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Proteolytic Ability of *Microbulbifer* Reveals Its Potential Application in the Detergent Industry

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Abstract
Protease-producing bacteria is a group of bacteria with potential for biotechnological applications, particularly in the detergent industry. In this study, an extracellular protease-producing bacterium strain CR4 was isolated from mangrove soil at Tanjung Piai National Park in Johor, Malaysia. The strain CR4 was identified as a member of the genus *Microbulbifer* via 16S rRNA gene sequencing analysis, with close sequence similarities (98.67%) to *Microbulbifer rhizosphaerae* strain Cs16b^T. The genome of strain Cs16b^T was examined to identify genes associated with proteolytic ability. Gene mining discovered 32 genes in its genome are related to proteolytic ability. Among these genes, 22 and 10 genes encode for endopeptidases (2 aspartic-, 14 serine- and 6 metallo-proteases) and exopeptidases (6 amino- and 4 carboxy-peptidases) respectively. This genome analysis combined with qualitative proteolytic ability screening may aid in the exploration of this genus for its proteinaceous stain removal application in the detergent industry.
Keywords: *Microbulbifer*; proteolytic ability; genome analysis; proteases

Introduction

Halophilic microorganisms have piqued the interest of many researchers as a potential source of industrial enzymes (Lam et al., 2018; Zakaria et al., 2020). A total of 60% production of proteolytic enzymes were applied in various industries, with 30% of the production of the protease contributed to the detergent industry (Haddar et al., 2009; Homaei et al., 2016). *Microbulbifer* is a genus that belongs to the family *Microbulbiferaceae* of the order *Cellvibrionales* (Spring et al., 2015). At the time of this writing, according to the List of Prokaryotic names with Standing in Nomenclature (LPSN), there are 25 members with validly published names in the genus *Microbulbifer*. Within this genus, the genome sequences with the elucidation of their algicidal, polysaccharides-degrading abilities, cold-adapted enzymes, agarolytic and comparative studies on the number of CAZymes presence were reported (Chen et al., 2019; Imran et al., 2019; Jung et al., 2018; Lee et al., 2016; Sun et al., 2014; D. Wang et al., 2021; J. Wang et al., 2022). However, to date, no genome analysis related to their proteolytic ability have been reported from this genus. This study explores the proteolytic ability of *Microbulbifer* for its potential application in the detergent industry.

Materials and methods

In this study, strain CR4 was isolated from the mangrove soil samples at Tanjung Piai National Park, Johor, Malaysia via serial dilution method on Marine agar 2216 (MA) (BD Difco, New Jersey, USA) and incubated at 30°C for 2 days. The strain was qualitatively screened for its ability to hydrolyse the peptide bonds that make up the casein protein on MA supplemented with 10% (v/v) of skim milk (Vashist et al., 2013). To identify the bacterium, the genomic DNA of strain CR4 was extracted using the Wizard® Genomic DNA Purification kit (Promega, Wisconsin, USA) following the manufacturer's instructions.

The extracted genome was subjected to a polymerase chain reaction (PCR) amplification process using the universal primers (27F and 1525R) (De Lillo et al., 2006). The PCR products were then purified and sent to Apical Scientific Pte. Ltd. (Seri Kembangan, Malaysia) for Sanger sequencing. The nearly full-length 16S rRNA gene sequence of strain CR4 was aligned and searched using the Basic Local Alignment Search Tool (BLAST) program on the EzBioCloud database (Yoon et al., 2017).

To investigate the genes related to proteolytic abilities in the genome of *Microbulbifer*, a genome sequence from this genus (*Microbulbifer rhizosphaerae* strain Cs16b^T =CECT 8799^T under NCBI accession number JACHWZ000000000) was retrieved from National Center for Biotechnology Information (NCBI) genome portal. The retrieved genome data was analyzed using NCBI Prokaryotic Genome Annotation Pipeline (PGAP) version 5.2 (Tatusova et al., 2016). All genes related to proteolytic ability were subjected to both SignalP version 6.0 (Teufel et al., 2022; Zakaria et al., 2020) and TMHMM Server version 2.0 (Möller et al., 2001) to predict the presence of signal peptides and the transmembrane helices in the proteins respectively. Then, the genes related to proteolytic abilities were further cross-checked using InterProScan version 87.0 (Jones et al., 2014).

