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Full Length Research Paper

Isolation and description of keratinase producing marine actinobacteria from South Indian Coastal Region

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A unique standard starch casein medium has been implemented for the isolation of actinobacteria from the south Indian marine sediments. A combination of techniques, morphological, physiological and biochemical tests provided the evidence for the isolated actinobacteria. All the 56 isolates were inoculated on milk agar and soya meal agar plates for the primary proteolytic screening and the proportional study was made by ANOVA. Among the 56 isolates, nine showed proteolytic activity in terms of making clear zone around their colony on the plates. Then, nine isolates were subjected to the secondary screening on feather broth where three isolates (IS -1, 2 and 18) showed degradation of feather between seven and ten days. The keratinolytic characters of crude enzymes were scrutinized by feather keratin as substrate and the protein concentration was determined. Then, the isolates were identified at molecular level by 16S rRNA gene amplification technique.

Key words: Actinobacteria, keratinase, milk agar, soya meal agar, 16S rRNA gene amplification.

INTRODUCTION

Keratin is an insoluble protein which is resistant to degradation by peptidases, such as trypsin, pepsin and papain (Riffel et al., 2007; Zaghloal et al., 2011). The protein chains are packed tightly either in α- helix (α-Keratin) or in β- sheet (β- Keratins) structures which fold into final 3-dimensional form (Kim, 2007). One of the vital features of keratin is that they possess high mechanical stability disulfide and hydrogen bonds (Korkmaz et al., 2004; Hof et al., 2005). Keratin forms a major component of the epidermis and its appendages via hair, feather, nails, horns, hoofs, scales and wool. Keratins are grouped into hard and soft keratins according to the sulfur content. Hard keratins are found in appendages; whereas, soft keratins like skin and callus have low content of disulphide bond (Scrooyen et al., 2001). Keratinases [EC 3.4.21/24/99.11] are large serine or metallo proteases capable of degrading the structure forming keratinous proteins (Ramani et al., 2005). Most of the purified known keratinases cannot completely solubilize native keratin (Gupta and Ramani, 2006). The applications are feather elimination, biodegradable films, glues and foils, cosmetics, leather industry and nitrogenous fertilizer for plants (Detoni et al., 2002; Gousteroa et al., 2005).

A number of keratinolytic microorganisms have been reported. Keratinolytic enzymes are produced by fungi, actinobacteria and bacteria which have been frequently isolated from soils where keratinous materials are deposited. Generally, an increase in keratinolytic activity is associated with thermophilic organisms, which allows decomposition of keratin wastes (Nam et al., 2002). Compared to other soluble proteins, keratin has high stability for the degradation by proteolytic enzymes such as pepsin, trypsin and papain (Xie et al., 2010). In the first report, actinobacteria strains from antartica were able to grow on keratin containing wastes for producing

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keratinolytic enzymes (Gushterova et al., 2005).

With this background, this study was designed to identify feather degrading actinobacteria among the 56 isolates and to assess the keratinase production.

MATERIALS AND METHODS

Sample collection

The actinobacterial isolates were recovered from marine soil sample collected from South Indian coastal region, Tamilnadu, India. A volume of one gram soil sample from each different collection area was vigorously shaking in 10 ml of sterile distilled water for 30 min on a shaker. Serial 1 in 10 dilutions were then made down to 10^{-8}. All the dilutions were plated on starch casein agar (SCA-ISP 4) medium for the actinobacteria isolation at room temperature for 7 days. The isolates were identified by various parameters such as colony morphology, physiological, biochemical and microscopic characterization (Lachevalier, 1989).

Maintenance of suspected actinobacterial isolates

Suspected actinobacterial isolates were maintained on ISP 4 medium. Inoculation of suspected actinobacterial isolates were done on solid medium surface and the plates were incubated at room temperature for 7 days.

Primary screening of keratinolytic actinobacteria

Milk agar and soya meal agar medium were used for the primary screening of keratinolytic actinobacteria (Riffel and Brandelli, 2006). After sterilization, the media were poured into sterile Petri dishes. Suspected actinobacterial isolates were inoculated and incubated at room temperature for 5 days and the plates were examined for clear zone formation on the agar plates.

