Isolation and Characterization of Psychrotolerant *Serratia Quinivorans* Strains Secreting β -D-galactosidase

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Abstract Cold adapted and extracellular β -D-galactosidase (EC 3.2.1.23) with high specific activity has potential in food industry. Two psychrophilic bacterial isolates (A5-2 and B8) were screened from soil collected from permanent glaciers of Himachal Pradesh, India. Both A5-2 and B8 isolates showed growth between 4-25°C, but not at temperature higher than 30°C, hence classified as psychrotolerants. Biochemical characteristics and 16S rDNA sequencing identified the isolates as *Serratia quinivorans* A5-2 and *Serratia quinivorans* B8, and deposited in NCBI GenBank under accession numbers KJ 176660 and KJ 176661, respectively. The cold active, extracellular β -D-galactosidase activity of A5-2 isolate was three fold higher compared to its intracellular activity. Comparatively, the B8 bacterial showed negligible intracellular activity, and its extracellular activity was two folds higher as compared to that of A5-2 isolate. Interestingly, growth and β -D-galactosidase activity of A5-2 was enhanced in lactose supplemented medium; whereas, growth of B8 isolate was unaffected and its β -D-galactosidase activity was enhanced when grown in lactose supplemented medium. β -D-galactosidase activity was also increased, when the isolates were grown in galactose supplementation medium, but decreased when grown in the presence of glucose. The presence of milk sugars like lactose, glucose, or galactose, or the milk metal ions, namely Ca^{2+} and Na^{+} ions did not inhibit the activity of β -D-galactosidase.

Keywords Psychrotolerant, *Serratia Quinivorans*, β -D-galactosidase Enzyme, X-Gal and ONPG Assay, 16S rDNA, Extracellular

psychrophiles, while cold-tolerant microbes as psychrotolerants [12-13]. Enzymes (example, proteases, lipases, amylases, cellulases, dehydrogenases, lactases/ β -D-galactosidases) produced by cold adapted microorganisms have been used in various industries such as detergents, food and dairy, cosmetics, textiles and biosensor applications [14]. Cold-active β -D-galactosidases have attracted the dairy industry for the production of lactose-free milk in cold storage to treat lactose intolerance. β -D-galactosidase hydrolyses lactose in milk into glucose and galactose, which increase the sweetness of milk and are also more fermentable than lactose. Hydrolysis of lactose from the whey generated in the cheese production process could reduce the pollution problem related to the dairy industry [15]. Cold-active β -D-galactosidases are useful for the synthesis of galacto- oligosaccharides (GO), which function as effective pre-biotics for humans. Although β -D-galactosidases from mesophilic *Kluyveromyces lactis* is commercially available as Lactozyme (Novo Nordisk Co.), cold adapted β -D-galactosidases have not been produced on industrial scale. The cold active β -D-galactosidase for such applications should be active in the presence of Na^{+} and Ca^{2+} ions/ lactose, galactose and glucose. In addition, intracellular enzymes pose additional cost for the recovery of enzymes, thereby increasing the demand for extracellular β -D-galactosidases. Cold-active β -D-galactosidases have been isolated from *Arthrobacter* sp. [16-18], *Pseudoalteromonas* sp. 22b [19], *Arthrobacter psychrolactophilus* [20-21], *Carnobacterium piscicola* [22], *Planococcus* sp. [23-24], *Pseudoalteromonas haloplanktis* [25] and *Pseudoalteromonas* sp. [26-27]. To the best of our knowledge, none of the β -D-galactosidases isolated from these sources have amenable potential for industries.

Therefore, the present study was undertaken to isolate psychrophiles for the production of cold-active extracellular β -D-galactosidase. We report the isolation of two psychrotolerant bacteria (A5-2 and B8) secreting cold-active, extracellular β -D-galactosidase enzyme. The biochemical tests and molecular characterization revealed that both A5-2 and B8 isolates belong to *Serratia quinivorans*. More importantly, the β -D-galactosidase activity of both the strains was constitutive and unaffected by calcium and

1. Introduction

A significant area of earth's biosphere is encompassed by cold regions with an average temperature of 4-5°C [1]. These regions are occupied by diverse organisms like arctic fish, terrestrial invertebrates, amphibians, arctic birds and microorganisms such as eubacteria [2-4], archaea [5-8] and yeasts [9-11]. The cold adapted microbes are classified as

sodium ions and sugars present in the milk.

