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Original Article

Computational analysis of collagenase from different Vibrio, Clostridium and Bacillus strains to find new enzyme sources

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Abstract

Collagenase is one the important enzyme, which is applied in varied fields ranging from tannery, food and cosmetic industries to clinical therapies. Currently, the commercially available collagenase enzyme has been produced by *Clostridium histolyticum* bacteria. In our study, in order to find new sources of collagenase producer, 30 collagenases from different species of *Clostridium*, *Vibrio* and *Bacillus* were evaluated from the view of phylogenetic relation, domain architecture and physiochemical features. Totally our results indicate that the non-pathogenic *C. novyi* (NT) with the aliphatic index (80.68), instability index (27), pI (6.54), Mw (112.838 kDa) and two PPC domain could be suggested as a potent bacteria for industrial production of collagenase.

Keywords: *Bacillus,* Collagenase, *Clostridium,* In silico features, Vibrio.

1. Introduction

Collagen is one of the most abundant proteins in our body. It is the main fibrous structure of extracellular connective tissue such as bone cartilage, skin and blood vessels. Whereas collagen has crucial functions in the body, it is a fairly simple protein. Briefly, collagen is constituted of three helically wound chains, two of which are identical (α1 chains), and another one is somewhat different in its biochemical constitution (α2 chain) (1).There are about 28 genetically distinctive kinds of collagen, each with particular physiological functions. Besides structural properties of collagen, it has an important role in cell adhesion, migration, and differentiation (2). Collagenases are unique proteolytic enzymes that are capable to specifically break the peptide bond in the triple helical domains of native and denatured collagen (3). Collagenase is vastly exploited in various areas, ranging from medical applications and food industries to molecular biology investigations (4-6). In clinics, collagenases are applied for enzymatic debridement of burn wounds, treatment of skin ulcers, Dupuytren's contracture, and Peyronie's disease (4, 7). Collagenase enzyme has two main sources: mammals and bacteria. Mammalian collagenases, which are a member of extracellular metalloproteinases family, cleave the native helical collagen chains at a single site. In contrast, bacterial collagenases attack many sites along the chain, producing small peptides (8). Collagenolytic proteases are categorized into two main subsets: metallocollagenases and serine collagenases. Amongst collagenolytic proteases from bacteria, metalloproteases are the most common, while serine proteases and other proteases are scarcely found. All metalloproteases include a conserved sequence for zinc protease, the HEXXH motif, which is able to cleave both denatured and native collagen (8). Bacterial collagenases have wider uses due to their ability to

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cleave the collagen at several sites. A variety of bacteria have been known as collagenase producers. Majority of them are pathogenic strains, applying the enzyme as the virulence factor to destruct the connective tissue barrier and penetrate to a target organ. Mainly three types of pathogenic bacteria are considered as collagenase origins: *Vibrio*, like V*ibrio alginolyticus* (9), *Vibrio* (*Grimontia*) *hollisae* (10) and *Vibrio parahaemolyticus* (11); *Porphyromonas*, such as *Porphyromonas gingivalis* (12); and *Clostridium*, like *Clostridium perfringens* (13) and *Clostridium histolyticum* (14).The well-studied bacterial collagenase is related to *Clostridium histolyticum*, presently manufactured and marketed as an injection by Auxilium Pharmaceuticals called Xiaflex® in the US and by Sobi named Xiapex® in Europe. An ointment dosage form, Santyl®, is also available. Collagenase *C. histolyticum* can be obtained in large amounts from the culture medium under standardized conditions by fermentation. Due to the pathogenicity of the above-mentioned bacteria, the usage of their collagenase would be limited. Recently, some microorganisms such as *Bacillus pumilus* (15), *Bacillus subtilis* (16), *Bacillus tequilensis* (17), *Streptomyces* sp. (18), and *Nocardiopsis dassonvillei* (19), which are usually considered safe to produce collagenase, were screened from water, caviar,

Table 1. List of 30 collagenase protein sequences from different species of *Clostridium*, *Vibrio* and *Bacillus*.

