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TRENDS IN BIOTECHNOLOGY: *Vibrio natriegens* AS POTENTIAL MICRO-FACTORY FOR VALORIZATION OF CRUSTACEAN WASTE

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ABSTRACT

Vibrio natriegens has recently been identified as a promising host for the biotechnology industry thanks to its inherent qualities, which include its fast growth rate, non-pathogenicity to humans, and versatility in using substrates. These advantages have led to the potential use of *V. natriegens* in the biosynthesis of several products. Basically, the industrial scale requires fermentation or cultivation processes to be conducted at high substrate or biomass concentrations to maximize the final retrieved product. However, studies on *V. natriegens* at high cell density are limited. Besides, the potential of *V. natriegens* to convert recalcitrant substrates such as chitin derivatives into biological products has not yet been understood. This review summarizes up-to-date information on the physiological characteristics, metabolism, genome, and genetic modification tools of *V. natriegens*. Subsequentially, statistics and analysis of research trends related to *V. natriegens* was presented. Finally, a discussion on the role of *V. natriegens* in converting chitin waste from the seafood processing industry into a culturing feedstock to achieve a circular economy and net zero emissions was provided.

Keywords: *Vibrio natriegens*, crustacean waste, chitin monomers, salt tolerance, trend in biotechnology.

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INTRODUCTION

The current trend in biotechnology is using *Escherichia coli* as a host for both molecular and synthetic biology (Valle & Bolívar, 2021). Lately, a new candidate for this position is *Vibrio natriegens*, a species with numerous advantages including i) being non-pathogenic for humans, ii) higher growth rate than *E. coli*, iii) can effectively use various carbon sources including sugars, organic acids and chitin monomers. Successful research has also been done on the protein expression and genetic modification systems of *V. natriegens*. Table 1 summarizes studies that have used *V. natriegens* as a host. In comparison to the conventional host *E. coli*, *V. natriegens* produce 2,3-butanediol and L-DOPA at a rate greater than 187% (Erian et al., 2020) and 400% (Liu et al., 2022), respectively. This productivity of 2,3-butanediol by using *V. natriegens* (Erian et al., 2020) was 230% higher titer than *Bacillus subtilis* (Biswas et al., 2012). L-alanine was obtained by a recombinant expression in *V. natriegens* with 9-fold higher than the reference using producer strain *E. coli* (Hoffart et al., 2017). It was also reported that the glucose consumption rate of a recombinant *V. natriegens* strain is 315% higher than *E. coli* (Erian et al., 2020). Zhang et al. (2021) successfully created an engineered *V. natriegens* strain that can produce 56.2 g/L of 1,3-propanediol. This result was 1.5 to 1.8-fold higher than the productivity of 1,3-propanediol using recombinant *E. coli* strains (Lee et al., 2018) but slightly inferior to *Clostridium beijerinckii* (Schoch et al., 2023). Considering indigoidine, a synthetic *V. natriegens* strain was reported with productivity of 29.0 g/L (Tian et al., 2023), which was 113% higher than *Pseudomonas putida* KT2440 (Banerjee et al., 2020). From these results, *V. natriegens* shows great potential to become a model for investigation and application in molecular biology, synthetic biology and related fields (Lee et al., 2016; Weinstock et al., 2016).

In Vietnam, large amounts of crustacean shell waste are being discarded, which not only pollutes the environment but also wastes natural resources. With up to 30% of chitin (Gbenebor et al., 2017), this waste stream can be converted chemically or biologically into smaller blocks such as GlcN and GlcNAc. These amino sugars are exploited as carbon and nitrogen sources for the biosynthesis of various compounds. This article aims to review the growth-related aspects, metabolism, and genetic characteristics of *V. natriegens* while evaluating the potential of this species in the production of value-added products from crustacean waste.

Vibrio natriegens as the potential host for future biotechnology

Quantitative analysis of bibliography on *V. natriegens*

264 articles were collected from the PubMed Central database using the term "natriegens" as searching keyword (in a specific field: title or abstract or keyword). This database generally shows that up to 153 publications have been produced between 2019 and 2023, which makes up 57.95% of all articles published between 1962 and 2023. This increasing trend indicates that *V. natriegens* is become a more and more interesting subject for research (Fig. 1).

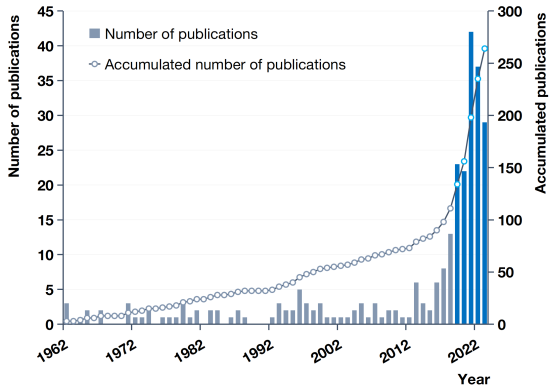

Figure 1. Annual and cumulative number of publications relating to the keyword "natriegens" (field: title or abstract or keyword), database: PMC

Figure 2 shows the distribution of studies across countries and continents. In this graph, the US and China contributed the largest number of publications followed by the UK, France, and Germany. The majority of the

nations that contribute to the studies on this bacterium are exposed to seas and oceans or have long coastlines.

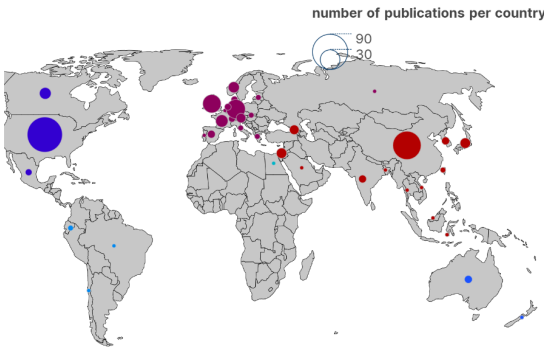

Figure 2. Geographic distribution of publications related to the keyword "natriegens" (field: title or abstract or keyword), database: PMC

In Figure 3, the distribution of publications by research field is presented, in which investigations on growth-related and genetic characteristics sectors predominate articles on fermentation, synthetic biology and genetic engineering. This result indirectly confirms that research on this species is still in their infancy. According to statistical analysis, studies on *V. natriegens* species are allocated in most continents except for Africa. Besides, countries tend to self-isolate and study on *V. natriegens* strains which are distinctive for their biodiversity.

