Master's Thesis of Engineering

# Isolation, identification, and characterization of a novel chitin metabolizing strain *Vibrio* sp. SP2

## 새로운 키틴 대사균주 *Vibrio* sp. SP2의 분리 동정 및 그 특성

August 2021

Graduate School of
Chemical and Biological Engineering
Seoul National University
Chemical and Biological Engineering Major

Mincheol Choi

# Isolation, identification, and characterization of a novel chitin metabolizing strain *Vibrio* sp. SP2

Submitting a master's thesis of Engineering

June 2021

Graduate School of
Chemical and Biological Engineering
Seoul National University
Chemical and Biological Engineering Major

Mincheol Choi

Confirming the master's thesis written by
Mincheol Choi
June 2021

| | |
|---|---|
| Chair | Byung-Gee Kim |
| Vice Chair | Sang Woo Seo |
| Examiner | Ji-Sook Hahn |

# Abstract

Chitin, the second most abundant biopolymer on Earth, is a sustainable resource with potential. However, chitin itself has limited applications due to its insolubility in water and resistance to transformation. Chitin metabolizing microorganisms exist which are able to degrade chitin using a group of enzyme called chitinases. Chitinases break the 1, 4 glycosidic bonds of N-acetylglucosamine units, changing chitin to a suitable form for intracellular use. Thus it would be beneficial if we could identify and engineer a fast-growing chitin metabolizing microorganism to convert chitin into useful chemicals.

In this study we isolated a bacterial strain capable of using colloidal chitin as the sole carbon source and named it *Vibrio* sp. SP2. Characteristics of *Vibrio* sp. SP2 were investigated based on the genomic data obtained by genome sequencing. Genetic tools useful for *Vibrio* sp. SP2 were identified by a series of experiment. Next, heterologous genes encoding proteins required for enhanced chitin metabolism and lycopene production were introduced under the control of synthetic expression cassettes. The engineered *Vibrio* sp. SP2 showed improved growth performance in colloidal chitin minimal medium.

**Keyword :** Chitin, Isolation, Genome analysis, *Vibrio* sp. SP2, Lycopene
**Student Number :** 2019-29816

# Table of Contents

# List of Figures

# List of Tables

# Chapter 1. Introduction

Chitin, a polysaccharide made from chains of N-acetylglucosamine, is the second most abundant biopolymer after cellulose. It is the major constituent of the exoskeleton of invertebrates and cell walls of fungi.

Currently, about 6 to 8 million tons of crustacean shell waste are produced by human diet annually worldwide [11]. Crustacean shells are composed of three industrial chemicals, namely calcium carbonate, chitin, and protein. Production of chitin from shell waste by traditional refinery methods involves excessive use of solvent comprised of hydrochloric acid and sodium hydroxide. Resulting waste water contains high levels of dissolved nitrogen and requires high treatment costs [12].

Chitin and its deacetylated form, chitosan, have received attention in the past decades as next generation base materials for industrial use due to their stable supply and relatively cheap price [36]. However, unlike chitosan, chitin is insoluble in water and cannot be directly absorbed to human body, limiting its range of application in food and pharmaceutical industries. Currently chitin is mainly used as coagulants for water treatment and additives in cosmetic products [37, 38].

Aside from human activity, more than 100 billion tons of chitin are naturally produced in the aquatic biosphere alone annually [13]. Chitin is a crucial nutrient source for marine microorganisms as marine environment has limited provision of carbon and nitrogen [14]. Many marine microbial genera such as *Bacillus*, *Vibrio*, *Acinetobacter* and *Streptomyces* enable the recycle of this water insoluble biopolymer by producing chitinases [15]. Chitinase is a glycosyl-hydrolase that can cleave the 1, 4 glycosidic bonds of N-acetylglucosamine units, hydrolyzing chitin to chitooligosaccharides.

Not much research has been done on bioconversion of chitin in fast-growing microorganisms. This is mainly because currently most-used platform organisms such as *Escherichia coli*, *Saccharomyces cerevisiae* and *Corynebacterium glutamicum* lack the ability to secrete extracellular chitinases [39]. A previous study was successful in converting colloidal chitin to ethanol in *Mucor* species, but the production rates were low [40].

The objective of this study was to check the possibility of using fast-

growing marine bacteria to convert chitin into useful chemicals. First, a native chitin metabolizing *Vibrio* strain was isolated among a bacterial consortium. Further experiment showed that it could also grow on a minimal media with colloidal chitin as the sole carbon source. Second, data acquired from whole genome sequencing proved existence of genes concerned with chitin metabolism and proposed several candidate chemicals for bioconversion from chitin. Following that, various genetic tools including selection markers, replication origins and promoters were tested for the applicability in gene engineering. Finally, we developed a recombinant strain harboring heterologous genes and tested its growth rate and lycopene production in colloidal chitin minimal media.

# Chapter 2. Materials and Methods

## 2.1. Culture medium preparation

The culture medium development process for chitinolytic bacteria is in two stages: preparation of colloidal chitin; and preparation of minimal medium containing colloidal chitin supplemented with mineral salts.

Colloidal chitin was prepared following the Wen et al. protocol, a modified version of the Roberts and Selitrennikoff protocol [4, 5]. Five grams of practically ground chitin from shrimp shells (C7170, Sigma-Aldrich Co., USA) were treated with 60 mL of concentrated HCl and incubated overnight at room temperature with vigorous stirring. Later it was added to 200 mL ice-cold 95% ethanol and incubated overnight once more. Its precipitate was harvested by centrifugation at 4000 g for 20 min, and sterile distilled water was used to wash the pellets until it reached pH 7.

Colloidal chitin minimal medium contained 10 g $L^{-1}$ of colloidal chitin, 30 g $L^{-1}$ of NaCl, 5 g $L^{-1}$ of $(NH_4)_2SO_4$, 1 mM of $MgSO_4$, 2 g $L^{-1}$ of $K_2HPO_4$ and 10 mL $L^{-1}$ of trace metal solution [Table S1].

## 2.2. Bacterial strain and culture condition

For bacterial isolation, a consortium extracted from the intestines of a starfish discovered from the seashores of Incheon, Korea, was aerobically cultured at 37 °C with shaking at 250 rpm in 20 mL of colloidal chitin minimal medium. After repeated subcultures of overnight culture broth, the saturated symbiotic culture was spread on an agar plate consisted of colloidal chitin for isolation.

## 2.3. Genome sequencing, assembly, annotation and taxonomic analysis

To make long reads, Genomic DNA from *Vibrio* sp. SP2 was extracted using genomic DNA extraction kit (Geneall Biotechnology, Seoul, Korea). The sequencing library was constructed by using SMRTbell Template Prep Kit (Pacific Biosciences, Melno Park, CA, USA), qualified by Bioanalyzer DNA 12000 chip (Agilent Technnologies, Santa Clara, CA, USA), and sequenced by the Pacific Biosciences (PacBio) RSII sequencer (Pacific Biosciences, Melno Park, CA, USA). Illumina short-read sequencer was used to mend putative errors in the draft genome. A gDNA library for short reads was prepared using the KAPA HyperPlus Kit (KAPA Biosystems, Wilmington, MA, USA) and sequenced by the MiniSeq 300-cycle mid-output kit (Illumina, San Diego, CA, USA). Paired-end reads obtained from MiniSeq were mapped to the genome generated from PacBio data using the breseq pipeline with default options [16]. The complete genome sequence in FASTA format was then uploaded to Rapid Annotation using Subsystem Technology (RAST) server [17] for gene annotation.

## 2.4. Plasmid construction and identification

Chemically competent *Escherichia coli* Mach-T1$^{R}$ was used as the cloning strain for every conventional DNA cloning process. Table S2 provides the list of strains and plasmids used throughout this study.

Recombinant plasmids constructed in this study can be classified into two groups according to their purposes. One is for identification of genetic tools applicable for *Vibrio* sp. SP2, plasmid origins and promoters in particular. The

other is for construction of an engineered *Vibrio* sp. SP2 strain suitable for lycopene production in colloidal chitin minimal media.

To test the availability of various types of plasmid origins, four different plasmids harboring one of four replication origins (p15A, CloDF13, RSF, and pUC) and a chloramphenicol resistance gene were made. To construct pCDF-C, chloramphenicol resistance gene from pACYCDuet-1 was amplified with AvrII_C-F and BamHI_C-R and inserted in pCDFDuet-1 vector. pRSF-C was prepared cloning pdCas9-derived chloramphenicol resistance gene amplified with BsaI_CAT_F and BsaI_CAT_R into pRSFDuet-1 vector amplified with BsaI_pRSF_F and BsaI_pRSF_R. For pUC-C preparation lacI-ECK120029600 terminator fragment was acquired by means of overlap extension PCR using Bsu36I_term_F, overlap_term_R, overlap_lacI_F and lacI_BamHI_R, followed by double cut ligation to amplified pMD19 backbone amplified with Bsu36I_pMD19_F and BamHI_pMD19_R. A multiple cloning site from pACYCDuet-1 was added to pMD19-lacI-ECK120029600 using Bsu36I_MCS_F and Duet_check_R for amplification. Next, a linear DNA fragment was amplified from the resulting vector using BsaI_pMD19_F and BsaI_pMD19_R then ligated with $Cm^R$ from pdCas9. Finally, by removing a repeated enzyme site through site directed mutagenesis using Site_deletion_F and Site_deletion_R, pUC-C was completed.