Bacterium	Entry num. (UniProt)	Identity (%)	Protein Length (aa)
Clostridium histolyticum	Q9X721(ColG)	100	1118
Clostridium histolyticum	Q46085 (ColH)	43	1021
Clostridium sordellii	A0A0A8W0C3	35	1025
Clostridium botulinum	A0A0E1KY83 (ColG)	48	1095
Clostridium perfringens	P43153 (ColA)	44	1104
Clostridium sporogenes	G9EY55	45	1217
Clostridium limosum	Q84IN0	40	1158
Clostridium argentinense	A0A0C1UGY8	44	1007
Clostridium tetani	Q899Y1	50	991
Clostridium novyi	A0PYC6	47	977
Vibrio alginolyticus	P43154	100	814
Vibrio vulnificus	Q8D4Y9	32	807
Vibrio neptunius	A0A0F4P2X5	67	821
Vibrio owensii	A0A0C1VUQ3	78	814
Vibrio parahaemolyticus	Q9AMB9	81	814
Vibrio ichthyoenteri	A0A0C1S4B1	65	842
Vibrio antiquaries	A7K0C6	99	814
Vibrio coralliilyticus	A0A097B074	67	823
Vibrio mimicus	D2YQE6	31	806
Vibrio cholera	A0A085RUN7	30	821
Bacillus cereus	C2N3Z1	100	971
Bacillus toyonensis	U5ZMJ0	94	971
Bacillus anthracis	Q81YG5	94	971
Bacillus bombysepticus	CY96_16640	99	971
Bacillus weihenstephanensis	ER45_13415	95	971
Bacillus thuringiensis	XI92_11040	61	965
Bacillus cytotoxicus	TU51_11700	61	967
Bacillus gaemokensis	BAGA_19830	85	972
Bacillus mycoides	A0A0B5SA80	61	965
Bacillus pseudomycoides	C3BGN8	57	975