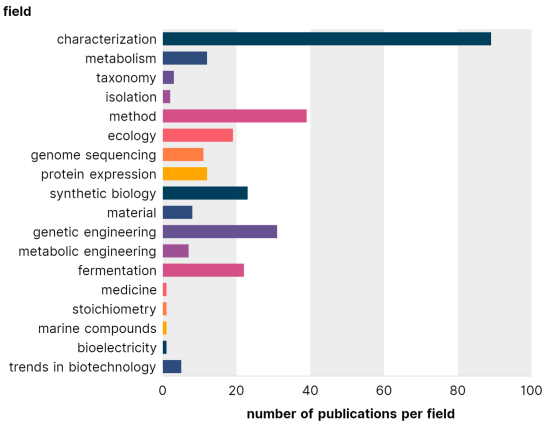

Figure 3. Distribution of number of publications by research field

General characteristics of *Vibrio natriegens*

V. natriegens was first mentioned by Payne (1958). This species had many names before finally being assigned as *V. natriegens*. The most studied strain was coded ATCC 14048 or DSM 759 or NBRC 15635. The taxonomy of *V. natriegens* can be found on NCBI database.

V. natriegens is a Gram-negative, rod-shaped, motile with polar flagella bacterium (Thoma & Blombach, 2021). It can grow in a wide range of pH between 5.5 and 10.0 but the optimal pH was reported at 7–8. The most well-known strain ATCC 14048 grows best at pH 7.5 (Payne et al., 1961; Örencik et al., 2019). *V. natriegens* is sensitive to low temperatures. This phenomenon is a common feature of the genus *Vibrio* (Oliver, 2010) and the mechanism for *V. natriegens* was assumed by the lack of intrinsic catalase activity making the cells vulnerable to hydrogen peroxide that naturally presented in the agar medium (Weinstock et al., 2016). *V. natriegens* ATCC 14048 can grow fastest at 37 ^{o}C (Payne et al., 1961; Örencik et al., 2019), however, a recent study reported higher cell density at 30 ^{o}C (Thiele et al., 2021). The metabolic activity of this species depends closely on Na^+ (Webb & Payne, 1971). Specifically, strain ATCC 14048 exhibited almost no grow at below 5 g/L or above 58.44 g/L NaCl. It grows well at NaCl concentrations ranging from 15 g/L to 30 g/L and is capable of growing in up to 40 g/L NaCl (Hoffart et al., 2017; Örencik et al., 2019). Thus, *V. natriegens* is clearly a more promising bacterium than *E. coli* for cultivation under high salt concentration-the case of chitin hydrolysate.

Culture conditions of *Vibrio natriegens*

Figure 4 shows the correlation between Na^+ concentration (mol/L) and Na^+/K^+ ratio used in *V. natriegens* studies. In general, most studies use media with favorable Na^+ concentration for this species. Only a few authors cultured *V. natriegens* at high Na^+ amount (1 mol/L equivalent to 60 g/L NaCl) (Huang et al., 2022; Meng et al., 2022; Coppens et al., 2023). Regarding the Na^+/K^+

ratio, a report by Payne (1960) showed that this ratio ≤ 100 is necessary for the growth of this species. Practically, most studies employ a Na^+/K^+ ratio ranging from 1 to 100.

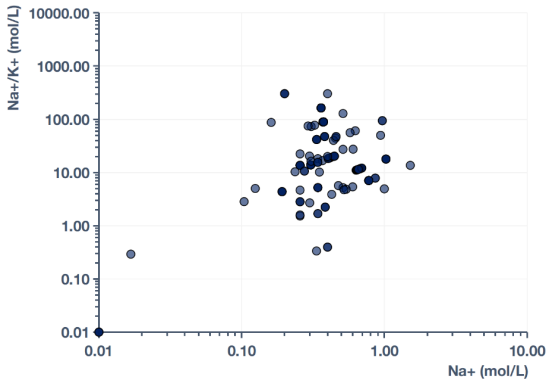

Figure 4. Correlation between moles of Na^+ and Na^+/K^+ ratio between studies

Figure 5a shows that ATCC 14048 is the most studied strain, accounting for 53% of the publications. In addition, some other *V. natriegens* strains have also been studied such as PWH3a (Zhong et al., 2023), WPAGA4 (Zhang et al., 2022). In Vietnam, the bacterial strains *V. natriegens* N5.3 and 10.3 were first announced in 2022 (Le et al., 2022; Le et al., 2023).

In terms of *V. natriegens* cultivation, the most commonly used medium is M9 (Wang et al., 2020) and its variants then come VN medium (Hoffart et al., 2017), LB medium, 2261E medium (Zhang et al., 2022) and their variations (Fig. 5b). Nutrient-rich media such as 2216E, 2xYT (Salomon et al., 2013), BHI and LB are used for preculture and are often applied in molecular biology while minimal mineral media such as VN, M9 and their corresponding variants are often employed in synthetic biology. The main difference between M9 and VN media is the significantly higher salt concentration in the M9 medium.

Figure 5c summarizes the frequency of common substrate utilized for *V. natriegens* culturing. Currently, most research utilizes nutrient sources (peptone, yeast extract, organic acids and easy-to-use sugar - Glc), and only 3.9% of the total mentioned publications use chitin monomers (GlcN, GlcNAc) as substrates. Moreover, these studies were limited to a preliminary level of evaluating growth parameters (Hoffart et al., 2017; Tschirhart et al., 2019; Conley et al., 2020) and no research on strain cultivation on the combination of two sugars GlcN/GlcNAc has been conducted.