Secondary screening of keratinolytic actinobacteria

All positive isolates obtained from the primary screening were subjected to secondary screening with the intention to isolate the feather degrading actinobacteria. Modified basal liquid medium ($MgSO_4.7H_2O$ 0.2 g/l; K_2HPO_4 0.3 g/l; KH_2PO_4 0.4 g/l; $CaCl_2$ 0.22 g/l) was supplemented with raw chicken feather and was used for the secondary screening. Whole fresh raw chicken feathers were collected from poultry farm and they were washed with tap water and distilled water to remove the blood strains, and they were dried in room temperature. 20 ml of modified basal liquid medium was taken in each boiling tubes and one cleaned, dried medium size chicken feather was added to each boiling tubes. Selected isolates were chosen based on their zone forming capability on the milk and soya meal agar media. The boiling tubes were incubated at room temperature and the tubes were examined weekly for two weeks and two days (Saber et al., 2010; Mona, 2008).

Determination of keratinolytic activity

Keratinase activity was determined by the method of Thanaa et al. (2011). The keratinase activity was expressed as one unit of the enzyme corresponding to an increase in the absorbance value of 0.01 h^{-1}.

Determination of protein concentration

The crude enzyme from each feather culture was used for the measurement of soluble protein using Lowry et al. (1951) method (bovine serum albumin as standard).

16s rRNA amplification and sequencing

The genetic level identification of potential actinobacteria were carried out. Genomic DNA was extracted according to the standard procedure. The 16s rRNA of the strains were amplified using the 16s rRNA primers, fD1 (5'-GAGTTTGATCCTGGCTCAG-3') and Rp2 (5'-ACGGCTACCTTGTTACGACTT-3'). PCR products were purified using QIAquick PCR purification kit. The purified DNA fragments were sequenced using sequencer model ABI 3100 sequencer according to the protocol provided by the manufacturer (ABI PRISM 3100 Genetic Analyzer User's Manual).The conservative part (16s rRNA) of three actinobacteria were aligned manually with available nucleotide sequences retrieved from the GenBank and the RDP using CLUSTRAL W (Maidak et al., 2000).

Phylogenetic analysis

16s rRNA sequence analysis was performed with the aid of molecular evolutionary genetic analysis (MEGA) by using 4 software package neighbour-joining method (Saitou and Nei, 1987). For the phylogenetic trees, only sequences from the type strains whose names have been validly published were taken into account.

RESULTS

Screening of keratinolytic bacteria

All the 56 isolates were subjected for primary screening on milk and soya meal agar plates and among the 56 isolates, IS-1, 2, 8, 18, 26, 34, 39, 46 and 51 were formed as the clear zone, which supported the degradation and utilization of casein (milk agar) and soya (soya meal) by the respective isolates. The zone diameters of the plates were clearly mentioned and the comparative study results were made by ANOVA statistical analysis (Table 1 and 2). Secondary screening was done to find out the feather degrading actinobacteria among the positive isolates and IS-1, IS-2, 8 and 18 were able to degrade the feather among the nine isolates selected through primary screening. All these four, IS-1, 2, 8, and 18 were found to degrade whole chicken feather in modified basal liquid medium after 7 to 10 days of incubation period (Figure 1).

Keratinolytic activity

Keratinolytic activities of the isolates were monitored during growth in feather meal broth. Keratinolytic activity was measured in the absorbance at 280 nm by the

Table 1. Primary screening of keratinolytic actinobacteria.

Isolate	Zone of Inhibition (Cm)	
	Milk agar medium	Soya meal agar
IS 1	8.0	7.8
IS 2	8.6	8.5
IS 8	7.5	7.2
IS 18	7.8	7.0
IS 26	7.0	6.7
IS 34	5.6	5.3
IS 39	4.8	4.5
IS 46	4.2	4.0
IS 51	3.0	3.1