We planned to test six different promoters in terms of superfolder-green fluorescent protein (sGFP) expression level in *Vibrio* sp. SP2, so six different plasmids with different promoters (J23100, J23108, J23117, UPJ23119, OXB20 and Ptac) and same pACYCDuet-1 backbone were made accordingly. As for constitutive promoters, sGFP was amplified with sGFP-F1 and consecutively amplified with J23100_BamHI-F2, J23108_BamHI-F2, J23117_BamHI-F2, UPJ23119_BamHI-F2, and OXB20_BamHI-F2, F3, all using sGFP_AvrII_R as the pairing primer. In the case of tac promoter, DNA fragment including both tac promoter and sGFP CDS was amplified with Ptac_BamHI-F and sGFP_AvrII-R. Finally, insert DNAs each including six different promoters and sGFP CDS were finally inserted into pACYCDuet-1 after digestion with BamHI and AvrII, resulting in six pACYC-sGFP with separate promoters.

To develop a recombinant strain optimized for production of lycopene from chitin medium, four heterologous genes (*chiP* encoding chitoporin, *crtE* encoding geranylgeranyl diphosphate synthase, *crtB* encoding phytoene

synthase and *crtI* encoding phytoene desaturase) with OXB20 promoter attached were ligated with pACYCDuet−1 backbone. *chiP* from *E. coli* K−12 W3110 was amplified with consecutive PCR using chiP_NdeI_F1, F2 and chiP_BglII_R. *crtE*, *crtB*, *crtI* from *Lamprocystis purpurea* were amplified with consecutive PCR from p1EBI plasmid using corresponding NdeI_F1, F2 and BglII_R primers. Four heterologous genes were then cloned into OXB20−sGFP backbone after digestion with NdeI and BglII. Four heterologous genes and pACYCDuet−1 backbone were amplified and assembled by using NEBuilder HiFi DNA Assembly Cloning kit.

All synthetic 5'−UTRs were designed with the aid of UTR Designer (http://sbi.postech.ac.kr/utr_designer) [22] and Salis Lab RBS calculator (https://salislab.net/software/design_rbs_calculator) [23]. Detailed sequence information of every primer mentioned above is organized in Table S3.

## 2.5. Transformation method of *Vibrio* sp. SP2

The existing electroporation method for *Vibrio natriegens* [24] turned out to be highly efficient in *Vibrio* sp. SP2 as well.

To prepare electrocompetent cell, a single colony of *Vibrio* sp. SP2 grown overnight was inoculated into a rich medium, LBv2. LBv2 is composed of 5 mL of Luria Broth (LB) and supplementary v2 salt (11.92 g $L^{-1}$ NaCl, 0.3 g $L^{-1}$ KCl, and 2.2 g $L^{-1}$ $MgCl_2$). The seed culture grown overnight was diluted to an initial optical density at 600 nm ($OD_{600}$) of 0.05 in 50mL of fresh LBv2 medium. Cell was harvested approximately at $OD_{600}$ 0.5 when the cell growth entered an exponential phase. Harvested cell was briefly incubated on ice, centrifuged at 4 °C for 10 minutes in 14000xg, and washed with a customized electroporation buffer (680 mM of sucrose and 7 mM of $K_2HPO_4$; pH 7.0) for three times. After the final washing procedure, electrocompetent cell was resuspended with 100 μL of electroporation buffer. Next, in a 1mm cuvette 1 μg of plasmid DNA was mixed with electrocompetent cell to reach final volume of 100 μL. At last, electric pulse of 1.1 kV, 25 μF and 200 Ω was applied to the cuvette using Gene Pulser Xcell (Bio−Rad, Hercules, CA, USA). After recovery for an hour in 700 mL of LBv2 medium, every cell was spread on colloidal chitin plate or LB agar plate supplemented with additional 5 g $L^{-1}$ NaCl (total of 15 g $L^{-1}$ NaCl)

containing antibiotics.

## 2.6. Plasmid copy number determination

Plasmid copy number (PCN) determination method was consulted from a preceding study on development of synthetic biology tools for *Vibrio natriegens* [25]. Absolute copy number of DNAs were measured via quantitative PCR (qPCR) using StepOnePlus Real-time PCR system (Thermo Fisher Scientific, Waltham, MA, USA) and primers amplifying each target. Purified DNAs and cell lysates were prepared as qPCR reaction mixtures using Accupower 2X greenstar qPCR Master Mix (Bioneer, Daejon, Korea). Cell were grown at 37 ℃ in a 250 rpm shaking chamber.

To develop standard curves, concentrations of freshly purified plasmids and PCR-amplified partial *rpoA* containing flanking regions were measured using Qubit 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) and serially diluted. All diluted standards were subjected to qPCR with qPCR_target_F and qPCR_target_R primers. Standard curves were constructed by plotting log 10 of template molecules on the x-axis and cycle of threshold (Ct) values on the y-axis.

To calculate plasmid copy number, *Vibrio* sp. SP2 strains transformed with plasmids harboring different replication origins were grown overnight in LBv2 with 6.8 mg $L^{-1}$ chloramphenicol. Seed cultures were then refreshed to an initial $OD_{600}$ of 0.05 and cultivated for 8 hours, a cultivation time considered enough for an ordinary *Vibrio* strain to reach stationary phase. After cultivation, cells within 1 mL of culture broths were harvested, washed with chilled electroporation buffer, diluted with deionized water to 20-100 fold and boiled for 10 minutes at 95 ℃ to make cell lysates. Cell lysates were subjected to qPCR and absolute copy number of plasmid or gDNA was obtained by substituting Ct values on standard curves. Finally, plasmid copy number of a plasmid with specific replication origin in *Vibrio* sp. SP2 was drawn by dividing absolute copy number of plasmid by that of gDNA.

## 2.7. Fluorescence measurement

Expression of sGFP in a group of *Vibrio* sp. SP2 strains each transformed with pTac-sGFP, pJ23100-sGFP, pJ23108-sGFP, pJ23117-sGFP, pUPJ23119-sGFP, and pOXB20-sGFP was measured using Hidex Sense Microplate Reader (Hidex, Turku, Finland). Strains were collectively cultivated overnight in 5 mL of glucose minimal medium (10 g $L^{-1}$ glucose, 10 g $L^{-1}$ NaCl, 5 g $L^{-1}$ $(NH_4)_2SO_4$, 1 mM of $MgSO_4$, 100 mM $K_2HPO_4$, 100 mM $KH_2PO_4$ and 10 mL $L^{-1}$ trace metal solution) supplemented with 6.8 g $mL^{-1}$ of chloramphenicol. 100 - fold diluent of overnight cultures were transferred into fresh medium and cultivated until $OD_{600}$ value reached 1.0. Next, cultures were refreshed once more in the same medium (initial $OD_{600}$ ~ 0.1). To induce tac promoter, 0.1 mM of isopropyl-D-1-thiogalactopyranoside (IPTG) was added when $OD_{600}$ reached 0.5. 150 $\mu$L of broth samples collected after 6 hours and 12 hours of cultivation were subjected to fluorescence measurement with excitation and emission wavelength of 485 nm and 535 nm, respectively. Cell density values of broth samples were measured in terms of $OD_{600}$ using Jenway 7300 Visible Spectrophotometer (Jenway, UK). With raw data being obtained, specific fluorescence was calculated by normalizing the fluorescence value by cell density and subtracting autofluorescence of cells with empty plasmid backbone.

## 2.8. Cell culture, extraction, and quantification of lycopene

Single colonies of *Vibrio* sp. SP2 strain including plasmid for production of lycopene in colloidal chitin medium (pACYC-chiP-crtEBI) and a negative control strain containing pACYC-chiP were inoculated in 250 ml baffled flask containing 20 mL of 10 g $L^{-1}$ colloidal chitin minimal medium and 6.8 mg $L^{-1}$ of chloramphenicol. Cells were cultured aerobically at 37 °C with continuous shaking (250 rpm). After 72 hours of cultivation, 1mL of culture broth was harvested from each flask to verify lycopene production. For comparison, same process was repeated with 20 mL of 10 g $L^{-1}$ glucose minimal medium.

For lycopene extraction, 1mL of absolute acetone was added to the harvested cell pellet making a cell lysate. Cell lysate in a micro centrifuge tube was incubated at 55 °C for 15 minutes with continuous vortexing. Incubated cell lysate was then centrifugated at room temperature with 17,000 x *g* for 15

minutes. The resulting supernatant was collected and subjected to further quantification process.

It has been shown that quantification of lycopene in a translucent sample is possible in a convenient manner by measuring the optical density at 450nm ($OD_{450}$) with UV/vis spectrophotometer [26]. Accordingly, lycopene production was quantified by measuring the optical density of supernatant of cell lysate at 450nm using Jenway 7300 Visible Spectrophotometer (Jenway, UK). The acquired optical density value was converted to lycopene concentration using a standard curve for lycopene at $OD_{450}$.