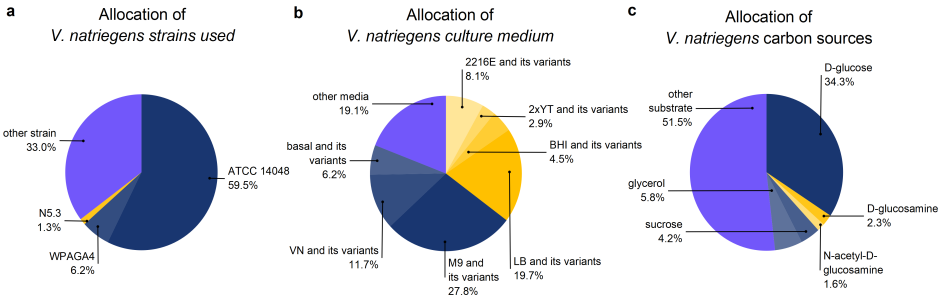

Figure 5. Distribution of Vibrio natriegens strains usage (a); Vibrio natriegens culture medium (b) and Vibrio natriegens cultivating substrates (c)

To increase product concentration, high cell density cultivation of production strain is required. Isabel Thiele et al. (2021) obtained 55 gDM/L biomass after 12 hours of cultivating on MSM (minimal salt medium) using the fed-batch method. In addition, the study also suggests that *V. natriegens* ATCC 14048 should be cultivated at 30 ^oC to minimize by-product secretion while lowering the risk of prophage activation (Thiele et al., 2021). Recently, research by Richard Biener and co-workers successfully achieved over 60 gDM/L of biomass by optimizing the medium composition and reducing Cl^- ion

accumulations during the culture process (Biener et al., 2023). Both abovementioned studies used Glc as a carbon source. Until now, there has been no study on high cell density cultivation of *V. natriegens* using chitin monomers as substrate.

Generally, although research on *V. natriegens* is increasing in number, most studies stay at the laboratory level, with batch fermentation on Glc or other nutrient sources. The majority of experiments used optimal salt concentrations ranging from 10 g/L to 30 g/L. It should be noted that no research has compared the growth rate, substrate consumption and product formation rate of the same *V. natriegens* strain on different minimal mineral media (M9 and VN). Besides, potential substrates such as GlcNAc, GlcN have not received enough attention.

Metabolic system of *Vibrio natriegens*

V. natriegens can use a variety of carbon sources such as citrate, D-glucose, D-mannitol, fructose, glycerol, L-rhamnose, sucrose, arabinose, L-arabinose, D-mannitol, GlcN, GlcNAc, maltose, gluconate, malic acid, citric acid and phenylacetic acid in either aerobic or anaerobic conditions (Hoffart et al., 2017). It can grow well on both a nutrient-rich medium (BHI, 2xYTN, LB) and a minimal mineral medium (VN, M9). Under optimal culture conditions (aerobic growth on BHI medium containing 15 g/L NaCl at 37 ^{o}C, and pH 7.5), *V. natriegens* doubles after every 9.4–9.8 minutes (μ = 4.24–4.42 h^{-1}). This is the highest growth rate that no other benign bacteria has achieved so far (Hoffart et al., 2017).

Figure 6 summarizes the aerobic metabolic pathway of *V. natriegens* for Glc, GlcN and GlcNAc from published studies. Long et al. (2017) reported that the glycolysis of *V. natriegens* with Glc includes the pentose phosphate, Entner-Doudoroff pathways and various catabolic reactions. This bacterium has a 250% higher Glc absorption rate than *E. coli*, however, the amount of carbon going through the pentose phosphate route is about 33% lower. Overall, with Glc as the sole carbon source, the carbon flux of the two bacteria is relatively similar: about 50% converted to biomass, 25% converted to acetate and the remaining 25% to CO_2.

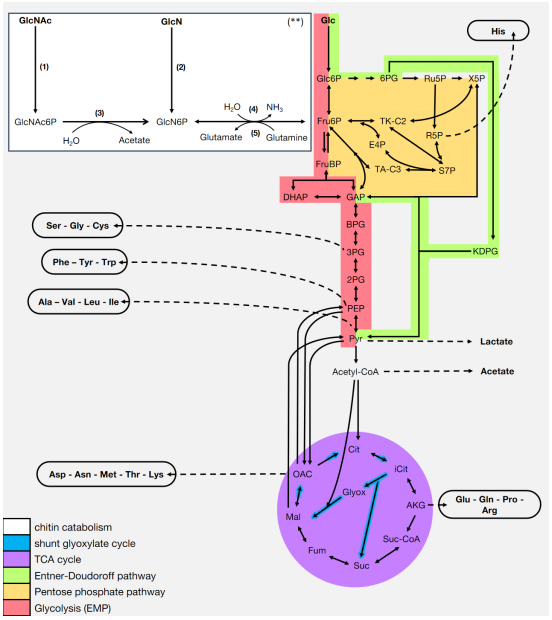

Figure 6. Substrate metabolism pathway of *Vibrio natriegens*. (**): hypothetical metabolic pathway with the participation of enzyme and gene, respectively: (1) enzyme II^{nag}, nagE; (2) enzyme II^{man}, manXYZ; (3) GlcNAc6P deacetylase, nagA; (4) GlcN6P isomerase, nagB; (5) GlcN6P synthase

Regarding the metabolism of chitin derivatives, research showed that *V. natriegens* DSM 759 strain exhibits a significantly higher grow rate on the GlcNAc (μ = 1.74 h^{-1}) than on GlcN (μ = 0.68 h^{-1}) (Hoffart et al., 2017). Similar conclusions were drawn with *V. natriegens* strain N5.3 isolated from Vietnamese seawater (Le et al., 2022). Until now, there have been no studies elucidating the metabolism of chitin monomers by *V. natriegens* to explain the difference in the growth rate on these substrates. In *E. coli*, a higher growth rate on GlcNAc over GlcN was reported (Alvarez-Anorve et al., 2005), which was explained by the role of GlcNAc6P in activating the *nagB* gene encoding GlcN6P isomerase, converting GlcN6P into Fru6P (Alvarez-Anorve et al., 2005). The similar

phenomenon observed in *Streptococcus mutans* is also explained by this mechanism (Moye et al., 2014). Therefore, it is possible that *V. natriegens* possesses a similar metabolic pathway for GlcNAc and GlcN compared to *E. coli* and *S. mutans*. In addition, a hypothesis chitin metabolic pathway in *Vibrio cholerae*, a closely related species, was also proposed (Hunt et al., 2008). From these pieces of information, Figure 6 (white close box) anticipates *V. natriegens'* GlcN and GlcNAc metabolisms.