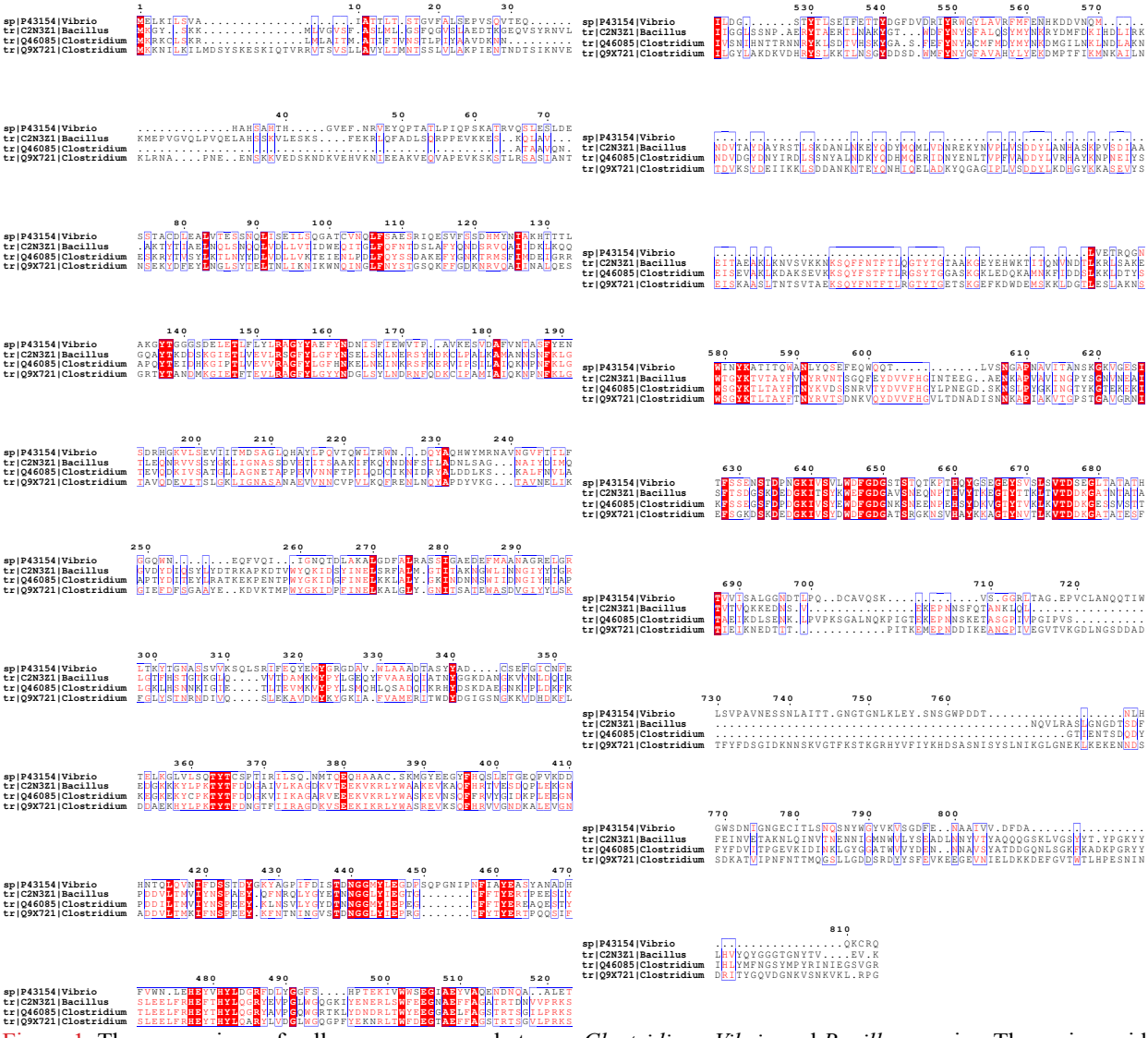

Figure 1. The comparison of collagenase enzyme between *Clostridium, Vibrio* and *Bacillus* species. The amino acid sequences from *V. alginolyticus*, *B. cereus*, *C. histolyticum* (ColH), and *C. histolyticum* (ColG) were aligned. The alignment was colored according to sequence conservation (CLUSTAL W matrix).Identical residues among the four sequences were colored by red and the consensus HEXXH sequences were surrounded by green box.

and soil. However, the collagenases of the safe microorganisms have low activity and restricted uses. Until now, few studies have been performed on safe bacteria as the collagenase producers.

In this study, with the help of bioinformatics tools, we aim to get a precise insight into phylogenetic relationship, structural, functional, and physicochemical properties of collagenases from various bacterial sources in order to find new safe strains with proper collagenase activity.

2. Methodology

2.1 Sequence collection and similarity search

The collagenase amino acid sequences with entry numbers Q9X721 (*Clostridiumhistolyticum*), P43154 (*Vibrio alginolyticus*), and C2N270 (*Bacillus cereus*) were retrieved via Uniport protein databank at http://www.uniprot.org as reference sequences and downloaded in FASTA format for further analysis. For each of *Clostridium*, *Vibrio* and *Bacillus strains* Protein BLAST (BLASTP) (20) against reference sequence database was done applying Blosum60 matrix to gather homologous sequences. The total 30 collagenase protein sequences with entry numbers from different species of *Clostridium*, *Vibrio* and *Bacillus* are listed in Table 1. The bold font in the Table 1 represents reference sequences.

