ISSN:2521-0858 (Print)
ISSN:2521-0866(Online)

Contents List available at RAZI Publishing

Science Heritage Journal / Galeri Warisan Sains (GWS)

Journal Homepage: http://www.razipublishing.com/journals/galeri-warisan-sains-gws

https://doi.org/10.26480/gws.01.2017.07.09

Isolation And Identification Of Halophilic Bacteria Producing Halotolerant Protease

Nadiah Syuhada Abd Samad[1], Azura Amid[2*], Dzun Noraini Jimat[2], and Nurul Aqilah Ab. Shukor[1].

[1]Department of Biotechnology Engineering, Faculty of Engineering, International Islamic University Malaysia, P.O. Box 10, 50728, Kuala Lumpur, Malaysia.
[2]Bioprocess and Molecular Engineering Research Unit, Department of Biotechnology Engineering, Faculty of Engineering, International Islamic University Malaysia, P.O. Box 10, 50728, Kuala Lumpur, Malaysia. *Corresponding author: azuraamid@iium.edu.my

ARTICLE DETAILS

Article history:

Received 27 September 2016
Accepted 13 December 2016
Available online 10 January 2017

Keywords:

Halotolerant Protease, Halophiles Bacteria, Bacteria Identification

ABSTRACT

Halotolerant proteases are known as one of the important groups of enzymes that have been used widely in various industries. However, high production cost of proteases in term of energy used for sterilization and high risk of microbial contamination during fermentation become the obstacles during upstream processing. Moreover, proteases that capable to withstand with harsh conditions of salinity, temperature and pH are required in industry. Therefore, this study presents newly isolated halophilic bacteria producing halotolerant proteases. Halophilic bacteria were isolated from fermented fish sauce. Screening of the bacteria producing halotolerant protease enzymes was carried out by using skim milk salt agar containing 10% NaCl at pH 7 and incubated under aerobic condition at 37oC for 2 days. The selected isolates were identified based on their morphology followed by 16S rDNA gene sequence analysis. 40 colonies of halophiles bacteria were isolated, however, only 20 of them showing proteolytic activity. All of 20 isolates are motile and gram positive bacteria. From 20 isolates, only 6 were chosen for further analysis. B7 showed the highest proteolytic activity compared with others. Results of 16S rDNA gene sequence analysis showed 98% homology to Bacillus amyloliquefaciens subsp. plantarum strain FZB42. Therefore, B7 is identified as Bacillus amyloliquefaciens strain B7.

1. INTRODUCTION

Halophilic bacteria are salt loving organisms that grow in saline environments and can be differentiated based on their requirements for sodium chloride. Ventosa et. al.[6] classified halophiles into slight halophiles grow optimally at 0.2-0.85M (1-5%) sodium chloride (NaCl); moderate halophiles grow optimally at 0.85-3.4M (5-20%) NaCl; and extreme halophiles grow optimally at 3.4-5.1M (20-30%) NaCl.

Previous research showed that the halophilic microorganisms as an ideal source to produce salt stable enzymes due to their ability to carry out catalysis under high salinity [4]. Furthermore, high salinity environments required by halophilic microorganisms may suppress the growth of other organisms and therefore, sterilization costs can be reduced. In addition, the isolation of halophilic bacteria which may be able to produce enzymes which are not only salt tolerant but also tolerate and active at high temperature and pH [14].

However, harsh operational conditions during the enzyme production such as high salinity and high temperature may cause the inhibition of the enzymes [4]. Thus, halotolerant proteases that are stable and active at a wide range of temperature, pH and ionic strength is highly demanded in industrial process. Besides that, high risk of microbial contamination and high energy consumption for sterilization during enzyme production [5] encourage the researchers to identify the most suitable protease enzyme that can tolerate to those disadvantages.