Genome characteristics of *Vibrio natriegens* and its adaptation to high growth rate at high salt tolerance

V. natriegens is considered safe for humans and is currently classified as biosafety level one (Thoma & Blombach, 2021). The genome of the wild-type *V. natriegens* ATCC 14048 was first published in 2013 and re-sequenced in 2015 to obtain the latest genetic map. *V. natriegens*' genome contains 4578 open reading frames (CDS), 11 rRNA operons and 129 tRNA encoding genes with a total GC content of 43.1–46.4%. The genome size is approximately 5.16 Mbp (larger than certain species of the same genus such as *Vibrio fischeri* ~ 4.2 Mbp, *Vibrio cholerae* ~ 4 Mbp), distributed on two circular chromosomes, a typical distribution pattern of fast growing *Vibrionaceae* family. Chromosome 1 (Chr1) and chromosome 2 (Chr2)' sizes are about ~ 3.24 Mb and 1.92 Mb respectively (Thoma & Blombach, 2021). However, 96% of the 587 essential genes required for rapid growth (Lee et al., 2019) are located on Chr1, of which many genes have copies on Chr2 but do not affect growth. However, due to containing 16 important genes, Chr2 still plays an essential role and is believed to be an evolutionary test version of the species (Lee et al., 2019; Hoff et al., 2020). Compared to *E. coli*, *V. natriegens* has only half the doubling time (~ 10 minutes) (Weinstock et al., 2016). One study suggests that this species has properly divided its genome into two chromosomes and replicated simultaneously to increase replication speed (Hoff et al., 2020). Through the crtS transcriptional activation site, Chr1 controls Chr2's replication, causing the two chromosomes to replicate in unison in *Vibrio*. In the *Vibrionaceae* family, this crtS position indicates that the replication of the two chromosomes terminates concurrently (Hoff et al., 2020). Besides genome organization, another possibility to explain the rapid growth rate of *V. natriegens* is that this species has a large number of ribosomes (Aiyar et al., 2002). In fact, the number of ribosomes and growth rate are known to have a linear relationship. *V. natriegens* contains approximately 115,000 ribosomes per cell during exponential growth with a generation time of 15 minutes, compared to *E. coli*, which contains 70,000 ribosomes with a generation time of 25 minutes (Aiyar et al., 2002). Large numbers of ribosomes are synthesized by the 11 rRNA operons (10 on Chr1, 1 on Chr2) located near the origin of replication (ori) on both chromosomes (Lee et al., 2019). Some rRNA promoters from *V. natriegens* were determined to have strengths comparable to the *E.coli* rRNA promoter (Aiyar et al., 2002) as well as possessing upstream elements that boost the corresponding promoter strength. These factors indicate a high capacity for protein production and a great potential for heterologous protein biosynthesis, with many membrane proteins of *V. cholerae* being successfully expressed in *V. natriegens*, demonstrating that this species is a suitable host to produce proteins of closely related pathogenic species (Lee et al., 2019). Furthermore, the commercial strain *V. natriegens* Vmax was shown to successfully produce several proteins that are either insoluble or just weakly expressed in other systems (Weinstock et al., 2016).

In addition to the remarkably high growth rate and substrate consumption rate that help *V. natriegens* become a potential host to replace *E. coli*, another notable feature of this species is its salt tolerance. Because high salt concentration (> 1% w/v) might influence cell function and diminish enzyme activity (Huang et al., 2022), exploiting autologous salt-tolerant microorganisms in high-salt settings is a reasonable and long-term strategy. In fact, *V. natriegens* has been studied and applied in degrading hazardous compounds in the sea

(environment with ~ 3% salt concentration) such as polyethylene terephthalate (PET), chlorpyrifos (CP) and hexabromocyclododecanes (HBCDs) (Huang et al., 2022).

In general, halotolerant bacteria have the capacity to accumulate organic osmotic solutes (OOSs) such as water-soluble sugars, alcohol-based sugars, many alcohols, amino acids and derivatives, ectoine, trehalose, glycine betaine, etc., that maintain osmotic balance in the cytoplasm and preserve cells in their normal biological mode. Transcriptomic analysis reveals the salt tolerance mechanism of *V. natriegens* Vmax is also linked to the secretion of the above solutes and enhanced Na^+ transport via elevated transcription levels of genes encoding transport channels. When responding to salt stress, the biosynthetic gene clusters ectoine *etcBACD*, proline/glycine betaine all increased 8–9 times in expression. The Na^+/H^+ transport gene cluster, which is linked to maintaining intracellular osmotic pressure, also showed a 4.49-fold increase in expression. Compared to other salt-tolerant bacteria such as *Bacillus halodurans* C-125, *Streptomyces coelicolor* A3, *Chromohabacterium salexigens* DSM 3043, the gene clusters linked to tolerance high salt concentrations in *V. natriegens* have its positional advantage due to being located next to each other and can be induced almost simultaneously, the promoters also have good sensitivity to salt induction, which greatly enhances the species' capacity to maintain osmotic balance (Huang et al., 2022).