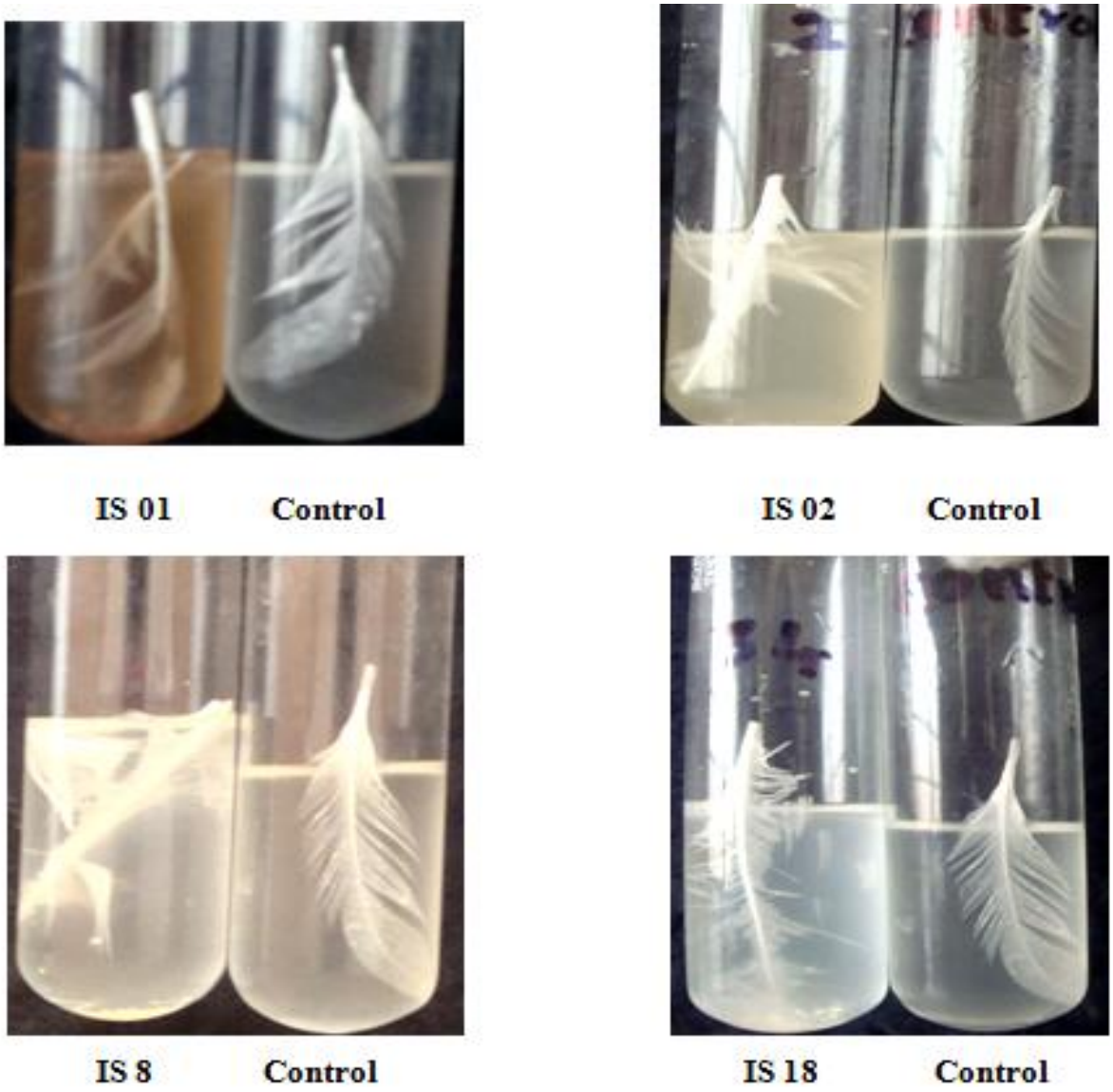

Figure 1. Feather degradation by actinobacteria.

Table 2. Comparative study results of proteolytic hydrolysis of actinobacteria on milk and soya meal agar in ANOVA analysis.