Halotolerant proteases are hydrolytic enzymes, which are mostly used in industries. For example, detergent industries add halotolerant proteases in their laundry detergent formulations to hydrolase proteinaceous stains [1]. The tannery industry uses halotolerant proteases to assist in de-hairing of animal hides and skin instead of using sodium sulphide treatment [2]. For therapeutic purpose, halotolerant proteases were used in the treatment of burns and purulent wounds. It is also acts as thrombolytic agent by showing fibrinolytic activity [3]. Besides that, in the food industry, halotolerant proteases play roles in meat tenderization and production of protein hydrolysates. Protein hydrolysates are used in hypoallergenic infant food formulations, in fortification of fruit juices or soft drinks and in manufacturing protein-rich therapeutic diets [2]. In the waste treatment, halotolerant proteases used to solubilize proteins in wastes such as waste feathers from poultry slaughterhouses.

Therefore, the aim of this study is to isolate and identify the halophilic bacteria from fermented food that could produce salt stable proteases using 16S rDNA as a molecular marker.

2. EXPERIMENTAL

2.1 Sample collection and isolation of bacterial strain

Sample of fermented fish sauce was cultured in nutrient broth and was incubated at 37oC in incubator shaker, at 250 rpm for 24h according to the method previously reported by Suganthi et al., (2013). Then, the sample was serially diluted and 100µl suspension was spread uniformly on nutrient agar plates with 5-10% NaCl. Colonies were picked based on divergence in morphology, size and color.

2.2 Screening of strain for extracellular protease activity

Forty colonies were selected and screened for protease activity. Proteolytic activity of the colonies was screened on skim milk agar containing 1.5% (w/v) skim milk, supplemented with 5-10% (w/v) NaCl for determining the hydrolytic activity of moderate halophiles [7]. The plate was incubated at 37oC for 24h. Colonies showing clear zone of skimmed milk hydrolysis were read as positive for protease production. However, from 40 colonies only 20 colonies showed the positive results and 6 isolates were selected for further analysis.

2.3 Protease assay

Quantitative protease assay (U/ml) was measured by using casein as a substrate at 37oC. 0.2ml of enzyme was mixed to 1ml casein (0.65% w/v in 50mM potassium phosphate buffer, pH 7.5) and the reaction mixture was incubated for 10 min. Then, 1ml trichloroacetic acid reagent (110mM) was added to stop the reaction and the mixture was incubated for 30 min. Then, supernatant was collected by centrifuging the mixture at 5000 rpm for 15 min. About 0.5ml of filtrate was mixed with 1.25ml sodium carbonate solution and 0.25ml Folin & Ciocalteu's Phenol Reagent. Absorbance was measured at 660nm. One unit of protease was defined as the amount of the enzyme required to liberate 1 µmol of tyrosine per minute under the defined assay conditions as reported by Shivanand & Jayaraman (2009). Protein concentration was determined by Bradford method using bovine serum albumin (BSA) as standard [9].

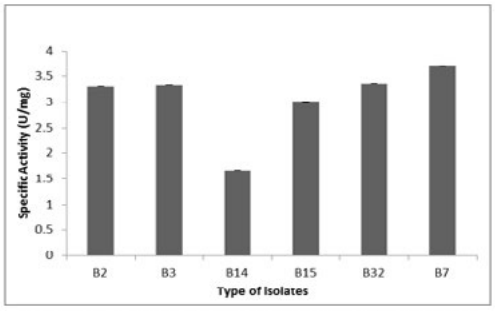

Figure 1: Specific activity (U/mg) on different isolates. The bars indicate the standard deviation of three replicate analyzed.

2.4 Identification of the bacterial strain

Morphology and molecular test were conducted on the B7 isolate. Gram staining was carried out to characterize the shape and type of the isolate while the genotype of protease producing strain B7 was identified by 16S rDNA gene sequencing. Genomic DNA was extracted according to the standard protocol by QIAGEN Genomic DNA handbook and the DNA was amplified by using universal 16S rDNA primers: forward primer 27F (5' AGAGTTTGATCMTGGCTCAG 3') and reverse primer 1492R (5' TACGGYTACCTTGTTACGACTT 3'). The amplified PCR product was sequenced by First Base Sdn. Bhd., Malaysia. The complete 16S rDNA gene sequence was compared with GenBank entries using Blast search [10]. For phylogenetic analysis, the complete gene sequence was aligned with closely related sequences, and a phylogenetic trees were constructed using neighbour-joining method.