# Chapter 3. Results and Discussion

## 3.1. Isolation and characterization of chitin metabolizing bacterium

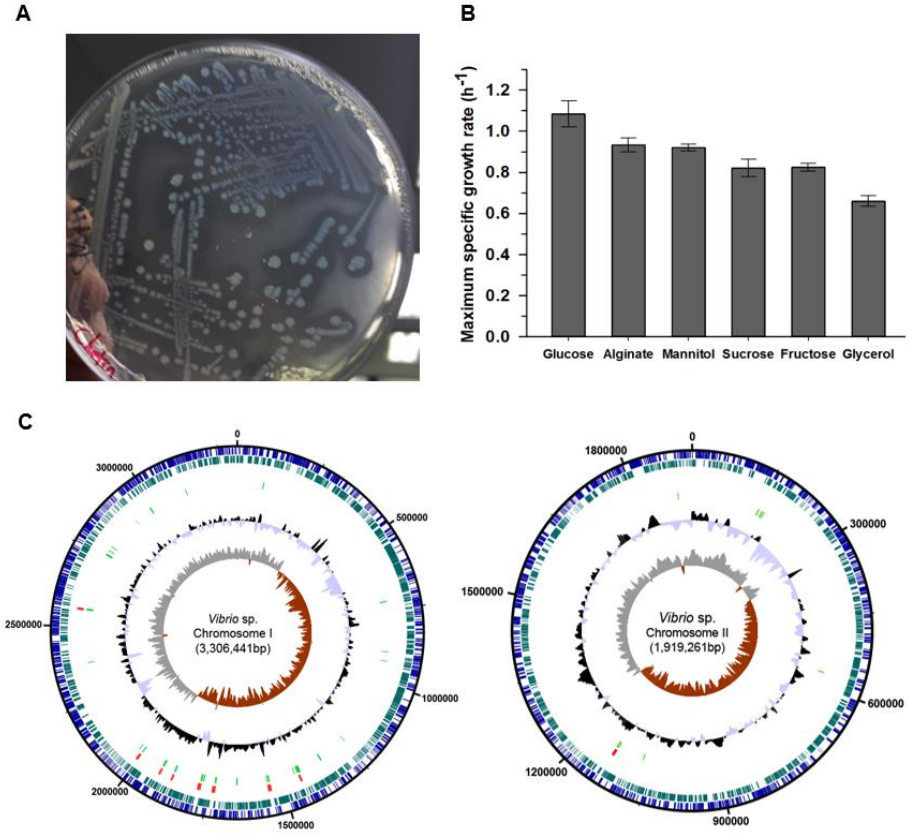
The earliest visible colony on colloidal chitin medium was isolated, cultivated overnight in a liquid medium, and was spread once more on the solid medium. After 24 hours of incubation at 37 ℃, a halo around colonies were observed, presumably a result of extracellular chitinase activity [Fig. 1A]. In addition, the isolated strain turned out to be capable of metabolizing other biomass-derived carbon sources such as glucose, alginate, mannitol, sucrose, fructose, and glycerol with high specific growth rate [Fig. 1B]. When cultured in rich media composed of BHI and v2 salt, this strain showed short doubling time of 19 min, faster than that of *E. coli* and similar to that of *Vibrio natriegens* ATCC 14048.

The draft genome contained two circular chromosomes each 3,306,441 bp and 1,919,261 bp long with GC content of 44.7% and 44.5%, respectively [Fig. 1C and Table S4]. The circular form of complete genome was visualized by DNAplotter [18]. The genomic contents traced by RAST annotation include 4,649 protein coding sequences (CDS), 130 tRNAs and 37 rRNAs [Table S4]. 1,803 genes out of total 4,649 annotated genes were categorized by specific subsystem features [Table S5].

RAST database server also provided a list of closest neighbors [Fig. S1A]. Based on this data the phylogenetic tree was drawn by UBCG [19] analysis and MEGA 7 [20] visualization tool [Fig. S1B]. The newly isolated strain was decided to be named as '*Vibrio* sp. SP2' according to the '*International Code of Nomenclature of Prokaryotes (2008 revision)*' [21].

With the aid of RAST annotation, endogenous metabolic pathways in *Vibrio* sp. SP2 were successfully organized. Such genomic data provided criteria for determining candidate genes for heterologous expression to develop a recombinant strain suitable for use.

**Fig. 1.** Characteristics of *Vibrio* sp. SP2. (A) The isolated strain *Vibrio* sp. SP2 showing halo formation around their colonies on 10 g $L^{-1}$ of colloidal chitin minimal medium at 37℃ after 24 hours of incubation. (B) Maximum specific growth rates of *Vibrio* sp. SP2 measured in various carbon sources. Error bars indicate the standard deviations of three independent cultures (n=3). (C) Circular map of *Vibrio* sp. SP2 genome visualized by DNAplotter. From the outer to inner circle: the direction of protein coding sequences (plus strand for blue and minus strand for blue−green), rRNAs (red), tRNAs (green), GC content (light purple/black) and GC skew (gray/brown).



Two major enzymes that take important role in the hydrolysis of chitin to N-acetylglucosamine (GlcNAc) are chitinase (EC 3.2.1.14) and $\beta$-*N*-acetylglucosaminidase (EC 3.2.1.30) [7]. Chitinases are enzymes that randomly cleave 1,4 glycosidic bonds of GlcNAc to produce soluble chitin oligosaccharides ($GlcNAc_{2-6}$), mainly chitobiose, which are further hydrolyzed to GlcNAc by $\beta$-*N*-acetylglucosaminidases [6]. Soluble chitin oligosaccharides produced by extracellular chitinases enter bacterial periplasm through an outer membrane diffusion channel termed chitoporin (ChiP). Starting from *V. furnissii*, the gene encoding ChiP has been found in most members of chitin metabolizing Vibrionaceae. A DNA microarray study conducted of *V. cholerae* showed that expression of the gene encoding chitoporin (*chiP*) in *V. cholerae* is induced by chitin oligosaccharides [1]. However, the exact mechanism of specific uptake of chitin oligosaccharides by chitoporin is yet unknown. While chitin oligosaccharides can be transported into the periplasmic space via chitoporin, smaller molecules like N-acetylglucosamine (GlcNAc) and N,N'-diacetylchitobiose ($GlcNAc_2$) enter the periplasm by nonspecific porins.

In the periplasm, chitodextrinases and $\beta$-N-acetylglucosaminidases degrade chitin oligosaccharides into GlcNAc and $GlcNAc_2$ which are transported through the inner membrane by ABC transporters and enter the cytoplasm [3]. After entering cytosol, $GlcNAc_2$ is converted to N-acetylglucosamine-6-phosphate (GlcNAc-6-P) with the help of chitobiose phosphorylase and phosphoglucomutase. Deacetylase converts GlcNAc-6-P to glucosamine-6-phosphate (GlcN-6-P) and deaminase converts GlcN-6-P to fructose-6-phosphate, which undergoes the glycolytic pathway.

We confirmed that *Vibrio* sp. SP2 possesses every gene responsible for chitin metabolism except the gene encoding chitoporin (*chiP*) [Fig. 2, Table 1]. A previous study on the heterologous expression of *chiP* in *Vibrio cholerae* showed that the *chiP* mutant strain restored its original growth rate in colloidal chitin medium when complemented with a plasmid encoding heterologous *chiP* [1]. Thus, we thought it would be possible to greatly enhance the growth rate of *Vibrio* sp. SP2 in colloidal chitin medium by heterologous expression of *chiP* encoded in a plasmid vector.

**Fig. 2.** Endogenous metabolic pathway of chitin in *Vibrio* sp. SP2 based on sequenced genome data. The red cross sign indicates the gene missing in *Vibrio* sp. SP2 genome.