In *V. natriegens*, the respiratory pump is a special enzyme system (with no homologous sequence in the *E. coli* genome). Na^+-translocating NADH: quinone oxidoreductase (Na^+-NQR) has an expression level of up to 300 mM in the proteomic system. In addition, Na^+-translocating oxaloacetate decarboxylase (Na^+-OAD), which functions as a membrane pump that interacts with the Na^+ gradient, is strongly expressed under high salt conditions. These pumping systems are important factors that help *V. natriegens* adapt and grow well in high salt environments (Coppens et al., 2023).

Tools for genetic modification of *Vibrio natriegens*

Since 2016, studies by Weinstock et al. (2016) and Lee et al. (2019) have made access to the genome of *V. natriegens* and paved the way for using this fast-growing bacterium in molecular biology applications. Genome editing and gene transfer techniques currently in use are summarized in Table 3.

The above genome editing methods have been applied to construct commercial *V. natriegens* strains Vmax™ express with good transformation efficiency and protein expression (Weinstock, 2018). However, as the genome of Vmax™ express is not available and many modifications are calculated for commercial criteria, Vmax™ is unlikely to become a platform for synthetic biology. Instead, this strain can be exploited for targeted plasmid transformation and protein production. Transformation of *V. natriegens* with plasmid DNA has been successfully performed using different methods. Many common plasmids are persistently maintained in *V. natriegens*, including a series of broad-spectrum transforming plasmids RSF1010 as well as the p15A and pMB1 variants and even some large ~ 100 kb plasmids (Conley et al., 2020). Although transformation in *V. natriegens* cannot be as effective as with *E. coli*, a variety of methods (Table 3) and continuous improvement of the protocol will be the key to developing *V. natriegens* as a common cloning host.

Another crucial factor when considering host switching is the transferability of the genetic construct, where the organism's ability to employ codons can affect the degree of expression and folding of the encoded protein (Quax et al., 2015). Codon usage frequency (occurrence of a specific codon per 1,000 codons) comparison between *V. natriegens* and *E. coli* shows that the structures designed for *E. coli* are often functional in *V. natriegens*. However, the frequency of codon usage can range up to approximately two times for some codons and the yield of expressed protein can vary significantly when using codon optimized sequences (Hoff et al., 2020).

Table 1. Some studies used Vibrio natriegens ATCC 14048 as the host

Vector backbone	Substrate	Product	Yield (g/L)		$Y_{P/S}$ ($g.g^{-1}$)	q_P ($g.L^{-1}.h^{-1}$)	q_S ($g.g^{-1}.h^{-1}$)	References
			Before	After				
pDM4	Glucose	L-alanine	0.31	n.a	0.81	0.56	n.a	(Hoffart et al., 2017)
pET-28a(+)	Catechol	L-DOPA(1)	0	54	n.a	5.40	n.a	(Liu et al., 2022)
p445_Ediss	Glucose	2,3-butanediol	0	15.89	0.22	0.28	1.26	(Erian et al., 2020)
pTrc99a-	Glycerol	1,3-propanediol	0	56.2	0.55	6.00	11.50	(Zhang et al., 2021)
pETRABC	Glucose	2,3-butanediol	0	41.27	0.39	3.44	n.a	(Meng et al., 2022)
pMMB67EH-tfox	Formate	Indigoidine	0	29	n.a	n.a	2.3 $g.L^{-1}.h^{-1}$	(Tian et al., 2023)
pACYC-GFP	Glucose	Pyruvate	31.3	54.22	0.57	3.39	n.a	(Wu et al., 2023)
pDM4	Glucose and $KHCO_3$	Succinate	6	60.4	1.46	20.8	1.33	(Thoma et al., 2022)
pUC19	BHET(2)	Decomposition time of 1 g/L PET(3)	2500 years	24 years	n.a	n.a	n.a	(Li et al., 2023)
pCB1D5-GFP	Glucose	Melanin	0.05	7.57	n.a	0.43	n.a	(Smith et al., 2023)

[1]: 3, 4-Dihydroxyphenyl-l-alanine, [2]: bis 2- hydroxyethyl terephthalates, [3]: Polyethylene terephthalate.

Table 2. Comparison of culture conditions and growth parameters of some studies (n.a: information is not available)

Strain	Medium	Carbon source	T (^oC)	pH	[Na^+] (mol/L)	$Y_{X/S}$ (g/g)	μ (h^{-1})	Reference
ATCC 14048	modified M9	Glc 7.6 g/L	37	n.a	0.77	0.44	n.a	(Long et al., 2017)
ATCC 14048	MSM	Glc 10 g/L	30	7.5	0.26	0.71	0.79	(Thiele et al., 2021)
ATCC 14048	MSM	Glc 10 g/L	37	7.5	0.26	0.36	1.36	(Thiele et al., 2021)
ATCC 14048	VN	Glc 15 g/L	37	7.5–8	0.26	0.71	1.03	(Stella et al., 2021)
ATCC 14048	VN	Glc 10 g/L	37	7–7.5	0.26	n.a	1.64	(Hoffart et al., 2017)
ATCC 14048	VN	GlcN 10 g/L	37	7–7.5	0.26	n.a	0.68	(Hoffart et al., 2017)
ATCC 14048	VN	GlcNAc 10 g/L	37	7–7.5	0.26	n.a	1.74	(Hoffart et al., 2017)
N5.3	modified MSM	GlcN 10 g/L	30	7.5	0.26	0.26	0.48	(Le et al., 2022)