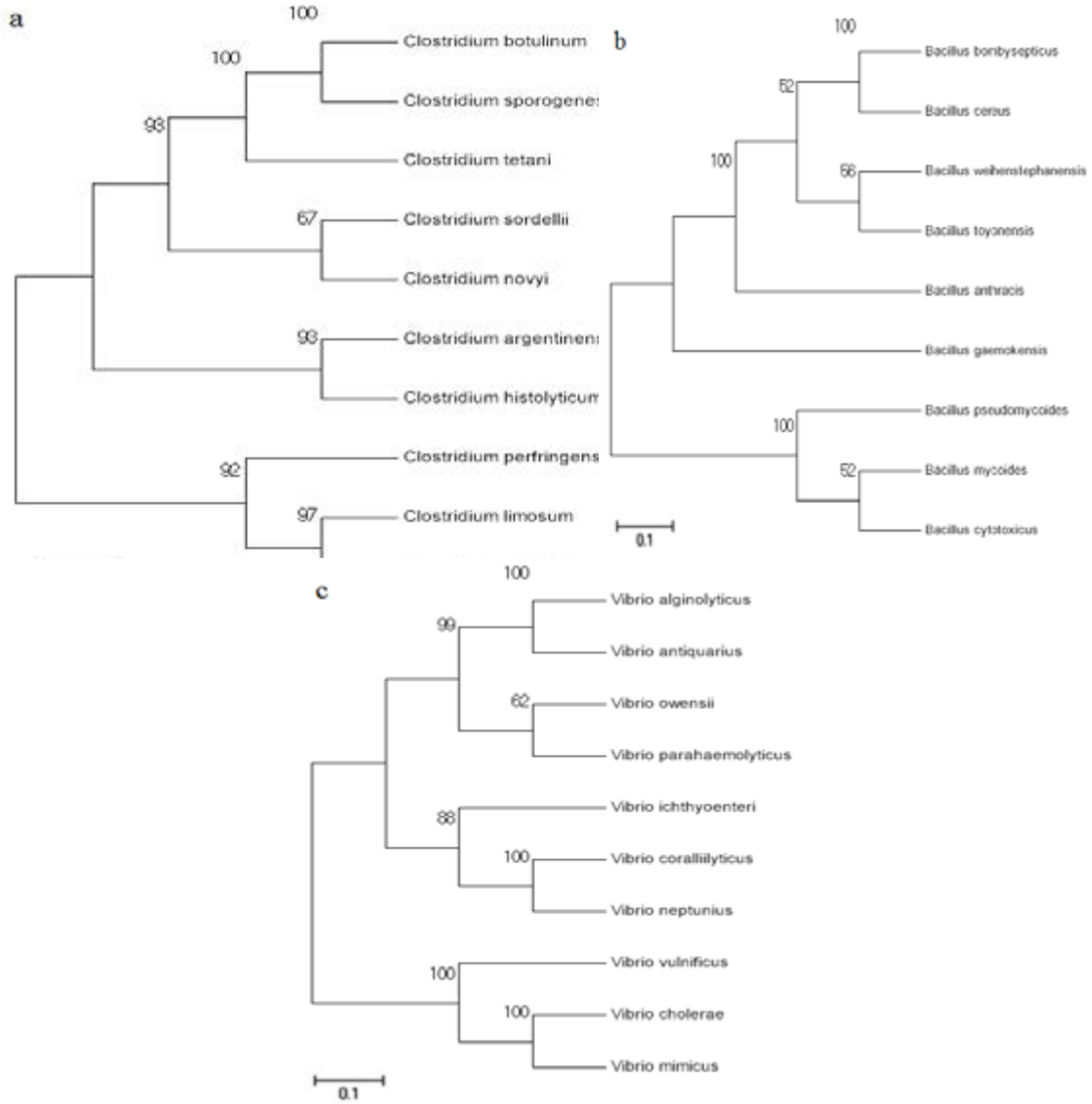

Figure 2. Phylogenetic trees were generated by Neighbor-Joining method based on (a) *Clostridium*, (b) *Bacillus*, (c) *Vibrio* collagenase from different species. Genetic distance and bootstrap values are indicated. Phylogenetic analyses were performed by MEGA 6.

2.2 Sequence alignment and phylogenetic analyses

For each above-motioned strain only the ten collagenase sequences that have identities higher than 30% with the reference sequences were selected. T-Coffee at http://www.ebi.ac.uk/Tools/msa/tcoffee/ and ESPript 3.0 at http://espript.ibcp.fr/ESPript/ESPript/ (21) were applied for performing alignment. Alignments can designate the conservancy of the amino acid residues among different strains. The phylogenetic trees were constructed applying Neighbor-Joining method in Mega 6 software (22).The bootstrap consensus tree resulted from 1050 replicates is taken to depict the evolutionary history of the taxa analyzed. For each node of branch the bootstrap value less than 50% are not considered as a reliable result. All positions including missing and gap data were deleted from the dataset.

2.3 Physicochemical features of sequences and domain identification

Various physicochemical data such as instability index, *in vitro* and *in vivo* half-life, theoretical isoelectric point (pI), aliphatic index, and molecular weight (Mw) were calculated by the ProtParam software applying EXPASY server at http://web.expasy.org/protparam/ (23). Conserved

Table 2. Biochemical properties and class ofcollagenase enzymes from differentspeciesof *Clostridium*, *Vibrio* and *Bacillus*.