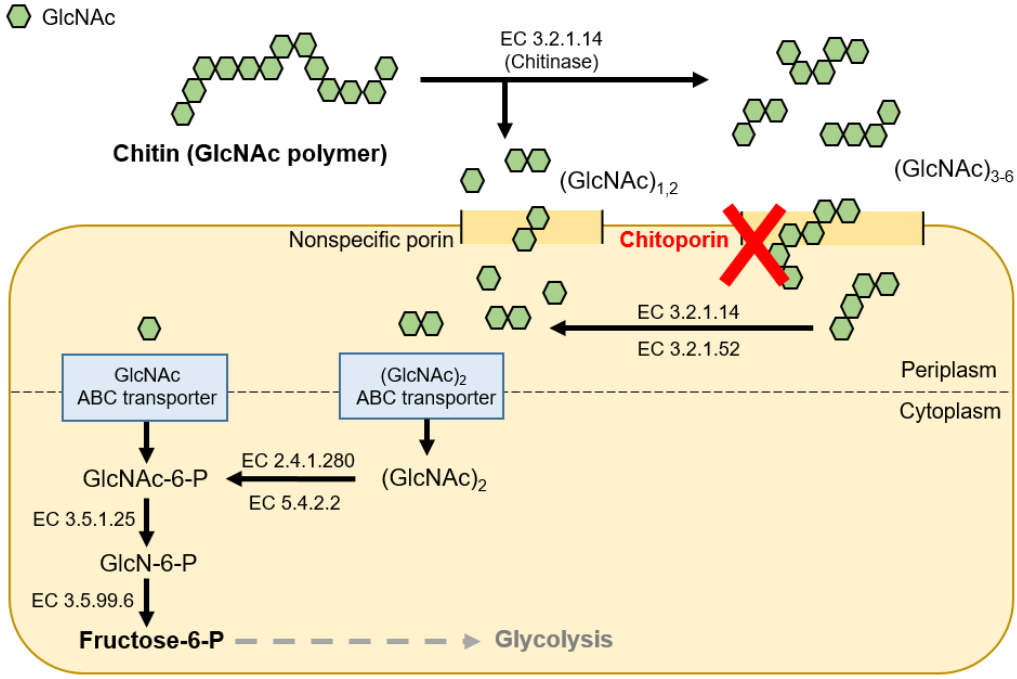


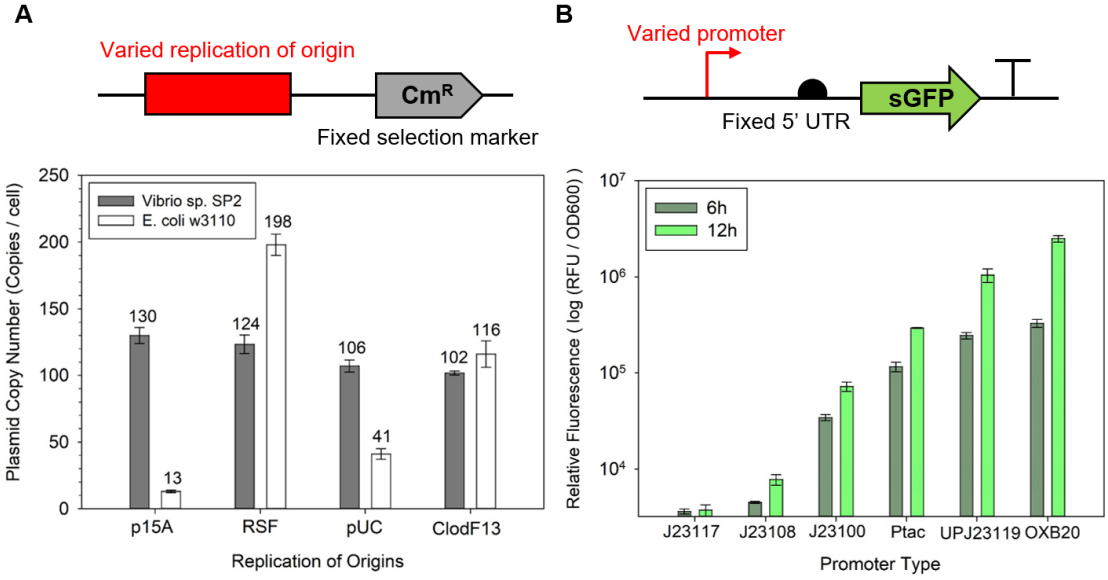

**Table 1.** List of endogenous genes in *Vibrio* sp. SP2 genome responsible for chitin metabolism.

| Gene number | Predicted function | Chromosome | Region (Start - Stop) | Strand | Gene length (bp) |
|---|---|---|---|---|---|
| peg. 1048 | Chitinase (EC 3.2.1.14) | 1 | 1,126,446-1,128,992 | + | 2,547 |
| peg. 2398 | | 1 | 2,643,790 - 2,645,493 | + | 1,704 |
| peg. 1093 | | 2 | 1,085,528 - 1,086,964 | + | 1,437 |
| peg. 378 | Chitodextrinase (EC 3.2.1.14) | 2 | 399,062 - 402,223 | - | 3,162 |
| peg. 1178 | N-acetyl-glucosaminidase (EC 3.2.1.52) | 1 | 1,277,095 - 1,279,014 | + | 1,920 |
| peg. 1583 | | 2 | 1,739,727 - 1,742,180 | + | 2,454 |
| peg. 1171 | Chitiobiose ABC transporter (EC 2.7.1.196) | 1 | 1,268,494 - 1,270,176 | + | 1,683 |
| peg. 2599 | N-acetylglucosamine ABC transporter (EC 2.7.1.193) | 1 | 2,867,024 - 2,868,517 | + | 1,494 |
| peg. 1175 | Chitobiose phosphporylase (EC 2.4.1.280) | 1 | 1,279,099 - 1,281,507 | + | 2409 |
| peg. 2607 | Phosphoglucomutase (EC 5.4.2.2) | 1 | 2,874,304 - 2,875,950 | + | 1647 |
| peg. 2598 | N-acetylglucosamine-6-phosphate deacetylase (EC 3.5.1.25) | 1 | 2,866,543 - 2,865,398 | - | 1,137 |
| peg. 1110 | Glucosamine-6-phosphate deaminase (EC 3.5.99.6) | 2 | 1,211,089 - 1,211,889 | + | 801 |

## 3.2. Performance test of genetic tools for *Vibrio* sp. SP2

Prior to gene engineering, a performance test of genetic tools such as selection marker, replication origins, and promoters must be conducted. To sort out antibiotics available as selection marker, minimal inhibitory concentration (MIC) of ampicillin, chloramphenicol, kanamycin, spectinomycin, tetracycline and erythromycin of *Vibrio* sp. SP2 in LBv2 medium were measured [Table S6]. Next, plasmid copy number (PCN) of plasmids with fixed selection marker and different origins were measured using standard curves [Fig. S2]. p15A, RSF, pUC, and CloDF13 had 129.6 ± 14.0, 123.8 ± 16.4, 106.2 ± 11.6, and 102.0 ± 5.6 copies per cell, respectively [Fig. 3A]. Different from *E. coli* K-12 W3110, p15A showed high PCN and stability in *Vibrio* sp. SP2. Finally, the sGFP expression level of a group of promoters consisted of five constitutive (J23100, J23108, J23117, UPJ23119 and OXB20) and one inducible (tac) were tested in *Vibrio* sp. SP2 [Fig. 3B]. After 12 hours of cultivation, *Vibrio* sp. SP2 transformed with pJ23117-sGFP, pJ23108-sGFP and pJ23100-sGFP, pTac-sGFP, pUPJ23119-sGFP, and pOXB20 came out with the expression level (RFU / $OD_{600}$) of 3412.9 ± 487.5, 4440.9 ± 668.2, 36320.5 ± 3725.1, 130306.3 ± 998.8, 227513.8 ± 9733.2, and 362797.5 ± 4212.7, respectively.

**Fig. 3.** Performance test of genetic tools for *Vibrio* sp. SP2. (A) PCN test of various replication origins in *Vibrio* sp. SP2 and *E. coli* W3110. (B) Evaluation of various promoters in terms of sGFP expression. Error bars indicate the standard deviations of three independent cultures (n=3).



## 3.3. Lycopene production from *Vibrio* sp. SP2 using colloidal chitin

After monitoring a number of candidate chemicals for production in *Vibrio* sp. SP2 in colloidal chitin minimal medium, lycopene was chosen as the primary target product. It was mainly because the MEP pathway required for lycopene production was already established inside the genome of *Vibrio* sp. SP2 [Fig. 4, Table 2]. To be specific, *Vibrio* sp. SP2 genome contains genes for metabolic pathway to generate farnesyl pyrophosphate (FPP) from glyceraldehyde-3-phosphate and pyruvate. The biosynthesis of lycopene from FPP required additional expression of three heterologous enzymes, crtE, crtB and crtI [Fig. 4]. To enhance growth rate of *Vibrio* sp. SP2 in colloidal chitin media, heterologous expression of chiP was required. Thus, OXB20 promoter was attached to genes encoding chiP, crtE, crtB and crtI and were assembled with pACYCDuet-1 backbone harboring p15A origin [Fig. 5A]. The completed recombinant plasmid was then transformed into *Vibrio sp.* SP2. The transformant was named chiP-crtEBI for further experiments.

Growth trend of chiP-crtEBI in 10 g $L^{-1}$ colloidal chitin minimal medium is presented in Fig. 5B. Because of the cloudy state of colloidal chitin medium, spectrophotometer was unfit for measuring cell density. Instead, serial dilutions of culture broth were spread on colloidal chitin agar plates and the number of visible colonies was converted into colony forming unit (CFU). After 72 hours of cultivation in colloidal chitin minimal medium, chiP-crtEBI reached colony forming unit of 724,000,000 ± 28,000,000 CFU $mL^{-1}$, more than 1000 times greater than wild type strain with backbone plasmid (682,000 ± 15,000 CFU $mL^{-1}$). Also, this growth rate equals to that of *Vibrio cholerae* strain harboring heterologous *chiP* from *Vibrio furnissii* grown in colloidal chitin minimal medium [1]. This is encouraging considering that *Vibrio furnissii* is one of the most well researched chitin metabolizing marine microorganism [2, 29, 33].

No lycopene production was detected from chiP-crtEBI grown for 72 hours in colloidal chitin minimal medium [Fig. 5C]. Compared to this, chiP-crtEBI grown in glucose minimal medium for the same amount of time showed 4.16 ± 0.79 mg $L^{-1}$ of lycopene production. The sole difference between two cases was the type of sole carbon source included in the medium, suggesting that the relatively slow growth rate of chiP-crtEBI in colloidal chitin minimal media to other minimal media might be the reason for poor production rate.

**Fig. 4.** MEP pathway and lycopene biosynthesis pathway of engineered *Vibrio* sp. SP2. Blue area represents endogenous MEP pathway found in *Vibrio* sp. SP2. Brown area represents the genes required for lycopene production.