2. Material and Methods

Collection of soil samples, isolation and screening of bacterial isolates

The soil sample was collected from Kafnu (altitude, 4500 m) and Sangla valley (altitude 5500 m) of Kinnour district of Himachal Pradesh, India. The soil samples were collected by using sterile spatula in polyethylene bags and stored at -20°C . 5 g of each soil sample was enriched in 1% lactose solution at 4°C . After 21 days of enrichment, 100 μl of soil free supernatant was spread on LB agar plates and incubated at 4°C till bacterial colonies were observed. The distinct colonies were purified by three successive streaking on LB agar medium and incubated at 25°C . The isolated colonies were subjected to Gram's staining and growth characterization at different temperatures i.e., 4°C , 10°C , 15°C , 30°C and 37°C by streaking on LB agar medium.

Qualitative and quantitative β -D-galactosidase enzyme assays

Both isolates were grown in LB broth medium for 24 h at 25°C . Cell cultures were centrifuged at 8000 rpm for 5 minutes. Cell lysate was prepared in Z-buffer (60 mM Na_2HPO_4 , 40 mM NaH_2PO_4 , 10 mM KCl and 1 mM MgSO_4 , pH 7.0) by adding 10 μl of chloroform and 10 μl of 10% SDS and incubation at 37°C for 15 minutes. The amount of total protein in whole cell lysate and cell free supernatant was estimated by Bradford method [28]. Two microgram of total proteins of whole cell extracts or cell free supernatant were spotted on LB agar medium supplemented with either X-gal (0.1 mM of X-gal in N, N dimethyl-Formamide), or X-gal and IPTG (25 mM) or X-gal and lactose (30 mM) and incubated at 25°C for 24 h and analysed for the formation of blue colored product.

For quantitative assay of β -D-galactosidase enzyme, bacterial cells were grown in LB broth medium for 96 h. The cell extracts and supernatant were assayed for β -D-galactosidase enzyme activity using 0.33 mM ONPG (ortho-nitrophenyl- β -D-galactopyranoside) as the substrate in Z-buffer [29]. The reaction was carried out at 30°C for 1 h and stopped by addition of 1 M Na_2CO_3 . The release of ortho-nitrophenol from ONPG was measured at 420 nm and estimated using standard graph of o-nitrophenol. The specific activity of β -D-galactosidase enzyme was calculated as μmoles of o-nitrophenol released per minute per microgram (U/mg) of total protein.

Effect of milk sugars and metal ions on growth and β -D-galactosidase activity of A5-2 and B8 isolates

To study the effect of lactose on growth and β -D-galactosidase production, both A5-2 and B8 isolates

were cultured either in LB broth or LB broth supplemented with 1% lactose and incubated at 25°C . Cell cultures were withdrawn at different time intervals (0 – 160 h). At each time interval, cell density was measured at 600 nm and β -D-galactosidase activity in the cell-free supernatant (5 μg total protein) was determined using ONPG as substrate. Similarly, the effect of glucose and galactose on the growth of the isolates and β -D-galactosidase activity was studied at different time intervals of growth (0-100 h).

To study the effect of milk sugars (glucose, galactose, and lactose) on β -D-galactosidase enzyme activity, A5-2 and B8 were grown in LB broth without supplementation of sugars and ONPG assays were performed using cell free supernatant (5 μg total protein) in the presence of different concentrations (2.5, 12.5 and 25 mM) of glucose, galactose or lactose. To study the effect of metal ions, such as Ca^{2+} and Na^{+} on the β -D-galactosidase activity, cultures were grown without supplementation of sugars as described above. ONPG assays were performed using cell free supernatant (5 μg total proteins) in the presence of different concentrations (5, 10, and 20 mM) of CaCl_2 and NaCl .