ANOVA: Two factor without replication	A	0.05	Average	Variance
Isolate	**Count**	**Sum**		
IS 1	2	15.8	7.9	0.02
IS 2	2	17.1	8.55	0.005
IS 8	2	10.8	5.4	0.08
IS 18	2	14.8	7.4	0.32
IS 26	2	13.7	6.85	0.045
IS 34	2	14.7	7.35	0.045
IS 39	2	9.3	4.65	0.045
IS 46	2	8.2	4.1	0.02
IS 51	2	6.1	3.05	0.005
Milk agar medium	9	56.5	6.277778	3.799444
Soya meal agar medium	9	54	6	3.465

ANOVA

Source of variation	SS	Df	MS	F	P-Value	F crit
Rows	57.87778	8	7.234722	243.4112	0.000	3.438101
Columns	0.347222	1	0.347222	11.68224	0.009	5.317655
Error	0.237778	8	0.029722			
Total	58.46278	17				

Reject null hypothesis because $p < 0.05$ (means are different); both row and column.

Table 3. Keratinolytic property of the isolates.

Isolate	Enzyme activity (U/ml)	Protein concentration (µg)
1	53.92	39.38
2	71.43	27.46
8	31.74	44.88
18	46.71	40.03

standard enzyme method. The keratinolytic activity of the isolates 1, 2, 8 and 18 was determined by one unit of the enzyme corresponding to an increase in the absorbance value of 0.01 h^{-1}.

The enzyme activities of the isolates and the protein concentration are shown in Table 3. Based on the results, IS 1, 2 and 18 were selected for further studies. The morphological and biochemical characterization of these three isolates were carried out and tabulated in Table 4.

These three isolates were full-fledged on ISP 7 medium where they showed white colonies (Plate 1) and they were observed under phase contrast microscope by cover slip method. All the cases substrate and aerial mycelium were observed clearly along with spores (Figure 2).

According to investigated results on the cultural, morphological and biochemical characteristics of the strains, strains 1, 2 and 18 were preliminarily classified to be Streptomyces genus. The analysis of the 16S rRNA gene is a more important tool for correct identification of microbial species; the 16S rRNA gene of strains 1, 2 and 18 were sequenced (waiting for Genbank accession no). The phylogenetic tree, which was constructed by neighbour joining method for comparison of the 16S r RNA gene sequences, indicated that strain 1, 2 and 18 belonged to the genus Streptomyces. The levels of similarity between the 16S r RNA gene of the Streptomyces species are shown in Figure 3. The National Centre for Biotechnological Information (NCBI) and European Molecular Biological Laboratory (EMBL) sequences data shows that isolate 1 (*Streptomyces acrimycini* NGP), isolate 2 (*Streptomyces albogriseolus* NGP) and isolate 18 (*Streptomyces variabilis* NGP) had 63, 100 and 56% similarity with EU570515, FJ775010

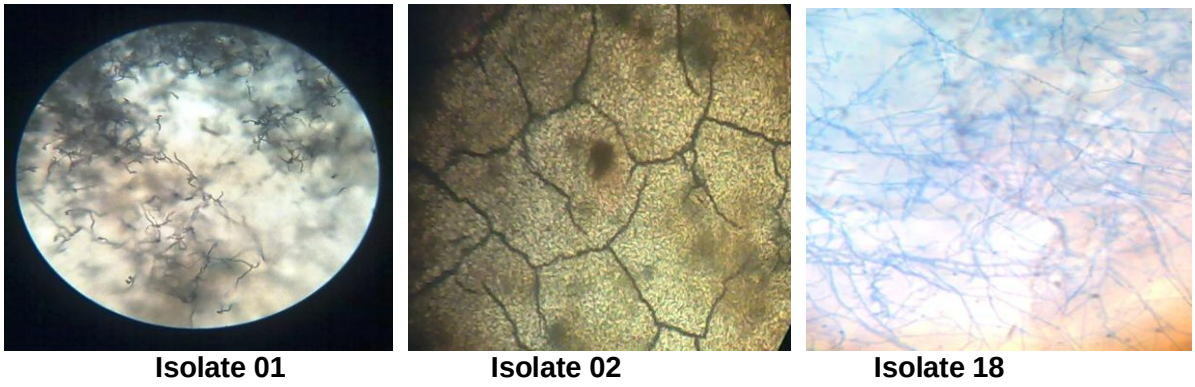

Figure 2. Microscopic characterization of the actinobacteria isolates.