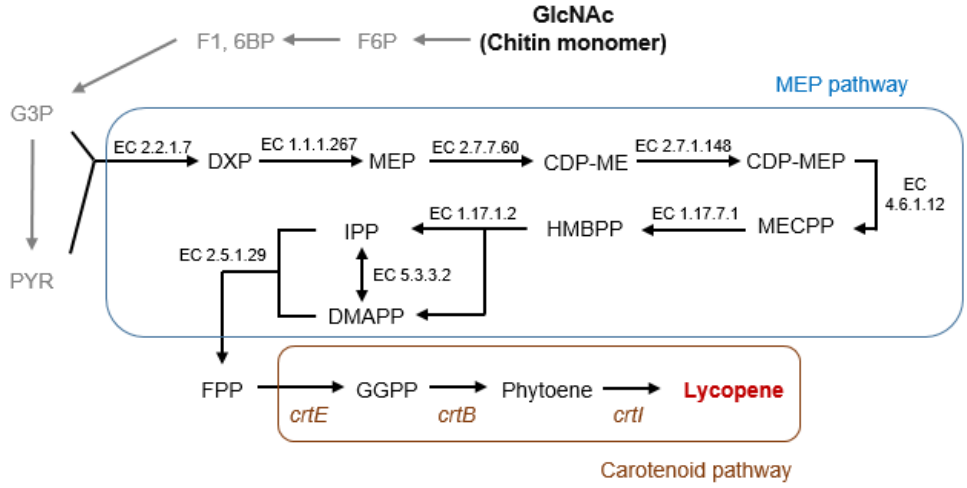
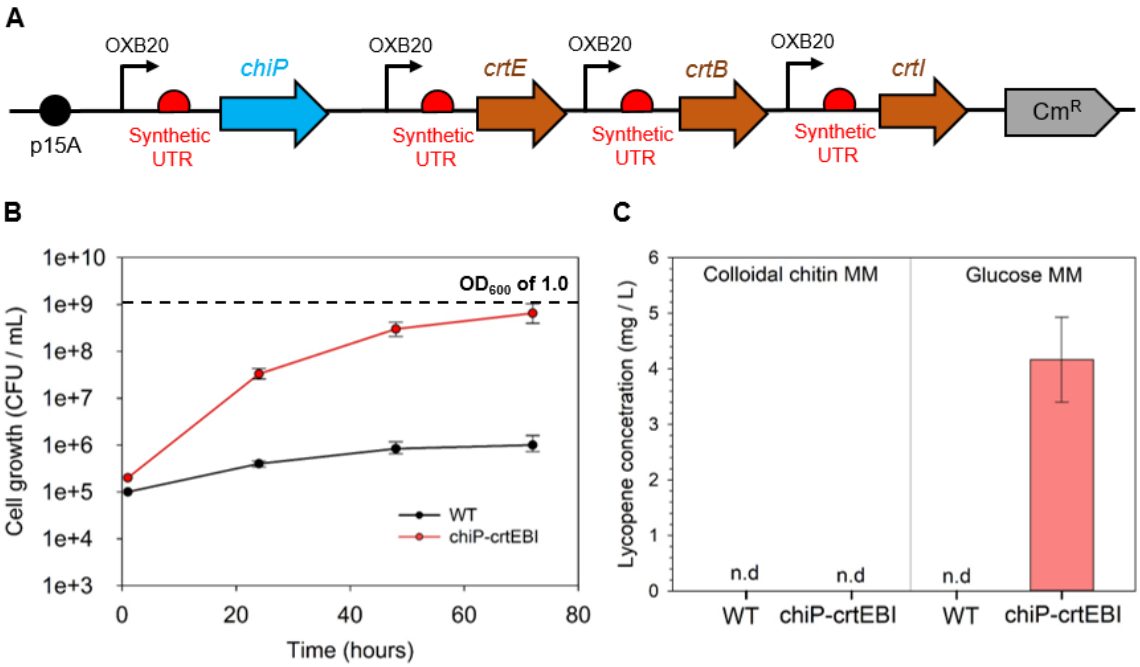



**Table 2.** List of endogenous genes in *Vibrio* sp. SP2 responsible for MEP pathway

| Gene Number | Gene function | Chromosome | Region (Start-Stop) | Strand | Length (bp) |
|---|---|---|---|---|---|
| peg. 1014 | 1-deoxy-D-xylulose 5-phosphate reductoisomerase (EC 1.1.1.267) | 1 | 1,095,132 - 1,096,337 | - | 1,206 |
| peg. 1247 | 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (EC 4.6.1.12) | 1 | 1,362,009 - 1,362,485 | - | 477 |
| peg. 1248 | 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase (EC 2.7.7.60) | 1 | 1,362,500 - 1,363,207 | - | 708 |
| peg. 2293 | 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (EC 1.17.1.2) | 1 | 2,608,861 - 2,609,979 | + | 966 |
| peg. 1892 | 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate synthase (EC 1.17.7.1) | 1 | 2,096,257 - 2,097,375 | + | 1,119 |
| peg. 2455 | 1-Deoxy-D-xylulose 5-phosphate synthase (EC 2.2.1.7) | 1 | 2,708,864 - 2,710,529 | - | 1,866 |
| peg. 2483 | Farnesyldiphosphate synthase (EC 2.5.1.10) / Geranylgeranyl pyrophosphate synthetase (EC 2.5.1.29) | 1 | 2,710,550 - 2,711-434 | - | 885 |
| peg. 2508 | 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase (EC 2.7.1.148) | 1 | 2,767,775 - 2,768,647 | - | 873 |
| peg. 340 | Isopentenyl-diphosphate delta-isomerase (EC 5.3.3.2) | 2 | 359,843 - 361,498 | + | 1,656 |
| peg. 812 | | 2 | 883,544 - 886,423 | - | 2,880 |
| peg. 888 | | 2 | 984,879 - 985,898 | - | 1,020 |

**Fig. 5.** Construction of an engineered *Vibrio* sp. SP2 strain and its performance. (A) Plasmid map of pACYC−chiP−crtEBI. (B) Growth of engineered *Vibrio* sp. SP2 in 10g $L^{-1}$ of colloidal chitin minimal media for 72 hours. Dotted line indicates the number of cells represented in CFU/mL corresponding to $OD_{600}$ of 1.0. (C) Lycopene production by engineered *Vibrio* sp. SP2 (chiP−crtEBI) after 72 hours of batch culture in minimal media using either 10 g $L^{-1}$ of colloidal chitin or glucose as the sole carbon source. WT, wild type bacteria with backbone plasmid; chiP−crtEBI, strain for chitoporin expression and lycopene production; n.d, not detected. Error bars indicate the standard deviations of three independent cultures (n=3).



# Chapter 4. Conclusion

In this study, we isolated a novel bacterium *Vibrio* sp. SP2 capable of metabolizing chitin and various carbon sources such as glucose, alginate, mannitol, sucrose, fructose and glycerol.

By introducing a recombinant plasmid encoding heterologous genes required for chitin metabolism and lycopene production, we were able to raise the growth rate of *Vibrio* sp. SP2 in colloidal chitin minimal media to a more competitive level compared to the two most well researched chitin metabolizing *Vibrio* species, *Vibrio cholerae* and *Vibrio furnissii*. Considering the virulence of two species, *Vibrio* sp. SP2 can be a safer choice for industrial use of chitin.

Despite such improvement, we failed to observe lycopene production from colloidal chitin minimal medium using engineered *Vibrio* sp. SP2 strain. We concluded that the relatively slow cell growth rate in colloidal chitin minimal medium is preventing *Vibrio* sp. SP2 from reaching a meaningful level of lycopene production. Several pilot tests to enhance growth rate by adjusting culture conditions (temperature, shaking speed, nitrogen source concentration etc.) were conducted but none showed positive results.

The next phase of our research would focus on developing a fast-growing strain in chitin medium. New measures would include additional heterologous expression of proteins involved in chitin metabolism (e.g. chitinases), development of a novel chitin medium, adaptive laboratory evolution, and so on. Introducing recently developed natural competent system in *Vibrio* species [34] would be helpful.

# References


[1] Meibom KL, Li XB, Nielsen AT, Wu CY, Roseman S, Schoolnik GK. The Vibrio cholerae chitin utilization program. Proc Natl Acad Sci U S A 2004;101:2524−9.

[2] Keyhani NO, Li XB, Roseman S. Chitin catabolism in the marine bacterium Vibrio furnissii. J Biol Chem 2000;275:33068−76.

[3] Hunt DE, Gevers D, Vahora NM, Polz MF. Conservation of the chitin utilization pathway in the Vibrionaceae. Appl Environ Microbiol 2008;74:44−51.

[4] Wen CM, Tseng CS, Cheng CY, Li YK. Purification, characterization and cloning of a chitinase from Bacillus sp. NCTU2. Biotechnol Appl Biochem 2002;35:213−9.

[5] Roberts WK, Selitrennikoff CP. Plant and bacterial chitinases differ in antifungal activity. J Gen Microbiol 1988;134:169−76.

[6] Keyhani NO, Roseman S. Physiological aspects of chitin catabolism in marine bacteria. Biochem Biophys Acta 1999;1473:108−22.

[7] Gooday GW. Physiology of microviiobial degradation of chitin and chitosan. In: Ratledge C. (eds) Biochemistry of microbial degradation. Springer, Dordrecht.

[8] Hjerde E, Lorentzen MS, Holden MTG, Seeger K, Paulsen S, Bason N, et al. The genome sequenc of the fish pathogen *Aliivibrio salmonicida* strain LFI1238 shows extensive evidence of gene decay. BMC Genomics 2008;9. https://doi.org/10.1186/1471−2164−9−616.

[9] Ma X, Gözaydin G, Yang H, Ning W, Han X, Poon N, Lian H. Upcycling chitin−containing waste into organonitrogen chemicals via an integrated process. PNAS 2020;117:7719−28.

[10] Soysa HSM, Suginta W. Identification and functional characterization of a novel OprD−like chitin uptake channel in non−chitinolytic bacteria. J Biol Chem 2016;291:13622−33.

[11] Food and Agriculture Organization of the United Nations. The State of World Fisheries and Aquaculture. 2014.

[12] Gortari MC, Hours RA. Biotechnological processes for chitin recovery out of crustacean waste: a mini-review. Electr J Biotechnol 2014;ISSN: 0717-3458 doi:10.2225. vol16-issue3-fulltext-10.

[13] Dhillon GS, Kaur S, Brar SK, Verma M. Green synthesis approach: extraction of chitosan from fungus mycelium. Crit Rev Biotechnol 2013;33:379–403.

[14] Johnstone J. Conditions of life in the sea. University of Cambridge 1908;332.

[15] Poulicek M, Gaill F, Goffinet G. Chitin biodegradation in marine environments. American Chemical Society 1995;163–210.