Biochemical characterization of A5-2 and B8 isolates

Both A5-2 and B8 isolates were characterized for their optimal growth at different temperature (4°C , 15°C , 25°C , 30°C and 37°C) and pH (4, 5, 7, 9 and 11) by streaking on LB agar medium or by measuring cell density at 600 nm. Biochemical tests were determined according to Bergey's manual of determinative bacteriology, which includes biochemical tests, such as coagulase, catalase, oxidase, growth on MacConkey agar, citrate utilization, lactose fermentation, indole test, MR-VP test and lysine decarboxylase assays. Utilization of glucose, galactose, lactose, sucrose, xylose, and trehalose was also studied [30].

Molecular identification of bacterial isolates A5-2 and B8 by 16S rDNA amplification and sequencing

Total genomic DNA from A5-2 and B8 isolates was isolated using genomic DNA extraction (Fermentas Co., USA). Amplification of 16S rDNA was carried out using 100 ng of genomic DNA as template and the universal primers 27F-(5' GAGTTTGATCCTGGCTCAG-3') [31] and 1492R (5'-GGTTACCTTGTTACGACTT-3') [32]. The annealing temperature was 45°C . After PCR amplification, reaction products were separated on 1.0% agarose gel and visualized in a gel documentation system. DNA sequencing was done on both the strands using 27F and 1492R primers at Eurofins Genomics Pvt. Ltd., Bangalore, India. The 16S rDNA gene sequence was subjected to BLAST analysis in the NCBI database (<http://www.ncbi.nlm.nih.gov>) and aligned using the Clustal W program [33]. The phylogenetic tree for the data set was constructed using the Phylip computer programme package drawgram 3.66 [34].

Figure 1. Phylogenetic analysis of 16S rDNA sequences of A5-2 and B8 psychrotolerant isolates. 16S rDNA sequences of A5-2 and B8 isolates were subjected to BLAST search. Phylogenetic tree was constructed for all the BLAST hits belonging to Enterobacteriaceae family (A) and *Serratia* species (B). The retrieved sequences were aligned using Phylip software and phylogenetic tree was constructed using Phylip computer programme package drawgram 3.66.

3. Results

3.1. PsychrotoLERANT *SERRATIA QUINIVORANS* STRains A5-2 and B8 Secrete Extracellular and Lactose Inducible β -D-galactosidase during the Decline Phase of growth

In order to isolate psychrophilic bacteria, we collected soil samples from Kafnu and Sangla valleys of the Himalayan range located in Kinnour District, Himachal Pradesh. The valleys are covered with snow throughout the year. The soil microflora were enriched for lactose degrading bacteria in 1% lactose solution. The enriched microflora were plated on LB agar medium supplemented with lactose and incubated at 4°C. Distinct colonies were observed on 7th day of plating (data not shown). 14 morphologically distinct bacterial colonies were picked and streaked by three successive streaking on LB agar medium and incubated at 4°C. To test the psychrophilic nature of bacterial isolates, all the isolates were streaked on LB agar medium and incubated at different temperatures ranging from 4 to 40°C (Table 5.1). Only two isolates, namely A5-2 and B8 showed visible growth at 4°C during the third day and no growth at temperature higher than 25°C. The optimum temperature for the growth of A5-2 was 20°C, and 25°C for B8 isolate (Table 5.2). The optimal pH for both the isolates was 7 (Table 5.2). Thus, A5-2 and B8 were classified as psychrotolerants. A5-2 and B8 isolates were further pursued for the biochemical characterization of the isolates and extracellular nature of β -D-galactosidase. The Gram's staining revealed that both isolates were Gram's negative and uniform rods (Table 5.2). Both isolates were coagulase negative, catalase positive, oxidase negative, lactose fermenting, indole negative, MR-VP negative, lysine decarboxylase positive, motile and sugars fermenting (Table 5.2). Based on biochemical tests, both A5-2 and B8 are identified as *Serratia sp.* belonging to Enterobacteriaceae family (Table 5.2).