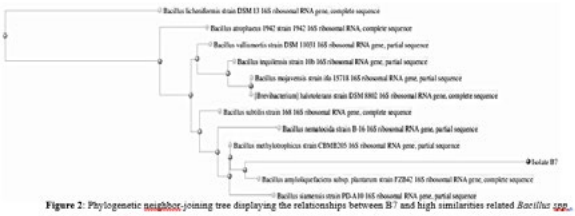

Figure 2: Phylogenetic neighbor-joining tree displaying the relationships between B7 and high similarities related *Bacillus* spp.

3. RESULTS AND DISCUSSION

Among 6 isolates which were identified as protease producers, B7 strain showed the highest specific activity on the proteolytic activity as presented in Figure 1. Thus, the strain B7 was selected as the strain that produced more of protease compared than other isolates. It was a gram-positive, aerobic, motile and rod-shaped bacterium. This strain grew well at various salt concentrations ranging from 5-10% NaCl. Morphological characteristics of the strain were listed in Table 1.

Morphology features	Characteristic
Bacterial Shape	Rods and chain in groups
Gram staining	Positive
Motility test	Motile
Colour	Cream
Opacity	Translucent
Forms	Irregular
Elevation	Flat

Table 1: Morphological characterization of B7 strain

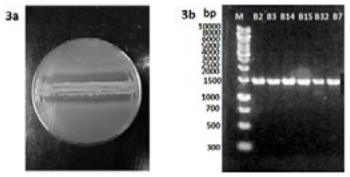

Figure 3a: B7 strain showing protease production by zone of hydrolysis around the colonies on skim milk agar plates. Figure 3b DNA of 6 isolates were amplified by using universal primers. The lane M is DNA ladder. The 1500bp product was obtained from each isolates.

For molecular characterization, a phylogenetic tree was constructed based on Blast (NCBI) search between B7 strains with other homologous sequences. From Figure 2, the isolate B7 positions related to cluster of type species of Bacillus amyloliquefaciens subsp. plantarum strain FZB42 and also showed 98% homology with that strain. Moreover, isolate was grouped with various species of Bacillus, thereby indicating the isolate is a member of the genus Bacillus. Thus, considering morphological and molecular analysis results, the isolate B7 was identified as Bacillus amyloliquefaciens B7 strain. According to Singh, et al, [3], bacteria from genus Bacillus, mostly B. subtilis and B. amyloliquefaciens have been found in many fermented products, especially from fermented soybean and they produced proteases that react specifically on fibrinolytic activity.

Based on the classification of halophilic bacteria, isolate B7 strain was moderate halophile which exhibit growth at 5-10% NaCl concentration. Formation of endospores gives an advantage to genus Bacillus to resist to a broad range of physiological stresses. Endospores is a dormant structure that is produced by some Gram-positive bacterial species that are exposed to harsh environmental conditions in order to survive such as in high concentration of salt environment [11]. Previously, another protease producing halotolerant bacterium B. licheniformis was isolated from saltern sediment and showed NaCl tolerance of up to 2M [10]. Furthermore, B. vietnamensis sp. nov., a moderately halotolerant, aerobic,endospore-forming bacterium was initially isolated from Vietnamese fish sauce [12]. B. horikoshii, B. atrophaeus, and B. methylotrophicus were also moderately halotolerant from genus Bacillus which have been reported to tolerate on 8–12 % NaCl concentration [13].