[16] Deatherage DE, Barrick JE. Identification of mutations in laboratory-evolved microbes from next-generation sequencing data using breseq. Eng. Anal. Multicell. Syst., Springer; 2014, p. 165–88.

[17] Aziz RK, Bartels D, Best AA, DeJongh M, Disz T, Edwards RA, et al. The RAST Server: rapid annotations using subsystems technology. BMC Genomics 2008;9:1–15.

[18] Carver T, Thomson N, Bleasby A, Berriman M, Parkhill J. DNA Plotter : circular and linear interactive genome visualization. Bioinformatics 2009;25:119–20.

[19] Na SI, Kim YO, Yoon SH, Ha S min, Baek I, Chun J. UBCG: Up-to-date bacterial core gene set and pipeline for phylogenomic tree reconstruction. J Microbiol 2018;56:281–5. https://doi.org/10.1007/s12275-018-8014-6.

[20] Kumar S, Stecher G, Li M, Knyaz C, Tamura K. MEGA X : Molecular Evolutionary Genetics Analysis across Computing Platforms | Molecular Biology and Evolution | Oxford Academic. Mol Biol Evol 2018;35.

[21] Parker CT, Tindall BJ, Garrity GM. International code of nomenclature of Prokaryotes. Int J Syst Evol Microbiol 2019;69:S1. https://doi.org/10.1099/ijsem.0.000778.

[22] Seo SW, Yang J, Kim I, Yang J, Min BE, Kim S, et al. Predictive design of mRNA translation initiation region to control prokaryotic translation efficiency. Metab Eng 2013;15:67–74.

[23] Salis HM, Mirsky EA, Voigt CA. Automated design of synthetic ribosome binding sites to control protein expression. Nat Biotechnol 2009;27:946-50.

[24] Weinstock MT, Hesek ED, Wilson CM, Gibson DG. Vibrio natriegens as a fast-growing host for molecular biology. Nat Methods 2016;13:849–51.

[25] Tschirhart T, Shukla V, Kelly EE, Schultzhaus Z, Newringeisen E, Erickson JS, et al. Synthetic Biology Tools for the Fast-Growing Marine Bacterium Vibrio natriegens. ACS Synth Biol 2019;8:2069–79.

[26] Aust O, Ale-Agha N, Zhang L, Wollersen H, Sies H, Stahl W. Lycopene oxidation product enhances gap junctional commnication. Food Chem Toxicol 2003;41:1399-1407.

[27] Beier S, Bertilsson S. Bacterial chitin degradation - mechanisms and ecophysiological strategies. Front microbiol 2013. https://doi.org/10.3389/fmicb.2013.00149.

[28] Pruzzo C, Crippa A, Bertone S, Pane L, Carli A. Attachment of Vibrio alginolyticus to chitin mediated by chitin-binding proteins. Microbiology 1996;142:2181-6.

[29] Bassler BL, Gibbons PJ, Yu C, Roseman S. Chitin utilization by marine bacteria - chemotaxis to chitin oligosaccharides by Vibrio furnissii. J Bio Chem 1991;266:24268-75.

[30] Jaeger T, Arsic M, Mayer C. Scission of the lactyl ether bond of N-acetylmuramic acid by Escherichia coli etherase. J Bio Chem 2006;280:30100-30106.

[31] Laura I, Calcagno ML, Plumbridge J. Why does Escherichia coli grow more slowly on glucosamine than on N-acetylglucosamine? Effects of enzyme levels and allosteric activation of GlcN6P deaminase (NagB) on growth rates. J Bacteriol 2005;187:2974-82.

[32] Swiatek MA, Urem M, Tenconi E, Rigali S, Wezel GP. Engineering of N-acetylglucosamine metabolism for improved antibiotic production in Streptomyces coelicolor A3(2) and an unsuspected role of NagA in glucosamine metabolism. Bioengineered 2012;3:280-5.

[33] Erken M, Lutz C, McDougald D. Interactions of Vibrio spp. with zooplankton. Microbiol Spectr 2015;3(3). doi: 10.1128/microbiolspec.VE-0003-2014.

[33] Bassler BL, Yu C, Lee YC, Roseman S. Chitin utilization by marine bacteria. Degradation and catbolism of chitin oligosaccharides by Virbio furnissii. J Bio Chem 1991;266:24276-86.

[34] Dalia TN, Hayes CA, Stolyar S, Marx CJ, McKinlay JB, Dalia AB. Multiplex Genome Editing by Natural Transformation (MuGENT) for Synthetic Biology in Vibrio natriegens. ACS Synth Biol 2017;6(9):1650–5.

[35] Shahidi F, Synowiecki J. Isolation and characterization of nutrients and value-added products from snow crab (Chinoecetes Opilio) and shrimp (Pandalus Borealis) processing discards. J Agric Food Chem 1991;39:1527-32.

[36] No HK, Meyers SP. Preparation and characterization of chitin and chitosan - a review. J Aquatic Food Prod Technol 1995;4:27-52.

[37] Hammami A, Hamdi M, Abdelhedi O, Jridi M, Nasri M, Bayoudh A. Surfactant and oxidant stable alkaline proteases from Bacillus invictae : characterization and potential applications in chitin extraction as a detergent additive. Int J Biol Macromol 2017;96:272-81.

[38] Kurita K. Chemistry and application of chitin and chitosan. Polym Degrad Stab 1998;59:117-20.

[39] Fracetic O, Belin D, Badaut C, Pugsley AP. Expression of the endogenous type 2 secretion pathway in Escherichia coli leads to chitinase secretion. Embo J 2000;19:6697-703.

[40] Inokuma K, Takano M, Hoshino K. Direct ethanol production from N-acetylglucosasmine and chitin substrates by Mucor species. Biochem Eng J 2013;72:24-32.

# Supplementary data

Table S1. Composition of trace element supplemented to carbon minimal media

| Composition | Amounts (g $L^{-1}$) |
|---|---|
| EDTA | 0.5 |
| $MgSO_4 \cdot 7H_2O$ | 3.0 |
| $MnSO_4 \cdot H_2O$ | 0.5 |
| NaCl | 1.0 |
| $FeSO_4 \cdot 7H_2O$ | 0.1 |
| $Co(NO_3)_2 \cdot 6H_2O$ | 0.1 |
| $CaCl_2$ (anhydrous) | 0.1 |
| $ZnSO_4$ | 0.1 |
| $CuSO_4$ | 0.010 |
| $AlK(SO_4)_2$ (anhydrous) | 0.010 |
| $H_3BO_3$ | 0.010 |
| $Na_2MoO_4 \cdot 2H_2O$ | 0.010 |
| $Na_2SeO_3$ (anhydrous) | 0.001 |
| $Na_2WO_4 \cdot 2H_2O$ | 0.010 |
| $NiCl_2 \cdot 6H_2O$ | 0.020 |

Table S2. Strains and plasmids used in this study

| Name | Relevant characteristics | Source |
|---|---|---|
| **Strains** | | |
| Mach-T1$^R$ | *E. coli* F$^-$ φ80(*lacZ*)ΔM15 Δ*lac*X74 *hsd*R(rK-mK+) Δ*rec*A1398 *end*A1 *ton*A | Invitrogen |
| K-12 W3110 | *E. coli* F$^-$ λ$^-$ rph-1 INV(*rrnD, rrnE*) | Invitrogen |
| *Vibrio* sp. SP2 | Novel strain isolated in this study | This study |
| chiP-crtEBI | *Vibrio* sp. SP2 / pACYC-chiP-crtEBI | This study |
| **Plasmids** | | |
| pACYCDuet-1 | Cm$^R$, p15A ori, LacI | Novagen |
| pdCas9 | Cm$^R$-lambda t0 terminator, p15A ori, dCas9 | Addgene |
| pCDFDuet-1 | Sm$^R$, CloDF13 ori, LacI | Novagen |
| pRSFDuet-1 | Kan$^R$, RSF ori, LacI | Novagen |
| pMD19 | linearized T-vector, Amp$^R$, pUC ori | Takara |
| pCDF-C | Cm$^R$, Sm$^R$, CloDF13 ori, LacI | This study |
| pRSF-C | Cm$^R$, RSF ori, LacI | This study |
| pUC-C | Cm$^R$, pUC ori, LacI-ECK120029600 terminator | This study |
| pTac-sGFP | Cm$^R$, p15A ori, $P_{tac}$ promoter (IPTG-inducible), LacI | This study |
| pJ23100-sGFP | Cm$^R$, p15A ori, J23100 promoter, LacI | This study |
| pJ23108-sGFP | Cm$^R$, p15A ori, J23108 promoter, LacI | This study |
| pJ23117-sGFP | Cm$^R$, p15A ori, J231017 promoter, LacI | This study |
| pUPJ23119-sGFP | Cm$^R$, p15A ori, UPJ23119 promoter, LacI | This study |
| pOXB20-sGFP | Cm$^R$, p15A ori, OXB20 promoter, LacI | This study |
| p1EBI | Cm$^R$, p15A ori, LacI $P_{tac}$_synUTR$_{crtE}$_crtE_$P_{tac}$_synUTR$_{crtB}$_crtB_ $P_{tac}$_synUTR$_{crtI}$_crtI_$T_{BBa_B1005}$ | This study |
| pACYC-chiP | Cm$^R$, p15A ori, LacI OXB20_synUTR$_{chiP}$_chiP_$T_{BBa_B1002}$ | This study |
| pACYC-chiP-crtEBI | Cm$^R$, p15A ori, LacI OXB20_synUTR$_{chiP}$_chiP_OXB20_synUTR$_{crtE}$_crtE_ OXB20_synUTR$_{crtB}$_crtB_OXB20_synUTR$_{crtI}$_crtI_$T_{BBa_B1005}$ | This study |