To confirm the identity of *Serratia sp.* at the molecular level, and examine their phylogenetic position, total genomic DNA was subjected to PCR amplification of gene encoding 16S rRNA. A PCR product of approximately 1500 bp was detected (data not shown). The 16S rDNA sequence thus obtained was subjected to homology search by BLAST search of the NCBI database and both the sequences (A5-2 and B8) showed 98% similarities with *Serratia quinivorans* strain 4364. Phylogenetic analysis revealed that A5-2 and B8 formed a distinct cluster from other *Serratia* spp. As well as from other members of Enterobacteriaceae family (Figure 1 A & B). Based on biochemical properties, 16S rDNA sequence and phylogenetic analysis, we classified isolate A5-2 as *Serratia quinivorans* strain A5-2 and B8 as *Serratia quinivorans* strain B8. The multiple sequence alignment of 16S rDNA of both the isolates showed 99% similarities with substitution at positions G528A, T584C, G642A and addition of G575 in A5-2 isolate, and addition of T607 and C670 in the sequence of B8 isolate (data not shown). 16S rDNA sequences of *Serratia quinivorans* strain

A5-2 and *Serratia quinivorans* strain B8 have been submitted to the NCBI GenBank database with accession number KJ 176660 and KJ 176661, respectively.

We assumed that both the psychrotolerant strains are novel, and therefore characterized them for the extracellular production of β -D-galactosidase. Qualitative assays showed the presence of enzyme activity in the whole cell extracts and cell free spent medium of both A5-2 and B8 isolates, when tested by spotting assay on LB agar medium supplemented with X-gal as a substrate and lactose or IPTG as inducer (data not shown). This suggests an intracellular and extracellular nature of β -D-galactosidase enzyme produced by A5-2 and B8 isolates. Moreover, β -D-galactosidase activity was detected in the absence of IPTG or lactose, suggesting an auto-inducible nature of the enzyme. Quantitatively, we observed approximately 10 folds increase in the extracellular activity (1800 U/mg) as compared to intracellular β -D-galactosidase activity (186 U/mg) in *Serratia quinivorans* strain A5-2 strain (Figure 2). Comparatively, very low amount of intracellular β -D-galactosidase activity (221 U/mg) was observed in *Serratia quinivorans* strain B8, and 14 folds more extracellular activity (2800 U/mg) (Figure 2). Due to the high levels of extracellular β -D-galactosidase production, A5-2 and B8 isolates were pursued for characterization of β -D-galactosidase.

Figure 2. Estimation of β -D-galactosidase activity in A5-2 and B8 isolates. Cells were grown at 25°C till A_{600} reached 1.0. Cell lysate (C) and supernatant (S) of A5-2 and B8 isolates were analyzed for β -D-galactosidase activity using ONPG as a substrate. The specific activity (U/mg) is plotted against the indicated samples.

3.2. Effect of Substrate and Products on the Growth and Production of β -D-galactosidase

To optimize the growth conditions for β -D-galactosidase production by A5-2 and B8 isolates, we studied the effect of lactose (substrate), glucose and galactose (products). Microbial growth of A5-2 and B8 isolates was markedly enhanced when cultured in the medium supplemented with lactose as compared to non-supplemented medium (Figure 3 A & B). In addition, the duration of stationary phase was prolonged in non-supplemented LB broth medium. In the case of A5-2 isolate, there was a marked increase in the β -D-galactosidase activity in the presence of lactose, with maximum activity (9000 U/mg) at 90 h of growth as compared medium without lactose supplementation, in

which maximum activity (5500 U/mg) was achieved at 140 h of growth for (Figure 3A). On the contrary, B8 isolate did not exhibit any significant difference in the growth pattern in the presence and absence of lactose, but showed a drastic increase in the β -D-galactosidase activity in lactose grown cultures (Figure 3B). The maximum β -D-galactosidase activity (23000 U/mg) of B8 isolate was observed at 100 h in the lactose grown cultures as compared to 8000 U/mg at 130 h in the absence of lactose (Figure 2.4.3B). The correlation of enzyme activity with the growth kinetics indicated that maximum β -D-galactosidase activity was observed during the late stationary phase of growth of both the isolates (Figure 3 A&B).

