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Isolation and molecular identification of α-amylase producing bacteria from hot water spring by 16S rRNA gene sequencing

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Among different types of microbial enzymes, amylases are the most widely used in industries as they are produced in large quantity and in an economic way as compared to plants and animals. Moreover, thermostable amylase has significance as compared to the amylase from mesophiles. Therefore, hot water springs are explored to dig into its bacterial diversity. In the current study, we tried to isolate amylase producing bacteria from the soil and water samples collected from the hot water spring in Rampur, Himachal Pradesh, India. The samples were serially diluted before plating on the Luria Bertani agar plates. A total of 42 bacterial morphotypes were isolated and were screened for amylolytic activity by starch agar plate method. Among the 42 bacterial isolates 25 showed amylolytic activity. Production of amylase was carried out at different temperatures and pH to optimize the temperature and pH conditions for each isolate. All the 25 isolates were characterized based on morphology, biochemical tests and molecular analysis. Sequencing of 16S rRNA gene for the 25 isolates followed by BLAST search revealed a majority of them (19) identified as *Bacillus licheniformis*. Other isolates were identified as *B. subtilis*, *B. safensis*, *B. halodurans*, *B. stratosphericus*, *Caldimonas hydrothermale* and *Exiguobacterium mexicanum*. An attempt was made to amplify *amyN* gene which codes for α-amylase but successful amplification was achieved only from *Bacillus licheniformis* alone.

Keywords: 16S rRNA, α-Amylase, *amyN* gene, MEGA6, Thermophillic

Microbiologists have estimated that only one percent of all microbial species have been identified and rest of the clan of the microbes remain undiscovered due to their presence in extreme environment like high or low temperature, pH and salt conditions, etc.[1]. The thermophilic organisms have the capability to produce enzyme which are thermostable and can maintain their activity in production processes for longer period of time[2]. The application of enzymes from thermophilic organism's increases rapidly with the discovery of thermostable enzyme as the processing is far more stable, faster and includes lesser cost[3]. Therefore, there is a continuous urge of the researchers to obtain thermostable enzymes[4].

Starch degrading amylolytic enzymes are most important in the biotechnology industries with its market estimated to 20–25% of the world enzyme market[5] making it the second biggest consumer among the other industrially important enzymes[6]. Amylolytic enzymes are exoacting, endoacting and debranching[7]. Unique and unconventional amylase is found in acidophilic, alkalophilic and thermo acidophilic bacteria[8]. Amylase is obtained from various origins like plant, animal, bacteria and fungi[9] but microorganisms are the significant producer of industrial enzymes because they are biochemically diverse, has high rate of production and can be engineered by genetic manipulation to obtain enzymes of desired characteristics[10].

Sea water was explored for α-amylase producing *Glaciecola amylolytica* sp. and antarctic ice cover for *Pseudoalteromonas* sp. M175 expressing Amy175 gene[11,12]. Several researchers reported amylases from *Bacillus* genus[13]. Diversity study of α-amylase from Unkeshwar hot springs (Maharashtra, India) reported 23 thermostable enzymes including 16 strains of *Bacillus*[14]. Amylolytic potential of two probiotic bacteria *Lactobacillus fermentum* and *Lactobacillus* sp. G3_4_1TO2 were checked for α-amylase production but the latter formed high amount of enzyme[15].

Amylase has applications in various industries like food, fermentation, paper, textile, pharmaceutical, fine-chemical. Its utility has also peeped into medical chemistry, clinical research and starch analytical chemistry, etc.[16]. These expanded the use of enzyme in

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different industries, thereby placing greater meaning to increase its production and efficiency[17].

With advances in DNA isolation, amplification techniques and bioinformatics tools, accuracy to taxonomically identify microorganisms have increased[18,19]. Efficient extraction and processing protocols are required to recover high quality/quantity of DNA which can be further identified by full length amplification of 16S rRNA gene followed by sequencing and phylogenetic profiling[20,21]. In this context, here, we explored the bacterial diversity (hot spring of Rampur, Himachal pradesh, India) based on 16S rRNA gene amplification. Further, we used *amyN* gene for identification of amylase producing bacteria in sampling sites.