Results and discussion

The strain CR4 was grown on an MA-skim milk medium and the bacterial colony was morphologically observed to be a coccus-shaped, brown-pigmented bacterium after 2 days of incubation (Figure 1A). The qualitative screening of its ability to hydrolyse the peptide bonds of protein molecules was tested positive through the formation of a clear halo zone around the bacterial colonies on the MA-skim milk medium. This indicates that strain CR4 was able to produce extracellular protease to degrade the casein (Figure 1B). The 16S rRNA gene sequence of strain CR4 has 98.67% similarities with *Microbulbifer rhizosphaerae* strain Cs16b^T. Based on these considerably high 16S rRNA gene sequence similarities, strain CR4 was identified as a member of the genus *Microbulbifer*.

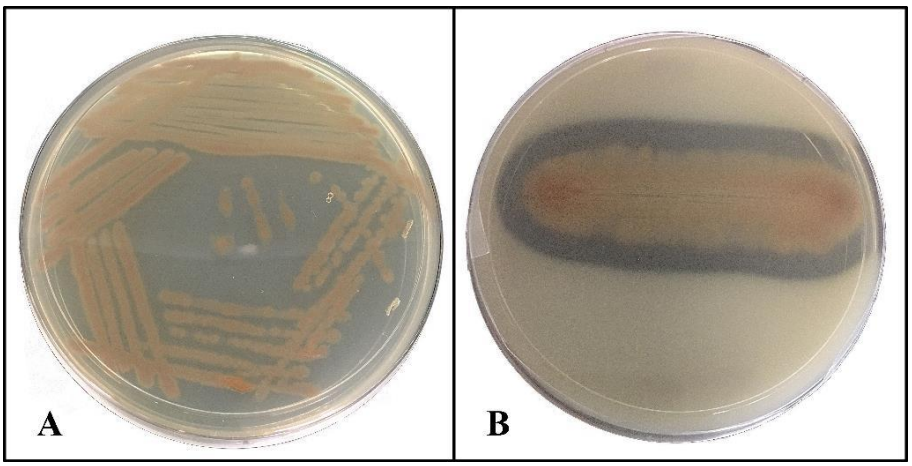

Figure 1 **(A)** The morphological characteristics of *Microbulbifer* sp. strain CR4 on MA after 2 days of incubation at 30°C and **(B)** the formation of a clear halo zone around *Microbulbifer* sp. strain CR4 on MA-skim milk medium after 2 days of incubation.

For the investigation of the key genes related to proteolytic ability in the genome of *Microbulbifer*, the genome sequence of *Microbulbifer rhizosphaerae* strain Cs16b^T (=CECT 8799^T) was used for analysis. A total of 32 genes were predicted to be involved in the production of extracellular proteases (Table 1). In detail, 22 and 10 genes encode for endopeptidases (2 aspartic-, 14 serine- and 6 metallo-proteases) and exopeptidases (6 amino- and 4 carboxy-peptidases) respectively. Collectively, both endo- and exo-peptidases played crucial roles in cleaving the peptide bonds at either N- or C-termini and within the polypeptide chains, respectively (Sharma et al., 2019). The activity of detergent-containing proteases was proven to be enhanced when both endo- and exo-peptidases were combined during the proteinaceous stain removal process (Valls et al., 2011). Therefore, the finding of this study showed that encoded proteolytic enzymes from *Microbulbifer* provide valuable information for future characterization, optimization and production of the proteases. It could serve as a potential candidate to be applied in the detergent industry.

Table 1: List of potential annotated genes related to proteolytic ability.