Table 3. Gene editing and transformation techniques currently popular

Citation	Method	Mechanism	Method characteristics
Genome engineering techniques			
(Lee et al., 2016), (Weinstock et al., 2016), (Tschirhart et al., 2019)	Conjugation	Homologous crossover with plasmid DNA obtained from *E.coli* cells through conjugation	Deleting segments, inserting new segments or replacing part of chromosomes with directional mutations. Require negative screening for the toxin gene encoding plasmid ccdB. Deletion up to 194 kb regions and fusion of a ~ 6 kb translation unit.
(Dalia et al., 2017)	Mutiplex genome editing by natural transformation (MuGENT)	Homologous crossover with linear DNA uptake from the environment through natural competency	V. natriegens requires over expression of the competent control element TfoX. Cells can uptake and integrate many foreign transforming DNA (tDNA) segments at the same time. Cells are incubated with two types of linear tDNA: (1) selected genes containing antibiotic resistance markers and (2) target products at one or more sites. Require very small amount of tDNA: 1 ng/108 CFU. The efficiency depends largely on the flanks' length, optimal is ~ 3 kb. Exchange, insert or remove large sequence regions.
(Lee et al., 2017)	Recombineering	Directional cross-over between the target sequence and a single or double-stranded recombinant DNA fragment	One-step cross-over with short homologous flanks, work with oligonucleotides as well. The mobile genetic element SXT (from V. cholerae) encodes a protein homologous to E. coli ssb (single-strand binding protein), λ-Beta (single-strand annealing protein) and λ-Exo (alkaline exonuclease protein). When overexpressed, these factors all increase the rate of DNA homologous recombination ~ 10,000-fold. Low genome editing efficiency.
Gene transfer method			
(Lee et al.,2016), (Weinstock et al., 2016)□	Electro-poration	Creation of pores in the cell membrane	Transformation of plasmids, oligos or recombinant constructs. Efficiency: 2 × 105–107 CFU/μg DNA (depending on protocol). Required ~ 10 ng DNA.
(Weinstock et al., 2016), (Tschirhart et al., 2019)□	Heat shock	Creation of pores in the cell membrane	Plasmid transformation. Efficiency: 105–106 CFU/μg DNA. Δdns (exonuclease) strain significantly increases transformation efficiency.

Citation	Method	Mechanism	Method characteristics
(Lee et al., 2016), (Weinstock et al., 2016)	Conjugation		Plasmid transfer. Homologous crossover. Applied to delete large gene fragment. Require donor strain E. coli S17-1 λpir. Effective. Time consuming.

To provide quantitative information for the design of complex genetic constructs, many studies have been conducted to characterize individual genetic components in *V. natriegens.* Thus far, various structural elements that function effectively in *V. natriegens* have been identified: inducible promoters (e.g., Ptet, PBAD), promoters induced by temperature and IPTG, and structural elements (e.g., ribosomal binding sites, RiboJ ribozyme, transcription termination sites, and proteolytic tails) (Tschirhart et al., 2019). In addition, many commonly used antibiotic resistance cassettes today have been successfully tested against *V. natriegens* such as chloramphenicol, kanamycin, ampicillin, carbenicillin and tetracycline despite significant differences in the concentrations used in the studies (Hoff et al., 2020). The genome editing tools and genetic components developed to date have created the foundation for the flexible use of *V. natriegens.*

Current trends in molecular biology techniques use of *Vibrio natriegens*

With its remarkably high growth rate and substrate consumption rate, *V. natriegens* is exploited as a host in many bioengineering processes. By using *V. natriegens*, a typical plasmid-based gene cloning protocol can significantly reduce time by nearly 40% (to 18 hours for a cloning process which is 10 hours faster than *E. coli* (Xu et al., 2022). The Marburg collection for *V. natriegens* has provided up to 191 genome components (promoters, antibiotic resistance genes, ribosome binding regions, etc.) optimal for this species. The above standard and optimal components when combined with an automated production system will greatly save time and effort in plasmid construction (Stukenberg et al., 2021).

Another trend favours the use of *V. natriegens* as a protein expression host. This bacterium functions well with the T7 RNA polymerase system and can express soluble proteins encoded by multiple gene sequences from different species. *V. natriegens* can effectively use the pET expression system (very common in *E. coli*) with 65% of the 196 pET plasmids produce soluble proteins, of which, 20 genes are expressed even better than those on *E. coli*. The reason for the lower expression of most genes in *V. natriegens* can be explained that the majority of genes have optimal codon sequences suitable for *E. coli* and the protein synthesis medium in the experiment is also a suitable medium for *E.coli* (Xu et al., 2021). When changing the protocol and optimizing the environment for *V. natriegens*, the expression efficiency increased and some encoded biocatalytic enzymes increased their activity by 50–128%. Besides comparison with *E. coli*, the host cell-free protein biosynthesis system isolated from *V. natriegens* also has high efficiency, producing GFP 11 times higher than *B. subtilis* (Wiegand et al., 2018).

In addition, *V. natriegens* is emerging as a potential host for metabolic engineering due to its versatility in utilizing different substrates and a metabolic pathway of many products similar to that of *E. coli*. Some commonly used strategies in metabolic engineering include (1) increasing the expression of genes encoding enzymes that have a feedback inhibitory mechanism at the key point of the biosynthetic pathway, (2) blocking competitive pathways, (3) expressing heterologous genes, and (4) implementation of enzymatic techniques (Kulkarni, 2016).

However, some challenges should be anticipated when using *V. natriegens* as "micro-factory". First, the lack of a reactive oxygen-detoxification system makes *V. natriegens* sensible to refrigeration (Weinstock et al., 2016), causing inconvenient in strains storage. Second, phages have been identified from *V. natriegens* genome (Pfeifer et al., 2019; Li et al., 2022) and this could lead to a risk of cell lysis during fermentation as reported by Thiele et al. (2021). Fortunately, deleting the prophage gene from *V. natriegens* genome will be a promising strategy to solve this issue (Clark et al., 2019; Pfeifer et al., 2019).