Materials and Methods

Sampling and isolation of bacterial strains

Soil and water samples were collected aseptically from various parts of hot water spring in Rampur district of Himachal Pradesh, India and carried back to laboratory in sterile plastic bags. Serial dilutions of samples were spread onto Luria Bertani (LB) agar plates for separation of bacterial isolates after incubation of plates at 50°C for 24 h. Isolates obtained were further sub cultured to obtain pure culture. Pure isolates on 1% w/v starch, LB and agar were maintained at 4°C.

Screening procedure for amylase producing bacteria by starch hydrolysis test

The pure isolates incubated at 50°C for 24h on starch agar plate were flooded with Lugol's reagent (mixture of iodine-potassium iodide). Formation of clear zones around the colonies indicated enzyme hydrolysis. Every isolate that showed a clear zone was selected as an amylolytic isolate.

Identification of amylase producing strains

A total of 42 isolates were obtained, out of which 25 amylolytic isolates were studied for morphological and biochemical characters[22] and identified *via* 16S rRNA gene sequencing[23,24]. The isolates were observed for colour, shape, size, margins, gram staining and negative staining. The bacterial isolates were characterized biochemically for amylase, catalase, oxidase, vogues, methyl red, indole, simmons citrate, urease, hydrogen sulfide production and motility[25].

Molecular identification of isolates

Genomic DNA extraction and 16S rRNA gene amplification

The pure cultures were inoculated separately in 20 mL of LB and incubated at 50˚C for 24 h. Bacterial cells were collected by centrifugation at 8000 rpm at 4°C for 10 min. Isolation of total genomic DNA was carried out as described[26] with little modifications. Agarose gel (0.8% w/v) in 1X-TAE buffer was prepared and 5 µL of each extracted DNA was electrophoresed at 75V. Molecular identification of the isolates was done by amplification of 16S rRNA gene with bacterial universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-CGGCTACCTTGTTACGACTT-3')[27]. PCR was carried out in a thermal cycler with total volume of 25 µL and initial denaturation at 94°C for 180 s, followed by 35 cycles of 94°C for 60 s, 50°C for 45 s and 72°C for 105 s with a final 72˚C extension for 5 min. Amplified product was then analyzed directly by electrophoresis on 1.5% w/v agarose gel.

Sequencing and phylogenetic analysis of 16S rRNA gene

Electroelution (gel extraction) of the 16S rRNA amplified DNA was done and purified using Qiaquick gel extraction kit (QIAGEN). Purified product was used as a template for sequencing. Sequencing was done using automated sequencer ABI 3130 by Bioserve Biotechnologies (India) Pvt. Ltd. Data was obtained in the form of chromatogram which was mined using the Bioedit software. The contigs were used for finding its homologs from NCBI and phylogenetic tree was constructed by MEGA6 software[28].

Molecular identification of amylase gene of isolates

Amplification of amyN gene and analysis of amplified product

The complete *amyN* gene and its flanking regions were amplified using upstream primer BLE1 (5'AAGTGAAGAAGCAGAGAGGC3') and downstream primer BLE2 (5'TTCAAACTGTCAGCAAGTCC3') to generate a fragment of approximately 1.8 kb. The amplification of *amyN* was done as reported[29]. Polymerase chain reaction was carried out in a thermal cycler with total volume of 25 µL and initial denaturation at 94°C for 3 min, followed by 30 cycles of 94°C for 45 s, 59°C for 45 s and 70°C for 2 min with a final 72°C extension for 5 min. Amplified product was then analyzed directly by electrophoresis on 1.5% w/v agarose gel. Electroelution of amplified DNA was done and purified by Qiaquick gel extraction kit (QIAGEN).

Sequencing and phylogenetic analysis of amyN amylase gene

Amplified *amyN* gene product was purified and used as a template for sequencing of both the strands. Sequencing was done using automated sequencer ABI 3130 by Bioserve Biotechnologies (India) Pvt. Ltd.