Table S3. Primers used in this study

| Name | Sequence (5' to 3') |
|---|---|
| AvrII_C-F | AAACCTAGGCTGATGTCCGGCGG |
| BamHI_C-R | TTTGGATCCGTCGAATTTGCTTTCGAATTTC |
| BsaI_CAT_ F | AATGGTCTCACGTAATTCTCACCAATAAAAAACGCC |
| BsaI_CAT_ R | CAAGGTCTCTATGCTGATCGGCACGTAAGAGGTT |
| BsaI_pRSF_F | TTGGGTCTCTGCATTGTATTTAGAAAAATAAACAAATAGGCAT |
| BsaI_pRSF_R | ATTGGTCTCATACGCGGTCGTCAGCTTGTCGTCG |
| Bsu36I_term_F | TAACCTCAGGTTGAGAAGAGAAAAGAAAACCGCC |
| overlap_term_R | GCGCAACGCAATTAATGTAAGTTAGCTCACTTCAGCCAAAAAACTTAAGACCGCC |
| overlap_lacI_F | GTGAGCTAACTTACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTC |
| lacI_BamHI_R | AAAAAGGATCCTAAGGGAGAGCGTCGAGATC |
| Bsu36I_pMD19_F | TAACCTGAGGTTAAGCCAGCCCCGACACCC |
| BamHI_pMD19_R | TAAGGATCCGCCTGGGGTGCCTAATGAGTGA |
| Bsu36I_MCS_F | TAACCTCAGGCCATGGGCAGCAGCCATCAC |
| BsaI_pMD19_F | ATTGGTCTCTTACGCTGTCAGACCAAGTTTACTCATATA |
| BsaI_pMD19_R | TTGGGTCTCGGCATTCACATTTCCCCGAAAAGTGC |
| Site_deletion_F | TCAGGCCATGGGCAGCAGC |
| Site_deletion_R | GTTAAGCCAGCCCCGACACC |
| sGFP-F1 | GCGGATAACAATTACGGAGGAAATCATGGCTAGCAAGGGCGAGGAGC |
| J23100_BamHI-F2 | AGCCAGGATCCTTGACGGCTAGCTCAGTCCTAGGTACAGTGCTAGCGCGGATAACAATTACGGAGG |
| J23108_BamHI-F2 | AGCCAGGATCCCTGACAGCTAGCTCAGTCCTAGGTATAATGCTAGCGCGGATAACAATTACGGAGG |
| J23117_BamHI-F2 | AGCCAGGATCCTTGACAGCTAGCTCAGTCCTAGGGATTGTGCTAGCGCGGATAACAATTACGGAGG |
| UPJ23119_BamHI-F2 | AGCCAGGATCCGGCGCGCCTCAAAAAGAGTATTGACAGCTAGCTCAGTCCTAGGTATAATGCTAGCTGCTAGCGCGGATAACAATTACGGAGG |
| OXB20_BamHI-F2 | GTTGTGAACCGATCCATCTAGCAATTGGTCCTAGCGCGGATAACAATTACGGAGG |
| OXB20_BamHI-F3 | AGCCAGGATCCAAGCTGTTGTGACCGCTTGCTCTAGCCAGCTATCGAGTTGTGAACCGATCCATCTAGCA |

| | |
|---|---|
| chiP_NdeI_F1 | ATTGGTC CATCGCAACTAGAAAGGAGGTTTTTTATGCGTACG TTTAGTGGCAAACG |
| chiP_NdeI_F2 | ATAATCATATGAAGTCCAATAACGGATGGCATCGCA ACTAGAAAGGAGG |
| chiP_BglII_R | TATTAAGATCTGCGAAAAAACCCCGCCGAAG |
| crtE_NdeI_F1 | GGACTAATACAAAATTAAGGAGGTATTTATGGTATC TGGCTCAAAGGCTG |
| crtE_NdeI_F2 | AATAACATATGCTCCGTCTCGAGACGGGGAGGACT AATACAAAATTAAGGAGG |
| crtE_BglII_R | TTATTAGATCTCGGATGGAGGAGCCACACCCTTAC |
| crtB_NdeI_F1 | GTACAGAACATCAATAAGGAGGTATTTTATGTCCCA ACCCCCCTTGCTAG |
| crtB_NdeI_F2 | AATATCATATGTATAGTCGCCCAGGGCCACTCAGTA CAGAACATCAATAAGGAGG |
| crtB_BglII_R | AATTAAGATCTGGGCTACGACGACGGCACCGTTAC |
| crtI_NdeI_F1 | TTCAATAAGGTATAGAAGGAGGTTTTTATATGAAAA AAACGGTTGTGATCGG |
| crtI_NdeI_F2 | TAATACATATGACGAGTAGTAATACTTCAATAAGGT ATAGAAGGAGG |
| crtI_BglII_R | TAATTAGATCTGCGGCGAAACCCCGCCGAAG |
| qPCR_p15a_F | AAGTCATGCGCCGGTTAAG |
| qPCR_p15a_R | GCTGACTTCAGGTGCTACAT |
| qPCR_cloDF13_F | TGTTCACTTGAGTCCAACCC |
| qPCR_cloDF13_R | TTCTGCGCGTAATCTTTTGC |
| qPCR_RSF_F | AAGCCGGAAAGAACATGTGA |
| qPCR_RSF_R | GAGAAAGGCGGACAGGTATC |
| qPCR_pUC_F | GCATCACAAAAATCGACGCT |
| qPCR_pUC_R | GAGAAAGGCGGACAGGTATC |
| qPCR_rpoA_eco_F | GGCGGTGAGAGTTCAGGGC |
| qPCR_rpoA_eco_R | CGTTCTCATCGGTCAGGTGGC |
| qPCR_rpoA_vib_F | GAGCTACTTAAAACGCCTAACC |
| qPCR_rpoA_vib_R | CAGGTGGCCAGTTTTCTAGG |
| Flank_rpoA_eco_F | GGCGGTGAGAGTTCAGGGC |
| Flank_rpoA_eco_R | GCTCAACTACGCGGCGCAGC |
| Flank_rpoA_vib_F | CCTGTGGTTGCAGGTGACAT |
| Flank_rpoA_vib_R | TTACTCAGCAGCTTCTTCAGCA |

Table S4. A summary of genomic contents of *Vibrio* sp. SP2

| Attributes | Chromosome | |
|---|---|---|
| | 1 | 2 |
| Length (bp) | 3,306,441 | 1,919,261 |
| GC content (%) | 44.7 | 44.5 |
| CDS | 2,942 | 1,707 |
| tRNAs | 117 | 13 |
| rRNAs | 34 | 3 |

Fig. S1. Taxonomic analysis of *Vibrio* sp. SP2.
(A) The list of closest strains of *Vibrio* sp. SP2 based on the genomic information according to RAST server. (B) The phylogenetic analysis utilizing UBCG (up-to-date bacterial core gene) was conducted and the outcome was visualized with MEGA 7 (Molecular Evolutionary Genetics Analysis).

A

Chromosome 1

| Genome ID | Score | Genome Name |
|---|---|---|
| 243277.1 | 523 | Vibrio cholerae O1 biovar eltor str. N16961 |
| 223926.6 | 497 | Vibrio parahaemolyticus RIMD 2210633 |
| 338187.4 | 468 | Vibrio harveyi ATCC BAA-1116 |
| 675814.3 | 464 | Vibrio coralliilyticus ATCC BAA-450 |
| 675812.3 | 437 | Grimontia hollisae CIP 101886 |
| 314292.23 | 389 | Photobacterium angustum S14 |
| 675817.3 | 387 | Photobacterium damselae subsp. damselae CIP 102761 |
| 312309.3 | 384 | Vibrio fischeri ES114 |
| 298386.1 | 377 | Photobacterium profundum SS9 |
| 316275.9 | 363 | Aliivibrio salmonicida LFI1238 |
| 882102.3 | 230 | Vibrio anguillarum 775 |
| 326297.7 | 207 | Shewanella amazonensis SB2B |
| 392500.3 | 201 | Shewanella woodyi ATCC 51908 |
| 550540.3 | 191 | Ferrimonas balearica DSM 9799 |
| 272620.3 | 186 | Klebsiella pneumoniae MGH 78578 |
| 498217.4 | 184 | Edwardsiella tarda EIB202 |
| 405955.9 | 184 | Escherichia coli APEC O1 |
| 316407.3 | 184 | Escherichia coli W3110 |
| 229193.1 | 181 | Yersinia pestis biovar Medievalis str. 91001 |
| 290338.6 | 180 | Citrobacter koseri ATCC BAA-895 |
| 273123.1 | 177 | Yersinia pseudotuberculosis IP 32953 |
| 393305.7 | 175 | Yersinia enterocolitica subsp. enterocolitica 8081 |
| 198215.1 | 173 | Shigella flexneri 2a str. 2457T |
| 218491.3 | 164 | Erwinia carotovora subsp. atroseptica SCRI1043 |
| 83333.1 | 164 | Escherichia coli K12 |
| 83334.1 | 163 | Escherichia coli O157:H7 |
| 511145.6 | 162 | Escherichia coli str. K-12 substr. MG1655 |
| 1157951.4 | 157 | Providencia stuartii MRSN 2154 |
| 218491.5 | 155 | Pectobacterium atrosepticum SCRI1043 |
| 693216.3 | 155 | Cronobacter turicensis z3032 |

