higher-order structures of morphology and different sizes. Hence, it is easier for them to deliver nutrients throughout the cells [5]. As for ammonium sulphate, it is less suitable due to its properties as an inorganic compound. Ammonium sulphate consists of 21% of nitrogen and 25% sulphur [8]. Since it is an inorganic compound, *B. cereus* 13BN cannot get any nutrients from this because *B. cereus* 13BN is a heterotroph [7]. After analysing all results, overall, it showed that maltose and casein have the highest caseinolytic activity among other types of sources. Hence, they were selected as the

main carbon and nitrogen source for the modified carbon-nitrogen NB.

3.3 Statistical Analysis for Modified Maltose-Casein Nutrient Broth (MMCNB)

The analysis of caseinolytic activities of *B. cereus* 13BN for MMCNB has been calculated using Central Composition Design (CCD). Based on the analyses, it is shown that the results are quadratic. The quadratic effect is used to determine whether the results can be used or not. The results are shown in **Figure 3.5**;

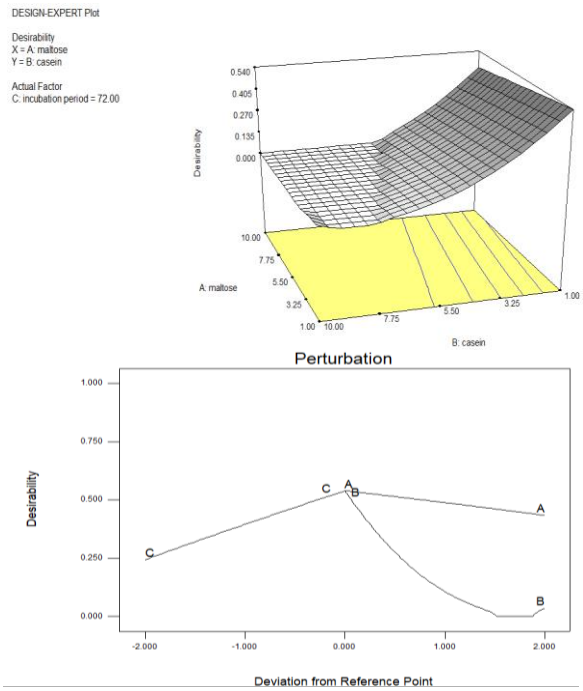

Figure 3.5 The perturbation and 3D-contour analysis for modified maltose-casein NB

Based on the analyses, it is shown that the caseinolytic activity has been produced the most during 72-hour incubation. The desirability shown from the calculations is 0.54, which is higher than the general nutrient broth. Although the general nutrient broth has more nutrients than MMCNB, it takes a longer time for *B. cereus* 13BN to digest the nutrients, and maybe has fewer desirable nutrients demanded by this type of microbe. Hence, it is shown that MMCNB is more suitable for *B. cereus* 13BN to increase the optimisation of the caseinolytic enzyme production.

3.4 The Relationship between Carbon and Nitrogen Sources with Incubation Time on Microbial Growth

Carbon sources have a very crucial part in microbial development. This is because microbes utilise carbon sources as their source of energy. Plus, the carbon source also plays a role as the foremost component for cellular materials [14]. On the other hand, carbon sources are also used by the microbes as the substrates of the metabolic pathway, which means they are degraded to provide groups of amino acids and other components, which build up the cell of the microbes [23]. Nitrogen sources are also very important in microbial growth.[22], Nitrogen sources are needed for supporting bacterial fast development and high cell yields [11][12][22]. Nitrogen sources are also crucial in giving nutrients to microbes since it is a growth-limiting nutrients. Plus, it can be acquired from both organic and inorganic compounds including proteins, nitrates, and molecular nitrogen [17]. Nitrogen sources are also required as the backbone for amino acids, nucleic acid nucleotides, and coenzymes in the cells as well [22].

Bacillus cereus 13BN strain is a Gram-positive, heterotroph, mesophilic, and spore-forming microbe, which uses organic carbon sources such as glucose as its energy source [7]. As for this research, this microbe utilised two types of carbon

sources: lactose and maltose. According to the results, it was shown that the *B. cereus* 13BN strain was interested more in maltose as its source of carbon, while casein was its favourable modified nitrogen source. Since glucose is an organic compound, it is understandable that the *B. cereus* 13BN strain easily utilised glucose as its main source of energy [14].