Current state of crustacean shell waste in Vietnam and the privilege of *Vibrio natriegens* in adapting with chitin valorization strategy

Actual situation in Vietnam

Crustacean (shrimps, crabs, lobsters, mollusks) with more than 300 species are extensively consumed worldwide (Gillett, 2008). Crustacean shells contain about 20–30% chitin, 30–40% protein, 30–50% calcium carbonate, and small proportions of other components (astaxanthin, canthaxanthin, lutein and β-carotene) (Gbenebor et al., 2017). Shrimp is one of two species in Vietnam's aquaculture development strategy, with more than 350 production facilities eligible for export (VASEP, 2023). Figure 7 illustrates the production of shrimp and shrimp waste in Vietnam during an 8-year period (General-statistics-office-of-Vietnam, 2013–2021). In the first quarter of 2023, shrimp production reached 152.5 thousand tons, equivalent to 15% of the total amount of aquaculture in the year (Directorate-of-fisheries, 2023). Farming output is estimated to reach 1019.4 thousand tons, a 3.2% increase compared to 2022 (Directorate-of-fisheries, 2023). The seafood processing industry will generate about 300 thousand shrimp waste tons by 2022 and is estimated to reach 500 thousand tons by 2025 (Ministry-of-Industry-and-Trade). Waste discarded (usually the head and shell) accounts for 44–60% of fresh weight containing approximately 30% chitin (Acosta et al., 1993). This material and its de-acetylated form (chitosan) are widely used in industry and also in scientific research (No & Meyers, 1995; Shahidi et al., 1999). Thus, it is important to proceed discarded crustacean shell cautiously while attempting to raise its added value.

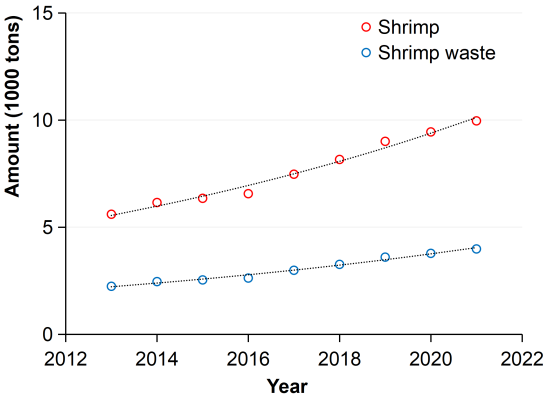

Figure 7. Amount of shrimp and shrimp waste accumulated by year

Advantages of *Vibrio natriegens* in response to different chitin valorization strategies

Figure 8 displays derivatives that can be obtained from chitin. Natural chitin degradation is complicated, requiring the interaction of the marine microbial community, and can take up to 150 days (Keyhani et al., 2000). The extraction process of crude chitin and its derivatives was proposed by Shahidi et al. (1999) whereby crustacean shells were deproteinized, demineralized, and decolorized. Crude chitin then can either be hydrolyzed to yield its monomers GlcNAc and GlcN, or deacetylated to obtain chitosan. Additionally, they can be partially hydrolyzed to produce chito-oligomers.

Chitin hydrolysis can be accomplished chemically using acids or biologically using enzymes. The acid hydrolysis of chitin is simpler to operate but requires high acid concentration and reaction temperature (~ 100 ^{o}C) that lead to the risk of equipment corrosion and fire hazard (Mohan et al., 2022). Following chemical hydrolysis, the neutralization of excess acid by NaOH leads to high salt concentration in the hydrolysate, up to 67 g/L NaCl (Pachapur et al., 2016) while the

sugar concentration is only about 90 g/L (Wahyuni, 2015). Generally, the conventional host *E. coli* grows at 0.5 g/L of NaCl (Ganjave et al., 2022); its growth rate decreased significantly at 40 g/L of NaCl (Kan et al., 2018). If the hydrolysate is diluted to a favor NaCl concentration for *E. coli*, it will lead to a low substrate concentration, which is not desirable. Thus, crustacean hydrolysate obtained chemically is not suitable for the cultivation of *E. coli* or other non-halophilic bacteria. Meanwhile, *V. natriegens* with its salt tolerance (mentioned above) showed a distinct advantage in adapting to chemical chitin hydrolysate.

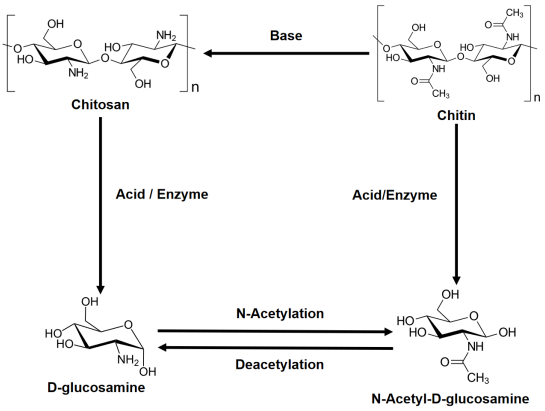

Figure 8. Derivatives retrieved from chitin (Shahidi et al., 1999)

Following biological method, the hydrolysis yield can be achieved at 76–97% (Liu et al., 2013; Bao et al., 2018) which is comparable to that of acid hydrolysis (71.5–98%) (Gözaydın et al., 2020). In addition, enzymatic hydrolysis of chitin has proved to be environmentally friendly (Liu et al., 2013). However, some drawbacks of the enzymatic process were pointed out such as long reaction time, high cost of enzymes (Broquá et al., 2019), high equipment requirements and complex operating processes (Mohan et al., 2022). For this biological approach, the *Vibrionaceae* family, and *V. natriegens* in particular also possess specific advantages for breaking the chitin substrate. The family harbours a variety of chitin-degrading genes, mostly via two pathways: chitin hydrolysis (glycosyl hydrolase- GH family), and chitin-oxidizing (Lytic polysaccharide monooxygenases LPMOs) (Jiang et al., 2022). However, there are only a few studies evaluating the potential of using *V. natriegens* for converting crustacean waste hydrolysates and chitin derivatives (Le et al., 2022; Le et al., 2023).