Category	Encoded Enzyme	Locus Tag
Endopeptidase	Aspartic protease	FHS09_RS03130
		FHS09_RS08125
	Metalloprotease	FHS09_001509
		FHS09_RS11935
		FHS09_RS15340
		FHS09_RS03955
		FHS09_RS15480
		FHS09_RS20860
	Serine protease	FHS09_RS02955
		FHS09_RS05445
		FHS09_RS01650
		FHS09_RS01980
		FHS09_RS02210
		FHS09_RS02750
		FHS09_RS05645
		FHS09_RS06245
		FHS09_RS06770
		FHS09_RS07090
		FHS09_RS11540
		FHS09_RS12045
		FHS09_RS16330
		FHS09_RS20505
Exopeptidase	Aminopeptidase	FHS09_RS07945
		FHS09_RS14575
		FHS09_RS14585
		FHS09_RS17040
		FHS09_RS20320
		FHS09_000630
	Carboxypeptidase	FHS09_RS02805
		FHS09_RS10105
		FHS09_RS18315
		FHS09_RS20285

Conclusion

The findings of this study demonstrated *Microbulbifer* could hydrolyse protein molecules by producing extracellular proteases. The genome of *Microbulbifer* sp. strain CR4 could be sent for whole-genome sequencing and the proteolytic genes encoded in its genome could be further characterized and potentially beneficial to the detergent industry for effective proteinaceous stain removal.

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References

Chen, Y. P., Wu, H. T., Wang, G. H., Wu, D. Y., Hwang, I. E., Chien, M. C., Pang, H. Y., Kuo, J. T., & Liaw, L. L. (2019). Inspecting the genome sequence and agarases of *Microbulbifer pacificus* LD25 from a saltwater hot spring. *Journal of Bioscience and Bioengineering*, *127*(4), 403–410. doi: 10.1016/j.jbiosc.2018.10.001

De Lillo, A., Ashley, F. P., Palmer, R. M., Munson, M. A., Kyriacou, L., Weightman, A. J., & Wade, W. G. (2006). Novel subgingival bacterial phylotypes detected using multiple universal polymerase chain reaction primer sets. *Oral Microbiology and Immunology*, *21*(1), 61–68. doi: 10.1111/j.1399-302X.2005.00255.x

Haddar, A., Agrebi, R., Bougatef, A., Hmidet, N., Sellami-Kamoun, A., & Nasri, M. (2009). Two detergent stable alkaline serine-proteases from *Bacillus mojavensis* A21: purification, characterization and potential application as a laundry detergent additive. *Bioresource Technology*, *100*(13), 3366–3373. doi: 10.1016/j.biortech.2009.01.061

Homaei, A., Lavajoo, F., & Sariri, R. (2016). Development of marine biotechnology as a resource for novel proteases and their role in modern biotechnology. *International Journal of Biological Macromolecules*, *88*, 542–552. doi: 10.1016/j.ijbiomac.2016.04.023

Imran, M., & Ghadi, S. C. (2019). Genome sequence analysis for bioprospecting of marine bacterial polysaccharide-degrading enzymes. In Advances in Biological Science Research: A Practical Approach (pp. 21–34). Elsevier Inc. doi: 10.1016/B978-0-12-817497-5.00002-1

Jones, P., Binns, D., Chang, H. Y., Fraser, M., Li, W., McAnulla, C., McWilliam, H., Maslen, J., Mitchell, A., Nuka, G., Pesseat, S., Quinn, A. F., Sangrador-Vegas, A., Scheremetjew, M., Yong, S. Y., Lopez, R., & Hunter, S. (2014). InterProScan 5: genome-scale protein function classification. *Bioinformatics*, *30*(9), 1236–1240. doi: 10.1093/bioinformatics/btu031

Jung, J., Bae, S. S., Chung, D., & Baek, K. (2018). Complete genome sequence of *Microbulbifer agarilyticus* GP101 possessing genes coding for diverse polysaccharide-degrading enzymes. *Korean Journal of Microbiology*, *54*(3), 299–301. doi: 10.7845/kjm.2018.8047

Lam, M. Q., Nik Mut, N. N., Thevarajoo, S., Chen, S. J., Selvaratnam, C., Hussin, H., Jamaluddin, H., & Chong, C. S. (2018). Characterization of detergent compatible protease from halophilic *Virgibacillus* sp. CD6. *3 Biotech*, *8*(2), 1–9. doi: 10.1007/s13205-018-1133-2

Lee, Y. S., & Choi, Y. L. (2016). Complete genome sequence of cold-adapted enzyme producing *Microbulbifer thermotolerans* DAU221. *Journal of Biotechnology*, *229*, 31–32. doi: 10.1016/j.jbiotec.2016.05.002

