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Purification and Characterization of chitinase from Thermophilic *Staphylococcus* sp.

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ABSTRACT

The objective of the research was to study the purification and partial characterization of thermophilic chitinase from the newly isolated *Staphylococcus* sp. The enzyme was purified. The enzyme was of 66 kDa as was evident by native PAGE. The protein was showing activity at pH 7 and 9. The optimum temperature activity was 60°C. Influence of metal ions such as calcium chloride ($CaCl_2$), Zinc sulfate ($ZnSO_4$), Magnesium sulfate ($MgSO_4$), Manganese sulfate ($MnSO_4$) was observed. Substrate specificity of the enzyme was also studied. Phylogenetic tree of the producer organism was also done.

Keywords:-Chitinase, Thermophilic, Purification, Metal ions, 16S rDNA sequencing

1. Introduction

Chitin, a β (1-4) polymer of N-acetyl –D-glucosamine (GlcNAc) is a major structural component of most of the biological systems such as mollusks, insects, crustaceans, fungi, algae and marine invertebrates (Bhushan, 2000). Chitin and its derivatives are of commercial and biotechnological interest.Chitinase are a group of enzymes that decompose chitin, the second most abundant polymer in nature and the most abundant in the marine environment (Alexander, 1977). Chitinases break down glycosidic bonds in chitin (Jollès and Muzzarelli, 1999). Chitinases breakdown chitin into a variety of products that include the deacylated oligomer chitosan $(GlcNAc)_n$, the disaccharide chitobiose $(glcNAc)_2$ and the monomer N-acetylglucosamine (Nawani et al. 2002).The seafood industry is a major source of chitinous wastes. The recycling of which is extremely important to retain the carbon nitrogen balance in the ecosystem (Fereidoon et al**.** 1999**)**. Chitin is a source of carbon and nitrogen. For that reason chitin was used as a sole source of carbon and nitrogen in the media to isolate chitinase-producing organisms. Chitinase producing organisms break chitin and use as a carbon and nitrogen source for their growth. Chitin is easily obtained from crab or shrimp shell and fungal mycelia. In the first case, chitin production is associated with food industries such as shrimp canning (Watanabe et al**.** 1994**).**

Chitin, a Greek word for envelope was discovered in 1811, as a substance occurring in mushrooms. It is the second most abundant natural biopolymer on Earth after cellulose, production 10^{10} to 10^{11} tons per annum. The omnipresence of chitin in the environment makes it important to understand the role and the underlying metabolic processes of chitin turnover in the environment. Chitin and its derived compounds have become of great interest not only as an under- utilized bio-resource, but also as a new functional material of high potential in various fields (Hobel Cedric , 2004). Phylogenetic origin of most of the microbial

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chitinases from family GH18 among *Bacteria* and *Archaea*, listed as such in GenBank (modified from Henrissat) (Henrissat, 1999).