4. CONCLUSION

This study showed the isolation of a strain that is able to grow and tolerate with 10% NaCl concentration which could reduce the risk of microbial contamination. Moreover, B7 could be used as a potential strain for production of protease under stress conditions of moderate salinity, thus give the high commercial value. The availability of halophilic bacteria producing halotolerant proteases gave an advantage in industrial processes where high salinity conditions could inhibit mesophilic enzymes. However, further studies in enzyme production, purification and characterization of the protease produced by B7 strain are still needed for future developments.

ACKNOWLEDGEMENT

The authors wish to acknowledge full gratitude to the Department of Biotechnology Engineering, IIUM for providing the necessary facility to carry out this work.

REFERENCES

[1] Anwar, A. & Saleemuddin, M. 1998. Alkaline proteases: A review. Bioresource Technology. 64: 175-183

[2] Kumar, C. G. & Takagi, H. 1999. Microbial alkaline proteases: From a bioindustrial viewpoint. Biotechnology Advances.17: 561–594.

[3] Singh, T. A., Devi, K. R., Ahmed, G., & Jeyaram, K. 2014. Microbial and endogenous origin of fibrinolytic activity in traditional fermented foods of Northeast India. Food Research International. 55: 356-362

[4] Karan, R., Kumar, S., Sinha, R., & Khare, S. K. 2012. Halophilic microorganisms as sources of novel enzymes. In Satyanarayana, T. (ed.) Microorganisms in Sustainable Agriculture and Biotechnology: Springer.

[5] Yin, J., Chen, J. C, Wu, Q., & Chen, G. Q. 2014. Halophiles, coming stars for industrial biotechnology. Biotechnol Adv. http://dx.doi.org/10.1016/j.biotechadv.2014.10.008

[6]Ventosa, A., Marquez, M. C., Garabito, M. J., & Arahal, D. R. 1998. Moderately halophilic gram-positive bacterial diversity in hypersaline environment. Extremophiles. 2: 297-304

[7]Sanchez-Porro, C., Mellado, E., Bertoldo, C., Antranikian, G., & Ventosa, A. 2003. Screening and Characterization of the protease CP1 produced by the moderately halophilic bacterium Pseudoalteromonas sp. Strain CP76. Extremophiles. 7: 221-228

[8]Shivanand, P., & Jayaraman, G.2009. Production of extracellular protease from halotolerant bacterium, Bacillus aquimaris strain VITP4 isolated from Kumta coast.Process Biochemistry.44:1088-1094

[9]Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry. 72(1-2): 248-254

[10] Suganthi, C., Mageswari, A., Karthikeyan, S., Anbalagan, M., Sivakumar, A., & Gothandam, K. M. 2013. Screening and optimization of protease production from a halotolerant Bacillus licheniformis isolated from saltern sediment. Journal of Genetic Engineering and Biotechnology. 11: 47-52

[11] Yadav, A. N., Verma, P., Kumar, M., Pal, K. K., Dey, R., Gupta, A., Padaria, J.C., Gujar, G., Kumar, S., Suman, A., Prasanna, R., & Saxena, A. K. 2015. Diversity and phylogenetic profiling of niche-specific Bacilli from extreme environments of India. Ann Microbiol. 65:611–629

[12]Noguchi, H., Uchino, M., Shida, O., Takano, K., Nakamura, L. K., & Komagata, K. 2004.Bacillus vietnamensis sp. Nov., a moderately halo tolerant, aerobic, endo spore-forming bacterium isolated from Vietnamese fish sauce. Int J Syst Evol Microbiol 54:2117–2120

[13]Yousuf, B., Sanadhya, P., Keshri, J., & Jha, B. 2012. Comparative molecular analysis of chemolithoautotrophic bacterial diversity and communitystructure from coastal saline soils, Gujarat, India. BMC

Microbiol.12:150–164
[14] Rohban, R., Ammoozegar, M. A., & Ventosa, A. 2009. Screening and isolation of halophilic bacteria producing extracellular hydrolyses from Howz Soltan Lake, Iran. J Ind Microbial Biotechnol. 36: 333-340