Table 1 — Biochemical analysis of 25 α-amylase positive isolates

Biochemical tests	α-amylase positive isolates
Catalase	RA3, RA5, RA6, RA8, RA11, RA12, RA13, RA16, RA17, RA19, TT22, TT23, TT25, HNC28, UWF29, RA31, RA33, RA34, HNC35, HNC36, HNC38, UWF42
Oxidase	RA2, RA3, RA5, RA6, RA8, RA11, RA12, RA13, RA16, RA17, TT23, TT25, UWF29, RA31, RA32, RA33, RA34, HNC35, HNC36, HNC38, UWF42,
Voges–Proskauer	RA1, RA2, RA3, RA5, RA8, RA13, RA16, RA17, TT23, UWF29, RA31, RA33, RA34, HNC35, HNC36
Methyl red	RA1, RA2, HNC28, UWF29, RA31, RA32, RA33, RA34, HNC38, UWF42
Citrate	RA5, RA6, RA8, RA11, RA13, HNC28, UWF29, RA31, RA33, HNC38, UWF42
Motility	RA1, RA2, RA3, RA5, RA6, RA8, RA11, RA12, RA13, RA16, RA17, RA19, TT22, TT23, TT25, UWF29, RA31, RA32, RA33, RA34, HNC35, HNC36, HNC38, UWF42

[RA, TT, HNC & UWF represents four different sampling sites 1, 2, 3 & 4 of hot spring, respectively]

Data from the chromotogram was mined using the Bioedit software and contigs obtained were used for finding its homologs from NCBI. A phylogenetic tree was constructed by MEGA6 software[28].

Enzyme assay

Bacterial culture broth was incubated for required time and temperature at 150 rpm and centrifuged at 8000 rpm for 20 min at 4°C. Cell free supernatant recovered after centrifugation was crude enzyme and used as enzyme sample for assay. One mL reaction mixture containing soluble starch (1% w/v) and 100 µL of the enzyme samples were incubated at 50°C for 10 min. 50 µL of each reaction mixture was used for the estimation of glucose by DNS method[30]. The amount of reducing sugars liberated during the assay was estimated by measuring the colour development at 540 nm by UV-VIS spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme which releases 1.0 µmol of reducing group per mL per minute.

Results and Discussion

The morphological examination of isolates revealed that 21 isolates were gram positive, 4 were Gram negative and 23 were identified as bacilli and 2 as cocci. Based on the biochemical characteristic 22 isolates were catalase positive, 21 were oxidase positive and all the isolates were negative for indole and hydrogen sulphide test (Table 1). Nineteen strains were *Bacillus licheniformis*, 1 isolate was *Bacillus subtilis* and others were *B. halodurans*, *B. stratosphericus*, *B. safensis*, *Exiguobacterium mexicanum* and *Caldimonas hydrothermale* as identified by 16S rRNA gene sequencing (Table 2).

Bacillus licheniformis and *Bacillus subtilis* are the major group of microbes isolated from the hot water springs for production, purification and characterization of α-amylase[31-34]. *Bacillus subtilis* RK6 producing thermoactive and alkali-tolerant amylase was isolated from three hot springs located in Munger, Bihar[33]. α-amylase producing *Bacillus licheniformis* was isolated from decaying soil surfaces of potatoes, sweet potatoes and enzyme was characterized (55°C and pH 8)[35]. G*eobacillus* sp. NMS 2 producing thermostable α-amylase was isolated from soil samples of a hot water spring (Nelumwewa of Sri Lanka)[36]. Partially purified amylases produced by *Bacillus* sp. from hot springs of Unkeshwar showed optimum catalytic activity and stability in a broad range of temperature (35-75°C)[14]. Whole genome sequencing of *B. safensis* has been done[37] and *B. safensis* has been isolated from spacecraft[38]. *B. halodurans* has been studied for the amylase production[39,40] and a possibility of different proteins

Table 2 — *16S rRNA gene* identification of α-amylase positive isolates and their amplification status *w.r.t. amyN* gene

Isolates	*16S rRNA* identification	Accession no.	*amyN* gene
RA1	*Bacillus licheniformis*	-	amplified
RA2	*B. licheniformis*	KJ680296	amplified
RA3	*B. licheniformis*	KJ680298	amplified
RA5	*B. licheniformis*	KU057000	amplified
RA6	*B. licheniformis*	-	amplified
RA8	*Exiguobacterium mexicanum*	KJ680297	not amplified
RA11	*B. licheniformis*	KJ680299	amplified
RA12	*B. licheniformis*	KJ748640	amplified
RA13	*B. licheniformis*	KU056999	amplified
RA16	*B. licheniformis*	KU157225	amplified
RA17	*B. licheniformis*	-	amplified
RA19	*B. subtilis*	KU157226	not amplified
TT22	*B. halodurans*	KJ873239	not amplified
TT23	*B. licheniformis*	-	amplified
TT25	*B. licheniformis*	-	amplified
RA31	*B. licheniformis*	KT989882	amplified
RA32	*B.licheniformis*	KJ867517	amplified
RA33	*B. licheniformis*	-	amplified
RA34	*B. licheniformis*	KT989883	amplified
HNC28	*B. stratosphericus*	KJ882999	not amplified
HNC35	*B. licheniformis*	-	amplified
HNC36	*Caldimonas hydrothermale*	-	not amplified
HNC38	*B. licheniformis*	KU157227	amplified
UWF29	*B. safensis*	KJ909969	not amplified
UWF42	*B. licheniformis*	KU133768	amplified