Table 1: Enzyme class order

Organism	Class or Order	Enzyme
Archaea		
Halobacterium sp. NRC-1	*Halobacteriales*	Chitinase
Thermococcus kodakaraensis	*Thermococcales*	Multidomain chitinase
Pyrococcus furiosus	*Thermococcales*	Multidomain chitinase
Thermococcus chitonophagus	*Thermococcales*	Multidomain chitinase
Bacteria		
Aeromonas caviae	*γ-Proteobacteria*	Chitinase A
Aeromonas sp 10S-24	*γ-Proteobacteria*	Chitinase 1
Alteromonas sp.	*γ-Proteobacteria*	Chitinase A
Arthrobacter sp.	*Actinobacteria*	Chitinase A
Bacillus circulans	*Firmicutes-Gram Positives*	Chitinase A1
Bacillus licheniformis	*Firmicutes-Gram Positives*	Chitinase
Bacillus thurigiensis	*Firmicutes-Gram Positives*	Chitinase
Clostridium paraputrificum	*Firmicutes-Gram Positives*	Chitinase B
Clostridium thermocellum	*Firmicutes-Gram Positives*	Chitinase
Enterobacter agglomerans	*γ-Proteobacteria*	Chitinase A
Flavobacterium meningosepticum	*Flavobacteriales*	Endo-N-acetylglucosaminidase F1
Flavobacterium sp.	*Flavobacteriales*	Endo-N-acetylglucosaminidase F1
Janthinobacterium lividum	*β-Proteobacteria*	Chitinase
Kurthia zopfii	*Firmicutes-Gram Positives*	Chitinase
Micobulbifer degradans	*γ-Proteobacteria*	Chitinase B
Pseudomonas sp. PE2	*γ-Proteobacteria*	Chitinase A
Ralstonia sp.A-471	*β-Proteobacteria*	Chitinase A
Rhodothermus marinus	*Sphingobacteria*	Family 18 chitinase
Serratia liquefaciens	*γ-Proteobacteria*	Chitinase B
Serratia marcescens	*γ-Proteobacteria*	Chitinase B
Stenotrophomonas maltophilia	*γ-Proteobacteria*	Chitinase A
Streptomyces coelicolor	*Actinobacteria*	Chitinase
Streptomyces erythraeus	*Actinobacteria*	Chitinase C
Streptomyces olivaceoviridis	*Actinobacteria*	Exo-chitinase
Streptomyces plicatus	*Actinobacteria*	Chitinase-63
Streptomyces thermoviolaceus	*Actinobacteria*	Chitinase
Vibrio fumissii	*γ-Proteobacteria*	Chitodextrinase
Vibrio harveyi	*γ-Proteobacteria*	Chitinase A

This article aims at the isolation and characterization of a thermostable chitinase isolated from a *Streptococcus* sp. of which the phylogenetic tree has also been detected.

2. Materials and methods

2.1 Growth and isolation of microorganisms

Diluted soil suspensions was allowed to mix aseptically with a sterile medium containing chitin and distilled water and kept it for 48 hours at 60ºC temperature. Only those which can utilize chitin as the carbon and nitrogen source and thermophiles will survive. After 48 hrs loop full of the media were taken from each of the conical flask and were stained following Lacto phenol-cotton blue staining. Loop full of the media that showed some existence of microorganisms on slides were streaked on Nutrient Agar plates and were allowed to grow at 60ºC temperature to observe the growth. The colonies came out after long period. Culture was maintained and grown on a modified media (Jollès and Muzzarelli, 1999) at 60ºC. Culture was grown also in aerobic and anaerobic condition. Culture was grown in different temperature and the cell numbers were counted by using Hemocytometer.

2.2 Enzyme Assay

The chitinase enzyme was estimated by three different methods. At first dimethyl aminobenzaldehyde (DMAB) was used for the estimation of chitinase activity (Bansode Vijay and Bajekal Shyam, 2006), next dinitrosalicylic acid (DNS) for the estimation of reducing sugar (Roberts and Selitrennikoff, 1988), which was produced by chitinase from chitin. Lastly ρ-Nitrophenyl-N- Acetyl –β-D-glucosamine (ρ-NNA β-D-G) was used as the substrate of chitinase (Roberts and Selitrennikoff, 1988).

3. Chitinase purification

The chitinase was purified by ammonium sulfate 20% to gradual increase up to 50% saturation using Dowex -50 and Sephadex G-200 chromatography. After ammonium sulfate saturation, protein precipitate was centrifuged at 10,000 x g for 15 min. The pellet was then dissolved in sterile buffer and dialyzed against 0.01 M dipotassium hydrogen phosphate buffer, pH-7, for over night at room temperature. Then dialyzed material was collected and passed through the ion exchange column. In this case Dowex 50 was used as an ion exchanger. Then passed solutions were collected and every fraction was estimated for the protein content and the enzyme activity. Then the fractions, which gave the activity, were pulled and were passed through Sephadex G-200 column equilibrated with buffer. Elute was collected and was assayed for chitinase activity. Pooled elute having chitinase activity was dialyzed and lyophilized. After lyophilization it was observed that sample lost activity. Therefore the following alternative method was followed.