Source of organisms	Molecular Weight (kDa)	Theoretical pI	Instability Index	Aliphatic index	Class of enzyme (MEROPS)
C. histolyticum(ColG)	126.241	5.62	26.70	72.19	M09.002
C. histolyticum (ColH)	116.377	5.82	35.44	70.93	M09.003
C. sordellii	118.450	5.18	31.37	70.54	M09.003
C. botulinum	124.847	5.57	29.42	70.68	M09.003
C. perfringens	125.935	4.93	24.13	72.74	M09.003
C. sporogenes	138.738	5.88	25.94	72.40	M09.003
C. limosum	133.184	6.49	34.14	71.95	M09.003
C. argentinense	114.560	5.34	25.93	73.57	M09.003
C. tetani	114.376	5.86	29.39	76.24	M09.003
C. novyi	112.838	6.54	27.00	80.68	M09.003
V. alginolyticus	89.962	4.51	35.24	74.08	M09.001
V. vulnificus	45.008	4.80	40.43	76.86	M09.004
V. neptunius	91.271	4.57	35.20	69.87	Not assigned
V. owensii	89.749	4.67	33.96	71.27	M09.001
V. parahaemolyticus	89.932	4.53	34.37	71.44	M09.001
V. ichthyoenteri	93.190	4.60	36.65	70.78	M09.001
V. antiquarius	89.853	4.52	34.94	74.78	M09.001
V. coralliilyticus	91.324	4.58	32.75	67.91	M09.001
V. mimicus	91.378	4.92	43.23	76.70	M09.004
V. cholerae	93.314	5.07	34.33	78.25	M09.004
B. cereus	109.805	5,62	24.92	71.79	M09.003
B. toyonensis	109.991	5.69	27.09	79.38	M09.003
B. anthracis	110.035	5.55	25.17	72.78	M09.003
B. bombysepticus	109.927	5.58	25.78	71.69	M09.003
B. weihenstephanensis	109.722	5.52	26.26	73.19	M09.003
B. thuringiensis	110.110	5.38	28.90	70.51	M09.003
B. cytotoxicus	110.950	5.39	28.57	67.16	M09.003
B. gaemokensis	110.492	5,47	26.28	72.62	M09.003
B. mycoides	109.873	5.29	28.06	69.62	M09.003
B. pseudomycoides	111.482	5.80	27.97	66.10	M09.003

structural and functional protein domains were determined by NCBI's CD (conserved domain)-Search tool at http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi (24). CD-search is the NCBI's interface for searching the Conserved Domain Database with nucleotide or protein query. It applies Reverse Position-Specific BLAST (RPS-BLAST), to quickly find a set of pre-calculated position-specific scoring matrices (PSSMs) with a protein query. High confidence relations among a query sequence and conserved domains are presented as specific hits. Moreover, the classification of bacterial collagenases was done using MEROPS Blast Server at http://merops.sanger.ac.uk/cgi-bin/blast/submitblast/merops/advanced (25), which links to MEROPS database. The MEROPS database is an information resource for peptidases and their inhibitory proteins.

Figure 3. The different domains of collagenase in various species of *Bacillus*, *Vibrio*, and *Clostridium*. Domains depicted: peptidase_M9_N, representing peptidase family M9 N-terminal; this domain is found in microbial collagenase metalloproteases; peptidase_M9, Collagenase; this family of enzymes break down collagens; PKD, polycystic kidney disease I domain; similar to other cell-surface moduleswith an IG-like fold; PPC, bacterial pre-peptidase C-terminal domain; this domain is normally found at the C-terminus of enzyme.

3. Results and Discussion

3.1 Sequence collection, similarity search and alignment

Microbial collagenases are important enzymes, which are exploited in various areas from food technology to cosmetic and medical industries (4-5, 26). But, undoubtedly, the most notable usage of bacterial collagenases is in the medical industry. Currently, bacterial collagenases are approved as therapeutic agents in a number of human diseases. Presently, collagenase produced by *C. histolyticum* is commercially used as a drug, due to its wide specificity to collagen, compared to other collagenase. Besides the prominent advantage of *C. histolyticum*, it has some deficiencies like inactivation after a 5-min incubation at 60 °C or pathogenicity (14, 27). In this regard, looking for new sources of enzyme with high stability, fewer pathogenicity and efficient enzyme activity is crucial. Searching for novel collagenase producers from various kinds of bacteria is time-consuming and tedious. To confer this obstacle, the bioinformatics tools are exploited. Nowadays, the on-hand bioinformatics methods, utilized in diverse biological areas (28-30), assist researchers to bypass some of these costly and laborious bench-work steps (31). So a series of wide studies were performed on varied properties of the collagenase enzyme of *V. alginolyticus* (9), *C. histolyticum* (14) and *B. cereus* (16), in this reason; they were selected as reference species in our research. A BLASTP search showed that more than 30% identity was observed in different species of *Vibrio*, *Clostridium* and *Bacillus*, so it could be concluded that, the collagenase from various sources possess similar structures and functions (Table 1). The most frequent bacterial collagenases are metalloproteases that share a

zinc-containing HEXXH motif sequence at their active sites and according to MEROPS database; they belong to the peptidase family M9 (25). The MEROPS M9 is subclassified into four subfamily types based on diversities in their amino acid sequences and catalytic function. The classifications of the studied collagenase enzymes are presented in Table 2. In our study, the amino acid sequences of *V. alginolyticus*, *C. histolyticum* (colH and colG), and *B. cereus* collagenases were aligned to find the identical residues (Fig. 1). Moreover, the HEXXH conserved motif is observed in all four sequences.