present in *B. halodurans* by using logistic regression and neural network model had been reported. *Exiguobacterium mexicanum* has been identified[41] and our results for *Exiguobacterium mexicanum* (optimum temperature 70°C and pH 8.0) were similar to that reported by[42] who isolated thermostable and alkaliphilic *Exiguobacterium mexicanum* from hot spring of Odisha, India. *Caldimonas hydrothermale* was isolated from thermal spring at Tozeur in southwest Tunisia[43] and reported it to be thermophilic with optimum temperature of 55°C and pH 7.0 for growth, whereas *Caldimonas hydrothermale* isolated by us had optimum temperature of 80°C and pH 8.0. *B. stratosphericus* has only been identified by 16S rRNA gene amplification[44] from air sample collecting tubes. There are no comprehensive studies so far that have assessed these novel microbes for their potential to produce amylase. Hence, we are reporting it for the first time that these isolates have the potential to produce amylase at high temperature and in alkaline conditions.

It was observed that *amyN* amylase gene is present in the *Bacillus licheniformis* isolates only and our results were same as reported[29]. So other genes apart from *amyN* are responsible for the production of α-amylase in other bacterial isolates stated above.

Isolates were screened for optimum temperature for the production of amylase enzyme (Table 3). All the isolates were enzymatically active in temperature range of 50-100°C but maximum activity was observed at 70°C for most of the *Bacillus licheniformis, Exiguo-bacterium mexicanum* (RA8), *B. halodurans* (TT22) and *Bacillus subtilis* (RA19), where as the optimum temperature of 80°C was recorded for *B. strato-sphericus* (HNC28), *B. licheniformis* (HNC35), *Caldimonas hydrothermale* (HNC36), *B. licheniformis* (HNC38), *B. safensis* (UWF29), *B. licheniformis* (UWF42). Isolates were screened for pH optimum for production of enzyme (Table 4). Similarly temperature optima (≥70°C) of the enzyme were reported[27,33,45-47]. There was no growth and activity in the acidic medium therefore data is not mentioned in the table. Growth and activity were observed in media with pH range of 7-10 and maximum activity was achieved in pH 8.0 after which there was a decrease in growth. These results were same as described[33,43,46].

Table 3 — Temperature profile of isolates producing α-amylase

	Relative enzyme activity (%)				
Isolates	50°C	60°C	70°C	80°C	90°C
RA1	46	70	100	86	76
RA2	30	60	100	89	80
RA3	49	62	100	88	87
RA5	64	88	100	93	82
RA6	67	77	100	83	70
RA8	65	76	100	89	77
RA11	20	60	100	67	64
RA12	72	74	100	90	81
RA13	82	88	100	78	79
RC16	80	86	100	89	66
RA17	91	95	100	99	88
RA19	58	74	100	66	48
TT22	75	77	100	82	57
TT23	73	78	100	93	84
TT25	53	90	100	86	88
RA31	82	94	100	99	85
RA32	80	89	100	96	81
RA33	69	83	100	61	58
RA34	60	82	100	95	55
HNC28	17	49	57	100	66
HNC35	19	68	71	100	70
HNC36	15	60	80	100	85
HNC38	29	78	65	100	63
UWF29	38	56	74	100	72
UWF42	15	71	56	100	53

[Each data point represents the mean of three independent experimental assays]