3.1 Alternative method for purification

At first the cultures were centrifuged and the supernatant was collected in a sterile conical flask. Then the conical flask was placed at 60ºC and the solutions were allowed to dry. Then the dried material was collected in sterile eppendorf tube. The dried protein was dissolved in buffer (1 mg/ml). The protein content was assayed and also the enzyme activity. Then the solution was allowed to run through the polyacrylamide gel using electrophoresis for purity testing of the protein.

4. Gel electrophoresis

The purity was checked by native PAGE and SDS-PAGE. Polyacrylamide gels were prepared by the method of Laemmli (1970). Gel was stained with coommassie Brilliant Blue R-250. The SDS-PAGE molecular weight markers of Sigma were Bovine serum albumin (66,000) and albumin egg (45,000).

4.1 Protein estimation

Protein was estimated according to the method of Lowry taking crystalline bovine serum albumin as the standard (Lowry et al. 1951).

4.2 Effects of metal ions and pH on chitinase activity

To determine the effect of chitinase activity, enzyme was preincubated with final concentration 2 mM for different metal ions such as $CaCl_2$, $ZnSO_4$, $MgSO_4$, $MnSO_4$, EDTA.

The activity was measured by dinitrosalicylic acid (DNS) method and pH optimum was observed (Koga, et al. 1999).

4.3 Kinetic study of purified enzyme

K_m and V_{max} of purified enzyme was done using different amount of substrate. Chitinase activity was determined by measuring the rate of hydrolysis of ρ-Nitrophenyl-N- Acetyl –β-D-glucosamine (ρ-NNA β-D-G) in phosphate buffer (pH-6.5, 50 mM)

4.4 Determination of K_m and V_{max}

A Lineweaver-Burk curve was drawn by plotting 1/ [substrate] (μM^{-1}) on the X-axis and 1/ [Sp.activity] (ΔO.D/mg pr/h) on the Y-axis.

5. Results and discussion

From the figure 1 it was clear that the enzyme activity was increased in two phases. Two phases were found for peak in activity – one after second day and another after fourth day.

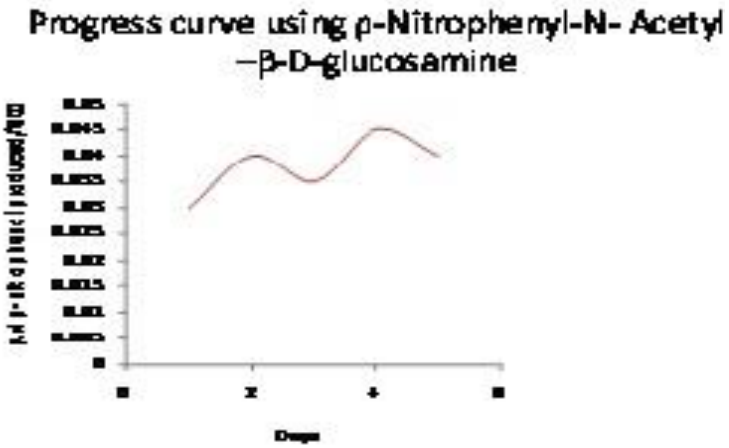


Figure 1: Progress curve using ρ-Nitrophenyl-N- Acetyl –β-D-glucosamine

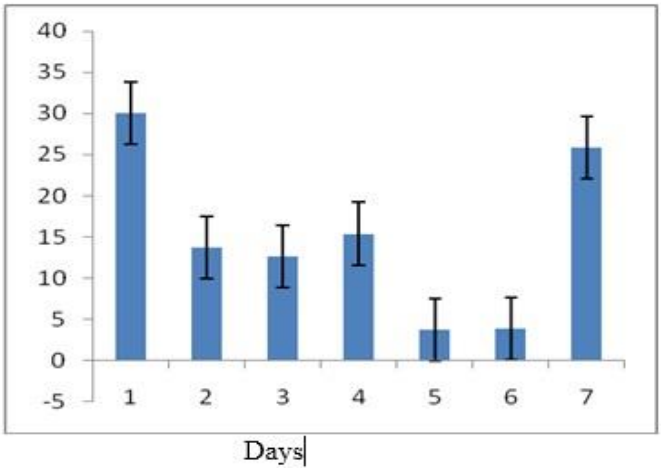


Figure 2: A specific activity (µg of NAG produced/mL/min/mg of protein) was measured in supernatant of culture media.