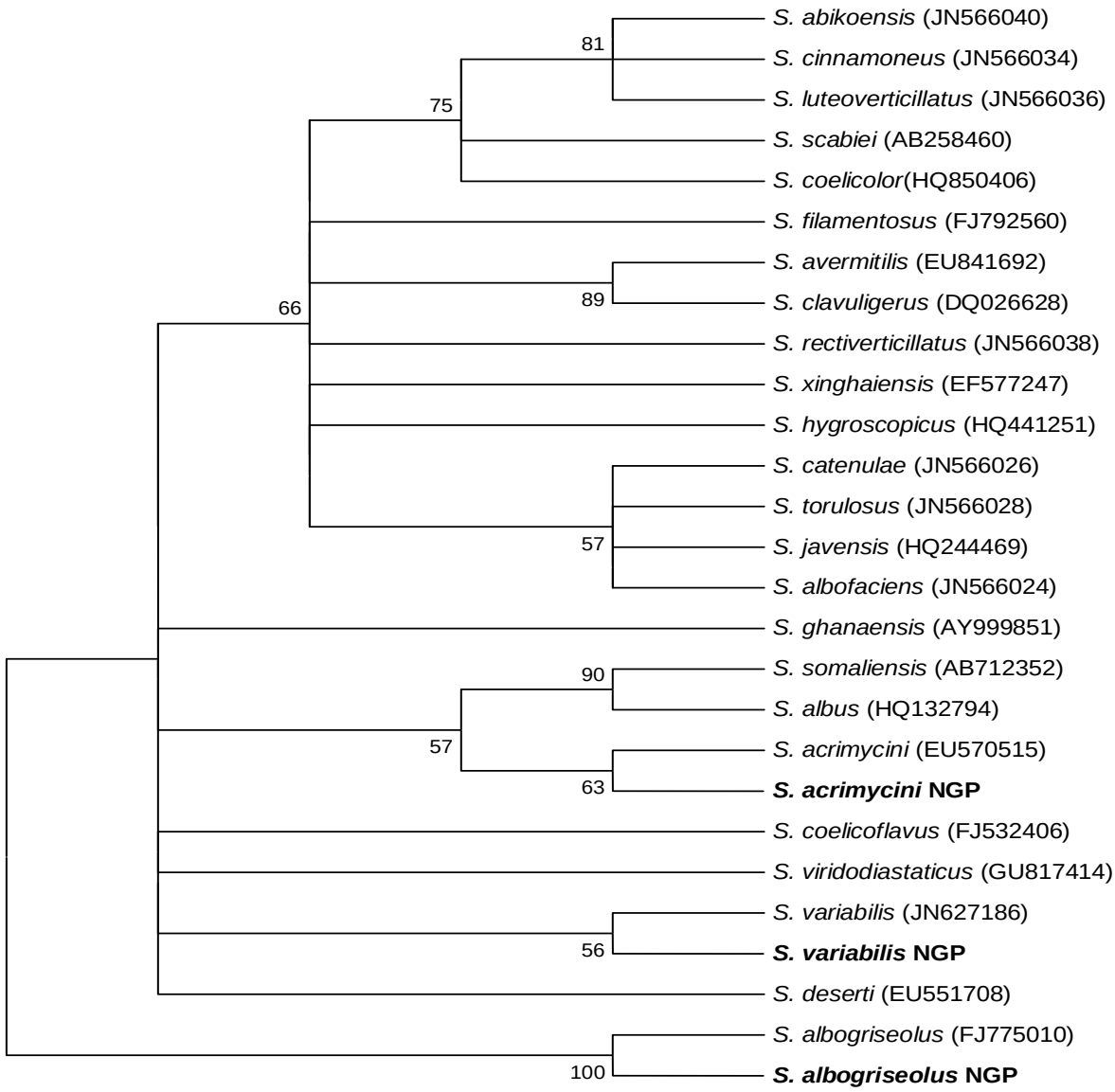

Figure 3. Neighbour joining phylogenetic tree based 16S rRNA gene sequences.

Plate 1. Isolated actinobacteria on SCA plates.

and JN627186.