Table 4 — pH profile of isolates producing α-amylase

	Relative enzyme activity (%)			
Isolates	pH7	pH8	pH9	pH10
RA1	84	100	97	79
RA2	88	100	92	66
RA3	95	100	97	89
RA5	83	100	71	50
RA6	79	100	83	69
RA8	85	100	69	61
RA11	80	100	84	69
RA12	80	100	58	47
RA13	95	100	71	63
RC16	91	100	77	74
RA17	89	100	68	65
RA19	95	100	75	61
RA31	73	100	79	65
RA32	81	100	60	57
RC33	95	100	79	71
RA34	81	100	77	71
TT22	91	100	94	91
TT23	90	100	68	68
TT25	94	100	82	76
HNC28	80	100	90	81
HNC35	87	100	90	87
HNC36	80	100	96	88
HNC38	89	100	96	87
UWF29	80	100	94	90
UWF42	80	100	89	85

[Each data point represents the mean of three independent experimental assays]

Genomic DNA was amplified for 16S rRNA gene with expected fragment of 1.5 kb (Fig. 1). The *amyN* amylase gene was amplified with expected fragment of 1.8 kb (Fig. 2). Sequences of 16S rRNA gene were submitted in NCBI database and accession numbers were provided to the sequences (Table 2). Based on the 16S rRNA sequences, multiple sequence alignment was done and the diversity of the sampling site was identified. Evolutionary analyses and phylogenetic trees were constructed in MEGA 6 software[48]. Fig. 3 shows the diversity of the hot spring from where samples were collected. The evolutionary history was deduced by using the maximum likelihood method. The bootstrap consensus tree with 500 replicates was taken to represent the evolutionary history of the taxa analyzed[49]. Initial tree(s) for the heuristic search were obtained by applying neighbour join and BioNJ algorithms to a matrix of pair wise distances estimated using the maximum composite likelihood approach, and then selecting the topology with superior log likelihood value. Codon positions included were $1^{st} + 2^{nd} + 3^{rd}$ + noncoding. All positions with less than 95% site coverage were eliminated. *i.e.* 5% alignment gaps, missing data, and ambiguous bases were allowed at any position.

Phylogenetic analysis of *amyN* gene was also done by multiple alignments of the homologous amylase genes (Fig. 4). The identity of *amyN* gene in terms of percentage is also is represented by blast search on NCBI (Table 5).

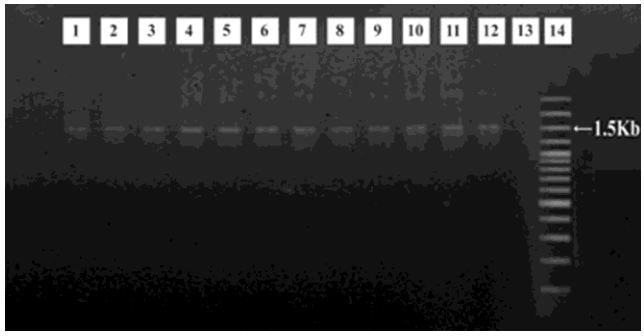

Fig. 1 — *16S rRNA* gene amplification of α-amylase positive isolates. [Lanes 1-3: RA1 isolate; Lanes 4-6: RA2 isolate; Lanes 7-9: RA3 isolate; Lanes 10-12: RA5 isolate; Lane 13: Negative control; and Lane 14: DNA ladder (SM 0321 Fermentas)]

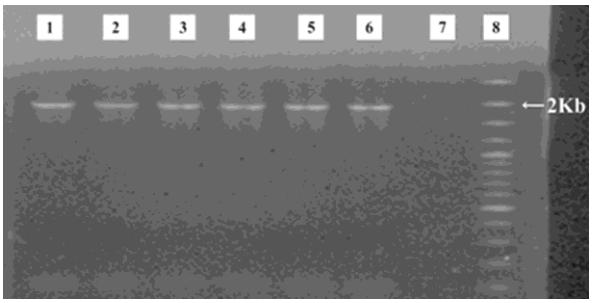

Fig. 2 — *amyN* gene amplification of α-amylase positive isolates. [Lanes 1-3: RA32 isolate; Lanes 4-6: RA33 isolate; Lane 7: Negative control; and Lane 8: DNA ladder 1.8 kb band.