Figure 3. Effect of lactose on growth of A5-2 and B8 isolates and their β -D-galactosidase production. Both A5-2 and B8 isolates were inoculated in LB broth medium (Lac⁻) or LB broth supplemented with 1% lactose (Lac⁺) and incubated at 25°C. Samples were harvested at indicated time periods of incubation and β -D-galactosidase activity was measured in cell free spent medium. At each time point, cell density was measured by measuring absorbance at A₆₀₀ nm. The β -D-galactosidase activity and the corresponding cell density (absorbance at 600 nm) for each time point are plotted against the time of incubation for A5-2 (panel A) and for B8 (panel B) isolates.

Similarly, we studied the effect of glucose and galactose supplementation in the growth medium and its correlation with microbial growth and β -D-galactosidase activity. There was a marginal increase in the growth of A5-2 and B8 isolates in the presence of galactose and glucose (Figure 4 A & C). β -D-galactosidase activity of both A5-2 and B8 was

enhanced when galactose was supplemented in the growth medium (Figure 4 B & D). While glucose had no effect on the β -D-galactosidase activity of A5-2 (Figure 4B), the activity was undetectable when B8 isolate was grown in the presence of glucose (Figure 4 D).

Figure 4. Effect of galactose and glucose on the growth and production of β -D-galactosidase of A5-2 and B8 isolates. A5-2 and B8 isolates were grown in LB broth supplemented with 100 mM of galactose or glucose. β -D-galactosidase activity and growth was measured at 24, 48, 72 and 96 h of incubation. The microbial growth and enzyme activity is plotted as indicated for A5-2 (A, microbial growth; B, β -D-galactosidase activity) and B8 (C, microbial growth; D, β -D-galactosidase activity) isolates.

3.3. Metal Ions and Sugar Constituents of Milk do not Affect β -D-galactosidase Activity *in vitro*

Since β -D-galactosidase has potential applications in dairy industries, it would be ideal if its activity is not inhibited by sugars (lactose, galactose and glucose) and metal ions such as Ca^{2+} and Na^{+} present in the milk and dairy products. To study the effect of sugars (galactose, glucose, and lactose) and metal ions on β -D-galactosidase activity, A5-2 and B8 isolates were grown in LB broth medium. The cell free spent medium was incubated with different concentrations of indicated sugars (2.5, 12.5, and 25 mM) or metal ions (10-30 mM), followed by β -D-galactosidase assay. There was a mild reduction (10-30% inhibition) in the β -D-galactosidase activity of both A5-2 and B8 isolates in the presence of lactose, glucose or galactose (Figure 5 A & B). More interestingly, the presence of metal ions such Ca^{2+} and Na^{+} at 10-30 mM did not affect the β -D-galactosidase enzyme activity of A5-2 and B8 isolates (Figure 6 A & B).

Figure 5. Effect of sugars (lactose, galactose and glucose) on β -D-galactosidase activity. A5-2 and B8 isolates were grown in LB broth for 96 h at 25°C. The cell free spent medium was incubated with various concentrations of lactose, glucose or galactose (0.1, 0.5% and 1%) for 5 minutes, followed by β -D-galactosidase assay. The relative specific activity was calculated and plotted against the concentration of sugars for A5-2 (A) and B8 (B) isolates.

These results clearly suggests that the presence of sugars in the production medium has greater role in either stimulating or inhibiting the β -D-galactosidase enzyme activity (3.2) as compared to the presence of sugars in the enzymatic assays (3.3).

Figure 6. Effect of Ca^{2+} and Na^{+} on β -D-galactosidase activity. The cell free spent medium was incubated with indicated concentrations of either CaCl_2 or NaCl for 5 minutes and β -D-galactosidase activity was measured by ONPG assay at 30°C. The relative β -D-galactosidase activity is plotted against the concentration of metal ions as indicated for A5-2 (A) and B8 (B) isolates.