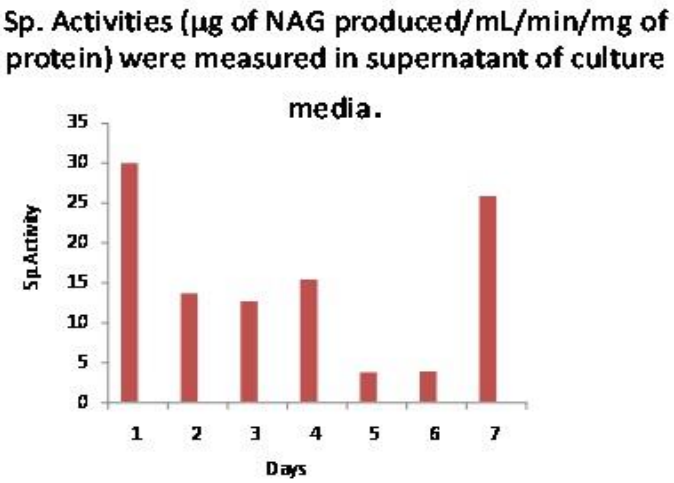


Figure 3: Sp. Activities (µg of NAG produced/mL/min/mg of protein) were measured in supernatant of culture media.

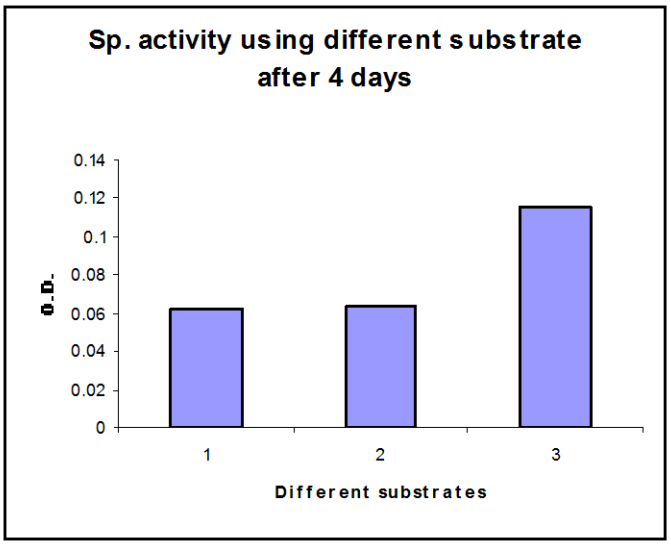


Figure 4: Specific activity using different substrate after 4 days

5.1 Substrate specificity

The result of the studies on substrate specificity of the enzyme from both the crude extract and post dialysis solution upon three different substrates: Dimethyl aminobenzaldehyde, dinitrosalicylic acid and ρ-Nitrophenyl-N- Acetyl –β-D-glucosamine respectively was showed in the Figure 4. The enzyme activity typically behaves on different substrates.

5.2 Thermo stability and pH stability

The enzyme remained stable at a temperature range 30°C to 80°C. above which the stability rapidly declined. The maximum activity was displayed at 60°C graph. The effect of pH on chitinase activity was characterized by stability from pH 5.0 to 10 after 72 h of incubation at 60°C -the optimum pH activities were at pH 7.0.