Chitin hydrolysis is carried out by chitinase, a complex system containing different subgroups classified based on function (endo-chitinases, exo-chitinases hydrolyzing β-1,4 glycoside bonds in chitin chains). In *V. cholerae* (a close relative of *V. natriegens*), the described complete chitin degradation is complicated and requires sequential steps (Hunt et al., 2008). This process is also conservative and is considered a key trait of the *Vibrionaceae* family (Hunt et al., 2008). For *V. natriegens* specifically, strain ATCC 14018 was demonstrated to carry the *chiA* gene encoding an extracellular chitinase on its genome that is crucial for the first stage of chitin breakdown. Given the importance and conservatism of the *chiA* gene, many studies have employed this gene as a chitinolytic capability marker (Hunt et al., 2008). However, despite having the *chiA* in its genome, *V. natriegens* ATCC 14048 exhibited minimal chitinolytic activity (Hunt et al., 2008), indicating that the mechanism of chitin degradation in this species requires further investigation.

Regarding the oxidative chitinolytic pathway, LPMOs break down polysaccharide chains through oxidation in crystalline regions. By disrupting the substrate crystalline areas, LPMOs enhance the efficiency of hydrolytic enzymes. In the CAZy (Carbohydrate-active enzyme) database, LPMOs are grouped into three auxiliary activity families AA10, AA11, and AA15, of which the majority of the known enzymes belong to the AA10 family (Mekasha et al., 2020). The chitinolytic oxidative pathway constructed for *Pseudoalteromonas prydzensis* ACAM 620 showed that in addition to the extracellular LPMO oxidizing chitin into oxidized chitooligosaccharides, strain ACAM 620 contains an eight-gene cluster (ong cluster)

essential for utilizing these subsequent oxidized substrate (Jiang et al., 2022). Genome analyses indicate that the oxidative chitin-oxidizing pathway may be relatively common in marine Gammaproteobacteria (Jiang et al., 2022). In comparison with *P. prydzensis* ACAM 620, the bacterial family *Vibrionaceae* has a complete collection of chitin-oxidizing genes with *V. cholerae* having the whole gene set of LPMO and *ong* gene cluster (Jiang et al., 2022). However, there has been no study to determine the oxidative chitinolytic ability of *V. natriegens.*

It can be seen that the *Vibrionaceae* family contains both hydrolytic and oxidative chitin degradation genes, in which the *V. cholerae* has been studied extensively and proven to be chitinolytic competent (Hunt et al., 2008; Jiang et al., 2022). In *V. natriegens* species, this capability is still limited and there has been no study that deeply analyzes this characteristic. As a species within the *Vibrio* genus, *V. natriegens* might possess additional chitinolytic genes, given the conservative traits of the family and the frequent occurrence of horizontal gene transfer, which is a common phenomenon in the *Vibrionaceae* (Le Roux & Blokesch, 2018). Additionally, the process of gene modification to enhance the chitinolytic capacity in *V. natriegens* can also be carried out promptly and conveniently due to the species' close relationship, such as gene transfer without the need for codon optimization and the potential presence of homologous flanks in the modified strain when using the MuGENT method (Table 3).

In conclusion, with its high salt tolerance and a potential chitinolytic enzyme system, *V. natriegens* clearly benefit from adapting to chitin valorizing substrate by both chemical and biological methods.

CONCLUSION

The role of *Vibrio natriegens* in the current context

V. natriegens is a benign bacterium with the shortest recorded generation time at room temperature. These characteristics render *V. natriegens* superior to *E. coli* as the future microbial host for the industrial production of a wide range of bioproducts. Besides, *V. natriegens* is highly salt tolerant and able to efficiently use chitin monomers (GlcN, GlcNAc) as nutrient sources. Therefore, this species is especially suitable for valorization of crustacean waste hydrolysates. The ability to culture recombinant *V. natriegens* strain without pasteurization creates new avenues for application by simplifying production on an industrial scale and may result in cost savings. One drawback of the high salt concentration fermentation is the electrochemical corrosion. However, this can be mitigated by utilizing single-use bioreactors made of ceramic or plastic that are disposable.

To enable the ability to biodegrade crustacean waste, the chitinolytic enzyme system found in *V. natriegens* should be considered as a key aspect. Although this system is incomplete and some studies indicate that *V. natriegens*' chitinolytic ability is limited, future research should focus on adding necessary genes to this species, aiming for a one-step process using chitin directly for growth and synthesizing bioproducts.

In the context of circular economy, *V. natriegens* appears to be a suitable host for the valorization of crustacean waste streams. This purpose completely fits the criteria of applying biotechnology for sustainable development, not only in Vietnam but also in other nations with long coastlines and economies reliant on aquaculture, fishing, and seafood processing. Besides, with the tropical climate and rich biodiversity, research may focus on isolating and selecting novel *V. natriegens* strains with beneficial characteristics to serve biotechnology.

Abbreviations: 2PG: 2-Phosphoglycerate; 3PG: 3-Phosphoglycerate; 6PG: 6-Phosphogluconate; Acetyl-CoA: Acetyl coenzymeA; AKG: α-Ketoglutarate; BPG: 1,3-Bisphosphoglycerate; Cit: Citrate; DHAP: Dihydroxyacetone phosphate; E4P: Erythrose-4-phosphate; Fru6P: Fructose-6-phosphate; FruBP: Fructose-1,6-bisphosphate; Fum: Fumarate; Glc6P: Glucose-

6-phosphate; GAP: Glyceraldehyde-3-phosphate; Glc: D-Glucose; GlcN: D-Glucosamine; GlcNAc: N-Acetylglucosamine; X5P: Xylulose-5-phosphate; Glyox: Glyoxylate; iCit: Isocitrate; KDPG: 2-Keto-3-deoxy-6-phosphogluconate; Mal: Malate; n.a: Not available; NCBI: National Center for Biotechnology Information; OAC: Oxaloacetate; PMC: Pubmed Central; PEP: Phosphoenolpyruvate; Pyr: Pyruvate; R5P: Ribose-5-phosphate; Ru5P: Ribulose-5-phosphate; S7P: Sedoheptulose-7-phosphate; Suc: Succinate; Suc-CoA: Succinyl-coA; TA-C3: 3-carbon intermediate of transaldolase; TK-C2: 2-carbon intermediate of transketolase.

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