Chromosome 2

| Genome ID | Score | Genome Name |
|---|---|---|
| 223926.6 | 525 | Vibrio parahaemolyticus RIMD 2210633 |
| 338187.4 | 463 | Vibrio harveyi ATCC BAA-1116 |
| 312309.3 | 365 | Vibrio fischeri ES114 |
| 675814.3 | 364 | Vibrio coralliilyticus ATCC BAA-450 |
| 243277.1 | 345 | Vibrio cholerae O1 biovar eltor str. N16961 |
| 316275.9 | 274 | Aliivibrio salmonicida LFI1238 |
| 392500.3 | 266 | Shewanella woodyi ATCC 51908 |
| 314292.23 | 263 | Photobacterium angustum S14 |
| 458817.3 | 261 | Shewanella halifaxensis HAW-EB4 |
| 380703.5 | 244 | Aeromonas hydrophila subsp. hydrophila ATCC 7966 |
| 357804.5 | 235 | Psychromonas ingrahami ingrahamii 37 |
| 298386.1 | 220 | Photobacterium profundum SS9 |
| 325240.9 | 206 | Shewanella baltica OS155 |
| 675817.3 | 177 | Photobacterium damselae subsp. damselae CIP 102761 |
| 211586.9 | 174 | Shewanella oneidensis MR-1 |
| 595494.3 | 170 | Tolumonas auensis DSM 9187 |
| 318161.14 | 168 | Shewanella denitrificans OS217 |
| 400668.6 | 164 | Marinomonas sp. MWYL1 |
| 882102.3 | 156 | Vibrio anguillarum 775 |
| 675812.3 | 152 | Grimontia hollisae CIP 101886 |
| 326297.7 | 150 | Shewanella amazonensis SB2B |
| 342610.6 | 133 | Pseudoalteromonas atlantica T6c |
| 203122.12 | 121 | Saccharophagus degradans 2-40 |
| 243365.1 | 111 | Chromobacterium violaceum ATCC 12472 |
| 160488.1 | 96 | Pseudomonas putida KT2440 |
| 208964.1 | 96 | Pseudomonas aeruginosa PAO1 |
| 498211.3 | 89 | Cellvibrio japonicus Ueda107 |
| 399741.3 | 86 | Serratia proteamaculans 568 |
| 326442.4 | 81 | Pseudoalteromonas haloplanktis TAC125 |
| 314283.5 | 80 | Reinekea blandensis MED297 |

B

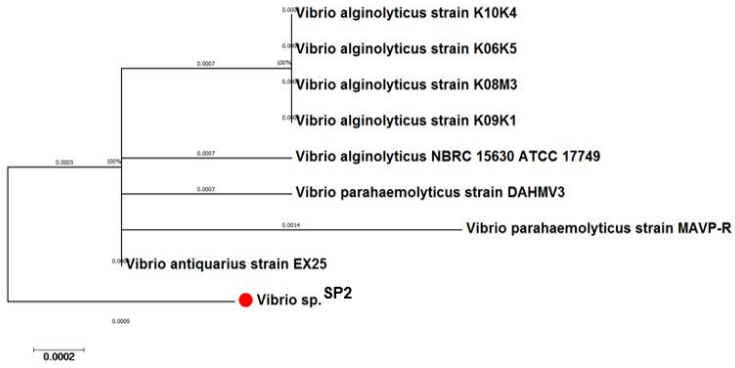


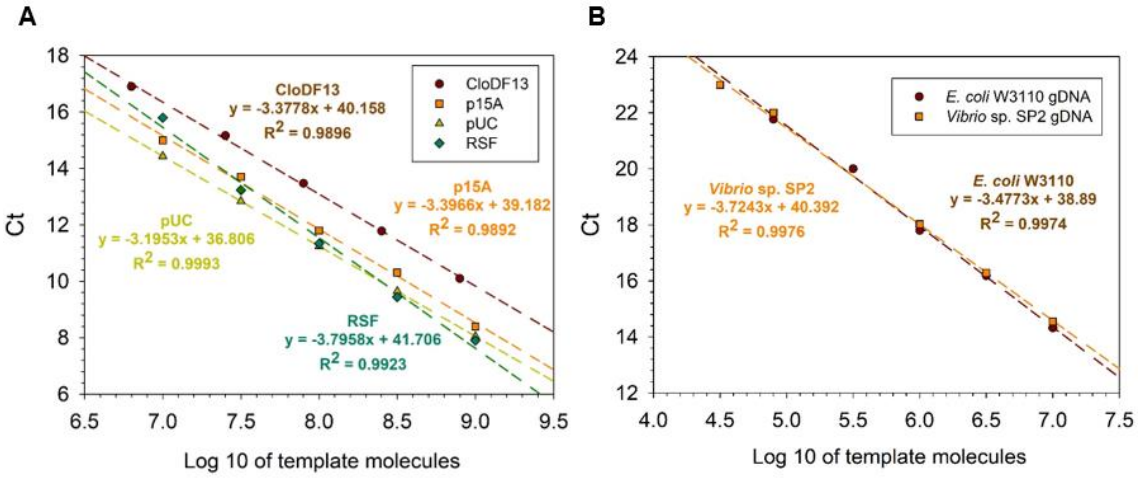
Table S5. Distribution of subsystem categories and features of *Vibrio* sp. SP2 genome

| Subsystem Features | Counts | Ratio (%) |
|---|---|---|
| Cofactors, Vitamins, Prosthetic Groups, Pigments | 149 | 8.26 |
| Cell Wall and Capsule | 26 | 1.44 |
| Virulence, Disease and Defense | 44 | 2.44 |
| Potassium metabolism | 9 | 0.05 |
| Photosynthesis | 0 | 0.00 |
| Miscellaneous | 10 | 0.06 |
| Phages, Prophages, Transposable elements, Plasmids | 4 | 0.02 |
| Membrane Transport | 151 | 8.32 |
| Iron acquisition and metabolism | 20 | 1.11 |
| RNA Metabolism | 51 | 2.83 |
| Nucleosides and Nucleotides | 70 | 3.88 |
| Protein Metabolism | 239 | 13.26 |
| Cell Division and Cell Cycle | 6 | 0.03 |
| Motility and Chemotaxis | 87 | 4.83 |
| Regulation and Cell signaling | 53 | 2.94 |
| Secondary Metabolism | 4 | 0.02 |
| DNA Metabolism | 73 | 4.05 |
| Fatty Acids, Lipids, and Isoprenoids | 54 | 3.00 |
| Nitrogen Metabolism | 40 | 2.22 |
| Dormancy and Sporulation | 2 | 0.01 |
| Respiration | 90 | 4.99 |
| Stress Response | 89 | 4.94 |
| Metabolism of Aromatic Compounds | 8 | 0.04 |
| Amino Acids and Derivatives | 286 | 15.86 |
| Sulfur Metabolism | 20 | 1.11 |
| Phosphorus Metabolism | 34 | 1.89 |
| Carbohydrates | 184 | 10.21 |
| Total | 1803 | 100 |

Table S6. Minimum inhibitory concentration of antibiotics applicable to *Vibrio* sp. SP2

| Antibiotics | Concentration |
|---|---|
| Ampicillin | Not applicable |
| Chloramphenicol | 3.4 μg $mL^{-1}$ |
| Kanamycin | (Solid) 200 μg $mL^{-1}$<br>(Liquid) 300 μg $mL^{-1}$ |
| Spectinomycin | 150 μg $mL^{-1}$ |
| Tetracycline | 1 μg $mL^{-1}$ |
| Erythromycin | 50 μg $mL^{-1}$ |

Figure. S2. Quantification of copy number of various replication origins.
(A) Standard curves of each origin and (B) *rpoA* of *Vibrio* sp. SP2 and *E.coli* W3110.



# Abstract in Korean

키틴은 지구상에서 두번째로 풍부한 생물자원으로 산업원료로서의 잠재력이 있다. 하지만 키틴의 물에 잘 녹지 않는 성질과 열과 물리적 충격에 강한 성질 때문에 응용가능한 산업분야가 제한돼 있다. '키티나아제'라는 이름의 효소를 분비하여 키틴을 분해 및 대사할 수 있는 미생물들이 존재한다. 키티나아제는 키틴을 구성하는 N-아세틸글루코사민 단량체 간의 글리코사이드 결합을 분해함으로써 키틴을 세포 안에서 대사가 가능한 형태로 전환시킨다. 따라서 만약 키티나아제를 분비하면서 키틴 배지에서 빠르게 자라는 미생물을 분리동정하여 키틴을 다른 유용한 화학물질로 생합성할 수 있다면 유용할 것이다.

본 연구에서는 콜로이덜 키틴이 유일한 탄소원인 배지에서 생장가능한 그람음성균을 분리동정하였다. *Vibrio* sp. SP2라고 명명한 이 균주의 게놈 분석을 통해 유전적 특성을 규명하였다. *Vibrio* sp. SP2에서 다양한 항생제, 복제원점, 프로모터 등의 유전학적 도구들의 작동 여부 및 성능이 테스트되었다. 다음으로 키틴대사경로와 키틴에서 라이코펜으로 이어지는 대사경로에 포함된 단백질들 중 *Vibrio* sp. SP2에 결여된 4개의 단백질을 특정하고, 이들을 암호화하는 유전자를 포함한 플라스미드가 형질전환된 신균주를 개발했다. 신균주는 콜로이덜 키틴 배지에서 향상된 성장속도를 보였다.

**주요어 :** 키틴, 분리동정, 게놈 분석, *Vibrio* sp. SP2, 라이코펜
**학번 :** 2019-29816