4. Discussion

β -D-galactosidase enzyme is an essential tool for the commercial production of lactose-free milk and related dairy products. The cold storage of milk and milk products necessitates the use of cold active enzymes for their processing, which are not inhibited by constituents of milk like sugars and metal ions. Moreover, extracellular enzymes are commercially more viable than the intracellular ones. β -D-galactosidases naturally produced by psychrophilic microorganisms are either intracellular or expressed at low levels. Therefore, the present study was undertaken to isolate the psychrophilic bacteria producing cold active, extracellular β -D-galactosidases. We report the isolation of two new psychrotolerant bacterial isolates A5-2 and B8 that could grow optimally at 25°C and exhibit no detectable growth at temperatures higher than 25°C, hence classified as psychrotolerant according to Morita [12]. Biochemical characterization of A5-2 and B8 isolates (Table 5.2) showed that both the isolates are Gram's negative rods and motile and belong to the genus *Serratia*. In agreement with the biochemical characteristics, 16S rDNA sequence analysis revealed their close identity (98% homology) to *Serratia quinivorans* strain 4364. Both the isolates are closely related to *Serratia quinivorans* strain 4364, but differ from each other at nucleotide positions 528, 575, 584, 607, 642, and 670

of the 16S rDNA. Thus the strains were named as *Serratia quinivorans* B8 and *Serratia quinivorans* A5-2. Isolates.. Phylogenetic analysis revealed that both the isolates form an independent cluster and are closely related to *Yersinia* sp., *Hafni* sp., *Rahnella* sp., and *Obesumbacterium* sp., as compared to the existing *Serratia* sp. Therefore, we conclude that *S. quinivorans* A5-2 and *S. quinivorans* B8 are novel members of the genus *Serratia*, which are also psychrotolerants. Very little is known about the existing *S. quinivorans*. It was originally classified as *Serratia proteamaculans* subsp. *Quinivora*, and later categorized as *Serratia quinivorans* [35,36].

To the best of our knowledge, there is no report of β -D-galactosidase from cold adapted *Serratia quinivorans*. Moreover, the extracellular nature of the enzyme makes it more important for industrial applications. Therefore, we further characterized and compared the β -D-galactosidase from *Serratia quinivorans* A5-2 and *Serratia quinivorans* B8 isolates.

Microorganisms that produce β -D-galactosidase have been extensively studied. β -D-galactosidases naturally produced by psychrophilic microorganisms are either intracellular or expressed at low levels. In contrast, bacterial isolates *S. quinivorans* A5-2 and B8 secrete extracellular β -D-galactosidase (8000 and 23000 U/mg respectively), which is constitutively expressed.

Traditionally, commercial β -D-galactosidases are produced from fungi of the genus *Aspergillus* and yeasts of the genus *Kluyveromyces* [37] and are sensitive to inhibition by the substrate (lactose) and reaction products (galactose and glucose). The activity of β -D-galactosidase from various microorganisms such as *Kluyveromyces lactis* [38], *Arthrobacter* sp. [16], and *Hymenaea courbaril* [39] was inhibited by galactose. On the other hand, there is only 10-30% inhibition of enzyme activity of A5-2 and B8 isolates in the presence of lactose and galactose. It was observed that. Nevertheless, inherent β -D-galactosidase activity was very high as compared to those of other psychrophilic bacteria like *Bacillus* sp. (0.5 U/mg) [40], *Arthrobacter* sp. 32c (155 U/mg) [41], *Arthrobacter* sp. ON14 (0.42 U/mg) [42], *Pseudoalteromonas* sp. 22b (114 U/mg) [19], *Rahnella aquaticus* (76.75 U/mg) [43], and *Hafnia alvei* (0.54 U/mg) [44]. More interestingly, the enzyme activity of *S. quinivorans* A5-2 and *S. quinivorans* B8 isolates was not inhibited in the presence of calcium and sodium ions, which are the most prevalent metal ions in milk.

5. Conclusions

In the present study, we isolated two novel species of psychrotolerant *Serratia quinivorans* which secrete cold active β -D-galactosidase. The β -D-galactosidase produced by *Serratia quinivorans* A-5-2 and B8 possesses two major advantages, 1) extracellular nature of the enzyme, and 2) enzyme activity not inhibited by the substrate (lactose), reaction products (glucose and galactose) and metal ions

(calcium and sodium) present in the milk. Due to these unique features, *Serratia quinivorans* A5-2 and *Serratia quinivorans* B8 would be advantageous over the existing isolates for the production of β -D-galactosidase and its application in milk and dairy industry. The purification and characterization of the β -D-galactosidase enzyme from both the isolates would be the immediate need for its commercial exploitation.

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