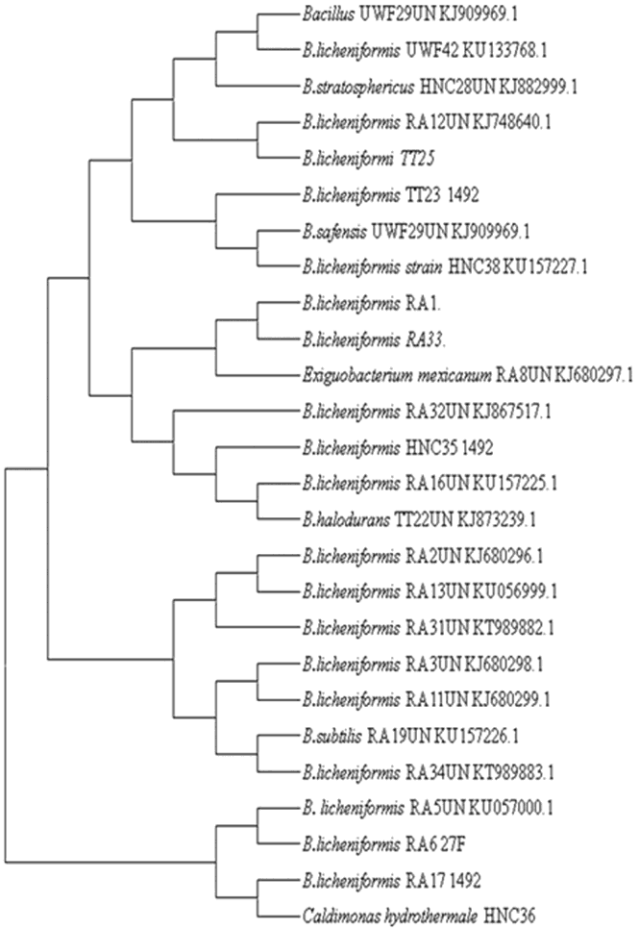

Fig. 3 — Evolutionary analyses (16S rRNA) and phylogenetic representation of diversity of α-amylase producing isolates from hot water spring in Rampur using MEGA6 software.

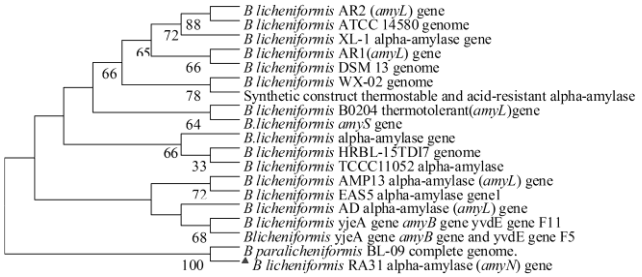

Fig. 4 — Evolutionary analyses and phylogenetic representation of *B. licheniformis* RA31 isolate for (*amyN*) amylase gene using MEGA6 software.