For nitrogen source, casein has been observed as this microbe's optimal source, due to the fact this microbe hydrolysed casein. For this reason, the production of caseinolytic enzymes by this microbe has been enhanced to the maximum [21]. Casein gives numerous supplements to the microbe such as amino acids and vitamins which can support microbial growth. Besides that, casein can also enhance the metabolic function and proteases activities of the microbe to the maximum, and act as an inducer for the production of the enzyme. The *B. cereus* 13BN strain utilised both k-casein and β-casein obtained from casein for a process called casein hydrolysis [14][16][24].

Compared to other types of bacteria, *B. cereus* can develop up to 30 minutes in a period, in 10-30℃ for mesophiles [13]. Since this microbe is also a spore-forming bacteria, it can also easily withstand its surrounding temperature until 45℃ and steadily grow as a thermophile. This microbe can also increase the colonies between 10℃-50℃ because this is the most perfect temperature for the microbe to develop [9]. This is also the main reason why the *B. cereus* 13BN strain still can live for 72 hours at 37℃ during incubation.

Carbon catabolite repression, known as CCR, is a regulatory mechanism through which the expression of genes needed for the usage of the secondary origin of carbon is averted beyond the presence of a desired substrate [15][20]. This allows the bacteria to double up their strength through optimising growth rates in natural environments requiring complex mixtures of nutrients. One of the main reasons bacteria preferred simple sugars, compared to complex sugar, is because of the utilisation of simplest sugar forms, especially for gene expressions and other activity of functions in the cells [10]. Most bacteria prefer glucose as the best carbon source, compared to other types of sugar. This is because glucose gives higher and faster growth to the bacteria, compared to other sugars [15]. Through this research, it is proved that CCR has occurred due to the inhibition of glucose and less production of caseinolytic enzymes by *B. cereus* 13BN strain happened during 24-48 hours of incubation. Since the *B. cereus* 13BN strain also is a spore-forming bacteria, the results also proved that the spore development had assisted this microbe to withstand the temperature for enhancing the production of caseinolytic enzymes during 72 hours of [18][19].

4. CONCLUSION

Based on the analyses, it showed that maltose and casein are the most preferable carbon and nitrogen source for optimising the production of caseinolytic enzymes from *B. cereus* 13BN. It is because both sources had shown a constant increase of caseinolytic activities and specific activities during incubation time, with desirability of 0.540 from the statistical analysis.

ACKNOWLEDGMENT

The authors acknowledge the Ministry of Higher Education (MOHE) for financial support and the Faculty of Biosciences and Medical Engineering, Universiti Teknologi Malaysia for facilities support. This study was made possible by UTM internal grant GUPQ.J130000.2645.14J42.