Möller, S., Croning, M. D. R., & Apweiler, R. (2001). Evaluation of methods for the prediction of membrane spanning regions. *Bioinformatics*, *17*(7), 646–653. doi: 10.1093/bioinformatics/17.7.646

Sharma, M., Gat, Y., Arya, S., Kumar, V., Panghal, A., & Kumar, A. (2019). A review on microbial alkaline protease: an essential tool for various industrial approaches. *Industrial Biotechnology*, *15*(2), 69–78. doi: 10.1089/ind.2018.0032

Spring, S., Scheuner, C., Göker, M., & Klenk, H. P. (2015). A taxonomic framework for emerging groups of ecologically important marine gammaproteobacteria based on the reconstruction of evolutionary relationships using genome-scale data. *Frontiers in Microbiology*, *6*, 1–17. doi: 10.3389/fmicb.2015.00281

Sun, C., Chen, Y. J., Zhang, X. Q., Pan, J., Cheng, H., & Wu, M. (2014). Draft genome sequence of *Microbulbifer elongatus* strain HZ11, a brown seaweed-degrading bacterium with potential ability to produce bioethanol from alginate. *Marine Genomics*, *18*(PB), 83–85. doi: 10.1016/j.margen.2014.05.009

Tatusova, T., Dicuccio, M., Badretdin, A., Chetvernin, V., Nawrocki, E. P., Zaslavsky, L., Lomsadze, A., Pruitt, K. D., Borodovsky, M., & Ostell, J. (2016). NCBI prokaryotic genome annotation pipeline. *Nucleic Acids Research*, *44*(14), 6614–6624. doi: 10.1093/nar/gkw569

Teufel, F., Almagro Armenteros, J. J., Johansen, A. R., Gíslason, M. H., Pihl, S. I., Tsirigos, K. D., Winther, O., Brunak, S., von Heijne, G., & Nielsen, H. (2022). SignalP 6.0 predicts all five types of signal peptides using protein language models. *Nature Biotechnology*. doi: 10.1038/s41587-021-01156-3

Valls, C., Pujadas, G., Garcia-Vallve, S., & Mulero, M. (2011). Characterization of the protease activity of detergents laboratory practicals for studying the protease profile and activity of various commercial detergents. *Biochemistry and Molecular Biology Education*, *39*(4), 280–290. doi: 10.1002/bmb.20488

Vashist, H., Diksha, S., & Avnet, G. (2013). A review on commonly used biochemical test for bacteria. *Innovare Journal of Life Sciences*, *1*(1), 1–7.

Wang, D., Wang, J., Shui, B., Zhu, L., Wang, J., Jin, L., & Qu, W. (2021). Complete genome sequence of *Microbulbifer* sp. YPW1 from mangrove sediments in Yanpu harbor, China. *Archives of Microbiology*, *203*(10), 6143–6151. doi: 10.1007/s00203-021-02578-y

Wang, J., Jin, L., Wang, J., Chan, Z., Zeng, R., Wu, J., & Qu, W. (2022). The first complete genome sequence of *Microbulbifer celer* KCTC12973^{T}, a type strain with multiple polysaccharide degradation genes. *Marine Genomics*, *62*(January). doi: 10.1016/j.margen.2022.100931

Yoon, S. H., Ha, S. M., Kwon, S., Lim, J., Kim, Y., Seo, H., & Chun, J. (2017). Introducing EzBioCloud: a taxonomically united database of 16S rRNA gene sequences and whole-genome assemblies. *International Journal of Systematic and Evolutionary Microbiology*, *67*(5), 1613–1617. doi: 10.1099/ijsem.0.001755

Zakaria, M. R., Lam, M. Q., Chen, S. J., Abdul Karim, M. H., Tokiman, L., Yahya, A., Shamsir, M. S., & Chong, C. S. (2020). Genome sequence data of *Mangrovimonas* sp. strain CR14 isolated from mangrove forest at Tanjung Piai National Park, Malaysia. *Data in Brief*, *30*, 1–5. doi: 10.1016/j.dib.2020.105658