Table 5 — NCBI Blast of *amyN gene* showing 98% identity with amylase *genes*

Description	Max score	Total score	Query cover	E value	Ident.	Accession
Bacillus licheniformis strain AMP13 α-amylase (*amyL*) gene, complete cds	3014	3014	96%	0.0	98%	KC176797.1
B. licheniformis yjeA gene (partial), amyB gene and yvdE gene (partial), strain F11	3014	3014	96%	0.0	98%	AM183788.1
B. licheniformis yjeA gene (partial), amyB gene and yvdE gene (partial), strain F5	3014	3014	96%	0.0	98%	AM183787.1
B. licheniformis WX-02 genome	3003	3003	96%	0.0	98%	CP012110.1
B. licheniformis strain AR1 α-amylase (amyL) gene, complete cds	3003	3003	96%	0.0	98%	KJ508878.1
B. licheniformis strain AR2 α-amylase (amyL) gene, complete cds	3003	3003	96%	0.0	98%	KJ508877.1
B.licheniformis α-amylase gene, complete cds	3003	3003	96%	0.0	98%	M38570.1
B. sp. 1s-1, complete genome	2998	2998	96%	0.0	98%	CP022874.1
B. licheniformis strain BL-010, complete genome	2998	2998	96%	0.0	98%	CP022477.1
B. licheniformis strain SCDB 14, complete genome	2998	2998	96%	0.0	98%	CP014842.1
B. licheniformis strain SCK B11, complete genome	2998	2998	96%	0.0	98%	CP014795.1
Bacillus sp. H15-1, complete genome	2998	2998	96%	0.0	98%	CP018249.1
B. licheniformis yjeA gene (partial), amyB gene and yvdE gene (partial)	2998	2998	96%	0.0	98%	AJ786636.1
B. licheniformis strain SRCM100027, complete genome	2992	2992	96%	0.0	98%	CP021677.1
B. licheniformis strain SRCM100141, complete genome	2992	2992	96%	0.0	98%	CP021669.1
B. licheniformis strain SCCB 37, complete genome	2992	2992	96%	0.0	98%	CP014794.1
B. licheniformis strain BL1202, complete genome	2992	2992	96%	0.0	98%	CP017247.1
B. licheniformis strain HRBL-15TDI7, complete genome	2992	2992	96%	0.0	98%	CP014781.1
B. licheniformis ATCC 14580, complete genome	2992	2992	96%	0.0	98%	CP000002.3
B. licheniformis DSM 13 = ATCC 14580, complete genome	2992	2992	96%	0.0	98%	AE017333.1
B. licheniformis strain ATCC 9789 chromosome, complete genome	2987	2987	96%	0.0	98%	CP023729.1
B. licheniformis strain B0204 thermotolerant α-amylase (amyL) gene, complete cds	2987	2987	96%	0.0	98%	DQ407266.1
B. licheniformis strain AD α-amylase (amyL) gene, complete cds	2981	2981	96%	0.0	98%	KJ508879.1
B.licheniformis amyS gene	2981	2981	96%	0.0	98%	M13256.1
B. licheniformis strain SCDB 34, complete genome	2920	3043	96%	0.0	98%	CP014793.1
B. licheniformis strain EAS5 α-amylase gene, complete cds	2883	2883	92%	0.0	98%	KJ786549.1
Cloning vector pAMY-em1 cat, amyL and ermAM genes	2868	2868	92%	0.0	98%	AJ243541.1
Expression vector pERM-ex1 ermAM and amyL genes	2868	2868	92%	0.0	98%	AJ243540.1
B. licheniformis strain ATCC 27811 α-amylase (amyA) gene, complete cds	2673	2673	86%	0.0	98%	AY630336.1
B. licheniformis strain XL-1 α-amylase gene, complete cds	2667	2667	86%	0.0	98%	KX034161.1
B. licheniformis strain AS08E plasmid pET28a α-amylase gene, complete cds	2667	2667	86%	0.0	98%	KC802019.1
Synthetic construct thermostable and acid-resistant α-amylase precursor, gene, complete cds	2667	2667	86%	0.0	98%	FJ792701.1
B. licheniformis strain DSM 8785 α-amylase (amyL) gene, complete cds	2667	2667	86%	0.0	98%	FJ556804.1
B. sp. 1-15 α-amylase gene, partial cds	2662	2662	86%	0.0	98%	JX090594.1
B. licheniformis thermostable α-amylase precursor, gene, complete cds	2662	2662	86%	0.0	98%	FJ792700.1
B. licheniformis strain TCCC11052 α-amylase gene, partial cds	2662	2662	86%	0.0	98%	EU231641.1
B. licheniformis strain RTS α-amylase gene, partial cds	2662	2662	86%	0.0	98%	EF644410.1
B. licheniformis strain BR1390 α-amylase (amyl) gene, partial cds	2645	2645	86%	0.0	98%	KR912177.1
B. licheniformis strain RH 101 α-amylase gene, complete cds	2639	2639	86%	0.0	97%	DQ517496.1
Uncultured bacterium clone pKSBS-4 putative α-amylase gene, partial cds	2418	2418	80%	0.0	97%	EU236765.1
B. licheniformis strain mk7 amylase A (amyA) gene, partial cds	2381	2381	79%	0.0	97%	KT693278.1

Conclusion

The bacterial diversity of hot water spring was identified by 16S rRNA gene sequencing studies. The phylogenetic tree revealed that the bacterial cells fit into an evolutionary cluster comprising members of *Bacillus licheniformis, B. halodurans, B. stratosphericus, B. safensis, B. subtilis, Exiguobacterium mexicanum*. Based on the above molecular study on *amyN* gene for amylase, it can be concluded that *Bacillus licheniformis* can be exploited fully for the production of amylase whereas the other noble bacterial strains needs the confirmation on the molecular level for other amylase genes.

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Conflict of Interest

The authors declare that there is no conflict of interests.

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