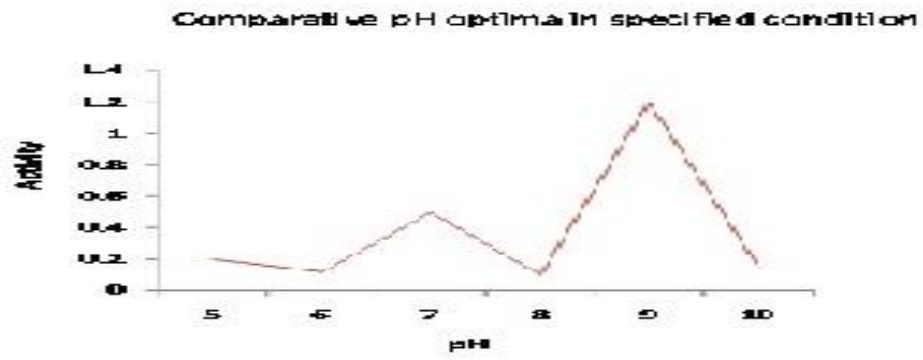


Figure 5: Comparative pH optima in specified condition

At pH 7 there was a peak which was of lower activity and the peak at pH 9 was of higher activity.

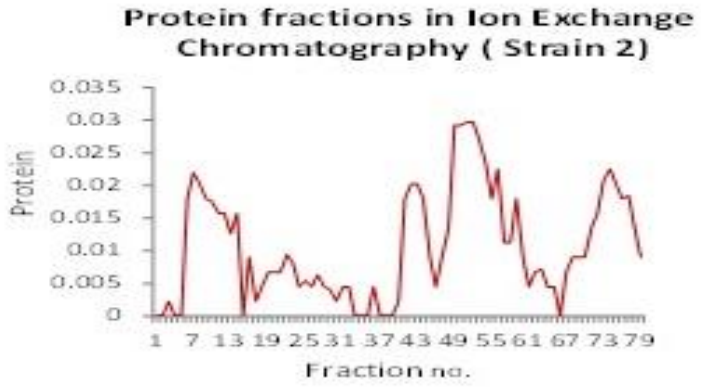
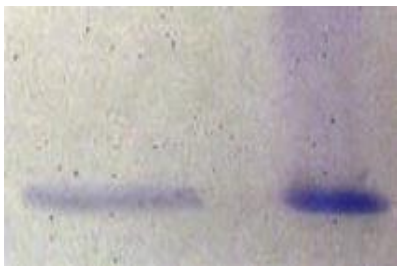


Figure 6: Protein fractions in Ion Exchange Chromatography (Strain 2)

Three peaks were available in ion exchange chromatography and that gave an idea of three protein subunits in the chitinase.



Isolated chitinase BSA

Figure 7: Native PAGE stained with coommassie blue

Native PAGE proved that the enzyme was purified properly giving a single band.

Figure 8: SDS-PAGE stained with Coommassie Blue

Three bands were observed in SDS-PAGE stained with Coommassie blue.

Figure 9: Silver Staining of SDS-PAGE

Three subunits in strain 2 chitinase as was observed by SDS- PAGE were corroborating three peaks for protein from strain 2 in ion exchange chromatography. Only one extracellular protein secreted in the medium having 66 kDa as was revealed from native PAGE using BSA as marker. Chitinase was of three subunits, all three subunits were found to have chitinase activity. Phylogenetic tree of the isolated organism was prepared after isolation of genomic DNA, sequencing of 1320 bp, using BLAST and the result was as follows. Tree had shown that our organism *Staphylococcus* sp.danc2 was closest to *Staphylococcus epidermidis*. Accession No:- BankIt1448813 Seq1 JF827032.