3.2 Phylogenetic and physicochemical analyses of different collagenases

Phylogenetic studies of different species of *Clostridium* indicated that *C. argentinense* and *C. limosum* are close to *C. histolyticum* (ColH) and *C. histolyticum* (ColG), respectively and instead of *C. histolyticum*, collagenase enzyme from *C. argentinense* and *C. limosum* could be used (Fig. 2a). Apart from *C. histolyticum* (ColG), which is a member of M09.002 subfamily, the remaining *Clostridium* species belong to M09.003subfamily. In the *Bacillus* phylogenetic tree, as shown in Fig 2b, *B. cereus* is very close to *B. bombysepticus* and close to *B. weihenstephanensis*, *B. toyonensis* and *B. anthracis*, but differerent from *B. pseudomycoides*, *B. mycoides* and *B. cytotoxicus*. All the *Bacillus* species belong to M09.003 subfamily. The phylogenetic tree of *Vibrio* species shows that, *V. mimicus*, *V. cholerae* and *V. vulnificus* are close to each other and belong to M09.004 subfamily, while the others are grouped into M0.001 subfamily (Fig. 2c). In addition, *V. alginolyticus* is very close to *V. antiquaries*. *Bacterial collagenases* contain four domains: peptidase-M9, matching with the activator domain (AD), peptidase-M9 domain; polycystic kidney disease (PKD) domain, and PPC, the bacterial pre-peptidase C-terminal domain (matching to CBD (collagen-binding domain)) that organized in distinct architecture in different species (Fig. 3) (24).Fig 3 indicates that collagenases from Clostridium share common domains with *Vibrio* and *Bacillus* collagenases. The *C. histolyticum* (ColG) collagenase possesses two tandems PPC domain allows it binding to all types of collagen fibrils; thus, this collagenase has been approved to indicate broad substrate specificity due to its PPC domains. In this regard, the collagen-binding domains that named PPC are really promising for applications. In our research, we found that *C. novyi* and *V. cholerae* both have two PPC domains. Although the *V. cholerae* is a pathogen but some non-pathogenic species of *C. novyi* are available such as *C. novyi*-NT. Molecular mass is an important characteristic of bacterial collagenase, which is varied in *Vibrio*, *Clostridium* and *Bacillus* species (Table 2). For instance, the molecular masses of collagenase from *C. histolyticum*, *V. alginolyticus*, and *B. cereus* are uncommonly high compared to other metalloproteases (8). The higher molecular masses of bacterial collagenases can be illustrated most likely by the presence of the CBD, as mentioned above. Stability is one of the important features for the industrial production of various peptidases. Although microbial collagenases have been produced by a broad range of mesophilic bacteria, the industrial scale uses of known bacterial collagenases have been hindered due to their insufficient stabilities. An instability value higher than 40 presents the protein as unstable. Our instability results indicate that all enzymes are stable (Table 2). As mentioned earlier, searching for bacterial collagenases with high catalytic activity and stability in thermoacidophilic environments (60 °C, pH 4.0) is pivotal in industrial applications. The high range of aliphatic indices shows that the proteins are thermostable. Moreover, from the view of pI index, the proteins locating in the range of 4 to 6 are acidic. The non-pathogenic *C. novyi* (NT) with the highest aliphatic index (80.68), instability index (27), pI (6.54), Mw (112.838 kDa), and two PPC domain is one of the potent bacteria for the industrial production of collagenase.

4. Conclusion

For finding new industrial sources of bacterial collagenase, the various features of collagenase such as instability index, pI, aliphatic index, and Mw were evaluated from different species of *Vibrio*, *Clostridium* and *Bacillus*. Our data shows that *C. novyi* is a potential bacterium for the industrial production of collagenase enzyme.

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Conflict of Interest

None declared.

5. References

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