DISCUSSION

Sums of 56 actinobacteria were isolated from South Indian Coastal Region. Among the 56, three were presented feather-degrading properties. Feather degradation by bacteria has been widely described for Bacillus spp., which appears to be ubiquitous bacteria in poultry waste and plumage of birds (Thys et al., 2004; Burtt and Ichida, 1999). Totally, 22 actinobacteria were isolated from feather, hair and poultry farm soil that owned keratinolytic activity and ability to degrade keratin wastes (Saha and Dhanasekaran, 2010). Other author discussed the microorganism isolation, growth condition and keratinolytic activity of the *Bacillus* spp., isolated from poultry processing plant wastewater JSC (Matikeviciene et al., 2009).

Preliminary screening test indicates that isolates 1, 2, 8, 18, 26, 34, 39, 46 and 51were capable to degrade casein and soya, which confirmed their proteolytic nature. Comparative study (ANOVA) results show that, milk agar medium was the best for the primary screening of keratinase. IS 1, 2, 8 and 18 grown on medium containing whole raw feather, could utilize feather as a unique carbon and nitrogen source. Plate assays were attempted on keratin powder plates by *Nocardiopsis* sp.TOA-1 where they formed extremely large ones on the plate (Mitsuiki et al., 2002). Milk agar medium was used for the primary screening of actinobacteria (Riffel and Brandelli, 2006). Another author discussed the primary screening of keratinolytic actinobacteria isolated from keratin waste dumped soil in Thiruchirapalli and Namakkal, Tamilnadu, India using milk agar medium (Saha and Dhanasekaran, 2010).

At last three isolates (1, 2 and 18) were reported the best keratinase producing isolates by performing the keratinase assay using chicken feather as substrate. Among them, isolate 2 will be used for the future applications. Keratinase producing active actinobacterial isolates were screened quantitatively in a basal salt medium containing feather as sole carbon and nitrogen sources (Kawato et al., 1959). The modified method for the determination of keratinase activity was carried out for *Alternaria tenussima* and *Aspergillus nidulans* isolated from poultry wastes (Aneja, 2003). On the other hand, the keratinolytic activity was assayed by incubating the *Bacillus licheniformis* enzyme solution in keratin solution (Vigneshwaran et al., 2010).

Biochemical and microscopic characterization studies were performed for the three isolates. Sangali and Brandelli (2000) performed biochemical studies for the identification of *Vibrio* sp, strain kr 2. The author characterized 47 actinobacteria based on morphological, physiological and biochemical studies (Arifuzzaman et al., 2010). Besides, unique technique such as coverslip method on solid medium enables to observe the actinobacteria. Observations on living colonies of *S.coelicolor* strain (A3) growing on cellophane films were made by phase contract microscopy (Donlan, 1983). The biochemical, physiological and morphological properties of the test strains are dependable with its assignment to the genus Streptomyces (Table 4). It is also clear that the organism forms a distinct phylogenetic line in the 16S rRNA which tree belongs to the group of Streptomyces (Kuznetsov et al., 1977; Lapage et al., 1976). The strains were further identified as *Streptomyces acrimycini* NGP, *Streptomyces albogriseolus* NGP and *Streptomyces variabilis*. The strain *S. albogriseolus* NGP will be used for further applications.

According to the results, it is promising to state that, the keratinase producing actinobacteria could be used in medical and industrial applications.

Table 4. Characterization of morphological, physiological and biochemical tests.

Isolate	IS-1	IS-2	IS-18
Morphological characteristics			
Colony colour	W	G	W
Colony shape circular entire margins	+	-	-
Rhizoid / filamentous margins	-	+	+
Physiological characteristics growth at			
25°C	+	+	+
37°C	++	++	++
45°C	+	+	+
55°C	-	-	-
Growth in presence of NaCl			
25 %	+	+	+
5%	+	+	++
10%	++	++	-
Biochemical characteristics			
Gram staining	+	+	+
Endosperm staining	C	R	R
In dole test	-	-	-
Methyl red test	-	-	+
Voges-Proskauer test	-	+	-
Caesinase test	-	+	-
Cellulase test	+	+	+
Deaminase test	-	-	-
Sugar fermentation	+	+	+
Nitrate reduction test	+	+	+
Geluting test	-	-	+

W, White; G, grey; +, normal growth; ++, good growth; -, no growth; C, cocci; R, rod.

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