ACGAGGAGCTTGCTCCTCTGACGTTAGCGGCGGACGGGTGAGTAACACGTGG
ATAACCTACCTATAAGACTGGGATAACTTCGGGAAACCGGAGCTAATACCGGAT
AATATATTGAACCGCATGGTTCAATAGTGAAAGACGGTTTTGCTGTCACTTATAG
ATGGATCCGCGCCGCATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCAAC
GATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGAACTGAGACACGG
TCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGGCGAAAGC
CTGACGGAGCAACGCCGCGTGAGTGATGAAGGTCTTCGGATCGTAAAACTCTG
TTATTAGGGAAGAACAAATGTGTAAGTAACTATGCACGTCTTGACGGTACCTAAT
CAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCA
AGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGTAGGCGGTTTTTTAAGTCT
GATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGAAAACTT
GAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGCAGAGA
TATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACGCTGA
TGTGCGAAAGCGTGGGGATCAAACAGGATTAGATACCCTGGTAGTCCAC

Figure 10: The forward sequence

Figure 11: The reverse sequence

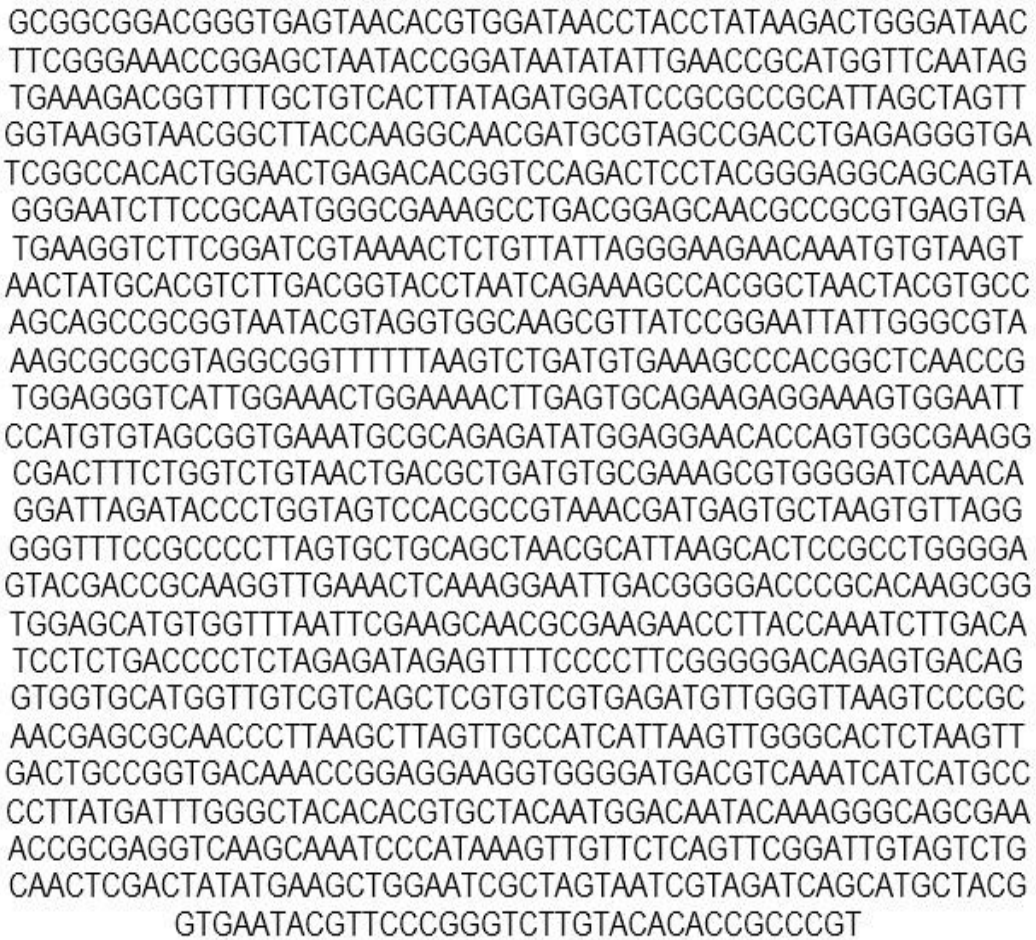


Figure 12: The complete sequence of the 16S rRNA

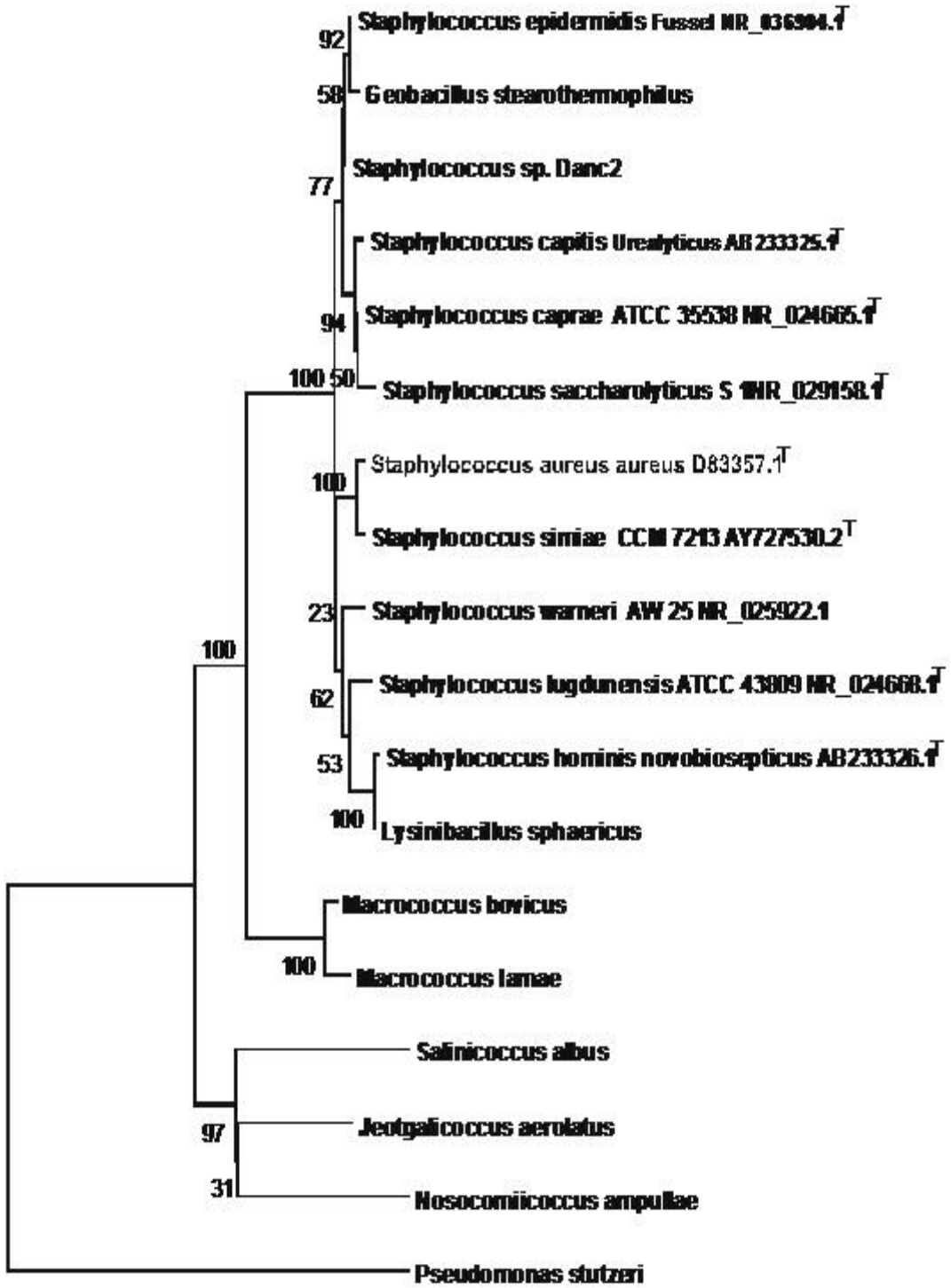


Figure 13: The phylogenetic tree

The 16S rRNA sequence of strain 2 was assigned to the taxonomical hierarchy proposed in Bergey's Manual of Systematic bacteriology. The result showed that strain 2 is assigned to genus *Staphylococcus*. Therefore, Strain2 was attributed to genus *Staphylococcus.*

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