REFERENCES

1. Bode, C., M.S. Runge, and R.W. Smalling. (1996). The future of thrombolysis in the treatment of acute myocardial infarction. *Eur. Heart J*. **17**, 55-60.
2. Engelking, L.R. (2015). Carbohydrate Structure. *Textbook of Veterinary Physiological Chemistry*. 3rd Edition.
3. Hornbuckle, W.E., Tennant, B.C. (2008). Gastrointestinal Function. *Clinical Biochemistry of Domestic Animals*. 6th Edition.
4. Sumi, H., Hamada, H., Tsushima, H., Mihara, H. & Murai, H. 1987 A novel ï¬Â• fibrinolytic enzyme (nattokinase) in the vegetable cheese Natto; a typical and popular soybean food in the Japanese diet. *Experientia*.43, 1110–1111.
5. Thorn, D.C., Carver, J.A. (2014). Polymorphism in Casein Protein Aggregation and Amyloid Fibril Formation. *Bio-nanoimaging*.
6. Wong, T.W., Tan, M.C., Ng, O.C., Joseph, A. and Hejar, A.R. (2014). Metabolic syndrome component prevalence of the cardiovascular disease among type 2 diabetic patients in Malaysia. *Southeast Asian J Trop Med Public Health.* 45(1):226-35.
7. Zakaria, Z., Salleh, M.M. and Abdul Rashid, N.A. (2015). Screening and identification of fibrinolytic bacteria from Malaysia fermented Seafood products. *Journal of Applied Pharmaceutical Science*. 5(10):022-031.
8. Zapp, K-H. (2012). Ammonium Compounds. *Ullmann's Encyclopedia of Industrial Chemistry*. 243.
9. Berthold-Pluta, Pluta, Garbowska, & Stefańska. (2019). Prevalence and toxicity characterization of Bacillus cereus in food products from Poland. Foods, 8(7), 269.
10. Bren, A., Park, J., Towbin, B. D., Dekel, E., Rabinowitz, J. D., & Alon, U. (2016). Glucose becomes one of the worst carbon sources for E.coli on poor nitrogen sources due to suboptimal levels of cAMP. Scientific Reports, 6(1). https://doi.org/10.1038/srep24834
11. Chai, J. M., & Adnan, A. (2018). Effect of different nitrogen source combinations on microbial cellulose production by Pseudomonas aeruginosa in batch fermentation. IOP Conference Series, 440, 012044.
12. Costa, E., Usall, J., Teixidó, N., Garcia, N., & Viñas, I. (2000). Effect of protective agents, rehydration media and initial cell concentration on viability of Pantoea agglomerans strain CPA-2 subjected to freeze-drying. Journal of Applied Microbiology, 89(5), 793–800.
13. Dion, M. P., Kapoor, M., Sun, Y., Wilson, S. M., Ryan, J., Vigouroux, A., Van Teeffelen, S., Oldenbourg, R., & Garner, E. C. (2019). Bacillus subtilis cell diameter is determined by the opposing actions of two distinct cell wall synthetic systems. Nature Microbiology, 4(8), 1294–1305.
14. Dake, M. S., Puntambekar, A. N. (2017). Isolation, purification, and optimization of thermophilic and alkaliphilic protease originating from

hot water spring bacteria. Asian Journal of Pharmaceutical and Clinical Research. 10(9), 284-291.
15. Görke, B., & Stülke, J. (2008). Carbon catabolite repression in bacteria: Many ways to make the most out of nutrients. Nature Reviews Microbiology, 6(8), 613–624.
16. Hidayat, M., Saud, H., & Samsudin, A. (2017). Isolation and Characterisation of Sulphur Oxidizing Bacteria Isolated from Hot Spring in Malaysia for Biological Deodorisation of Hydrogen Sulphide in Chicken Manure. Media Peternakan, 40(3), 178–187.
17. Li, P. (2021). Photosynthetic hydrogen production bacteria breeding technologies. Waste to Renewable Biohydrogen, 179–199.
18. Mehta, D., Metzger, L., Hassan, A., Nelson, B., & Patel, H. (2019). The ability of spore formers to degrade milk proteins, fat, phospholipids, common stabilizers, and exopolysaccharides. Journal of Dairy Science, 102(12), 10799–10813.
19. Seung-Ki Kim, Jin-Woo Kim, Song-Yi Kim, Jae Min Jo, & da Hae Gam. (2018). Isolation of Protease Producing Bacillus from Korean Bean Paste and Optimization of Protease Production Conditions. Journal of Advanced Engineering and Technology, 11(3), 205–211.
20. Stülke, J., & Hillen, W. (1999). Carbon catabolite repression in bacteria. Current Opinion in Microbiology, 2(2), 195–201.
21. Suleiman, A. D., Abdul Rahman, N., Mohd Yusof, H., Mohd Shariff, F., & Yasid, N. A. (2020). Effect of Cultural Conditions on Protease Production by a Thermophilic Geobacillus thermoglucosidasius SKF4 Isolated from Sungai Klah Hot Spring Park, Malaysia. Molecules, 25(11), 2609.
22. Todar, K. (2012). Nutrition and growth of Bacteria. Online Textbook of Bacteriology. 1-6.
23. Wang, X., Xia, K., Yang, X., & Tang, C. (2019). Growth strategy of microbes on mixed carbon sources. Nature Communications, 10(1).
24. Janštová, B., Dračková, M., & Vorlová, L. (2006). Effect of Bacillus cereus Enzymes on Milk Quality following Ultra High-Temperature Processing. *Acta Veterinaria Brno*, *75*(4), 601–609.