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ORIGINAL PAPER

16S rRNA gene sequences analysis of *Ficus elastica* rubber latex degrading thermophilic *Bacillus* strain ASU7 isolated from Egypt

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Abstract A thermophilic *Bacillus* strain ASU7 was isolated from soil sample collected from Assiut governorate in Upper Egypt on latex rubber-containing medium at 45 °C. Genetically, the 16S bacterial ribosomal RNA gene of the strain ASU7 was amplified by the polymerase chain reaction (PCR) and sequenced. The sequence of the PCR product was compared with known 16S rRNA gene sequences in the GenBank database. Based on phylogenetic analyses, strain ASU7 was identified as *Bacillus amyloliquefaciens*. The strain was able to utilize *Ficus elastica* rubber latex as a sole source for carbon and energy. The ability for degradation was determined by measuring the increase in protein content of bacterium (mg/g dry wt), reduction in molecular weight (g/mol), and inherent viscosity (dl/g) of the latex. Moreover, the degradation was also confirmed by observing the growth of bacterium and formation of aldehyde or keto group using scanning electron microscopy (SEM) and shiff's reagent, respectively.

Keywords 16S rRNA gene sequencing · Phylogenetic analysis · Natural rubber · Biodegradation · Parameters of degradation · Thermophilic *Bacillus*

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Introduction

More than 12,000 plant species yield latex containing rubber; the rubber-producing plant families are Apocynaceae, Asteracceae, Asclepiadaceae, Euphorbiaceae, Loranthaceae, Moraceae, and Sapotaceae (Archer and Audley 1973). Rubber fig or rubber tree (*Ficus elastica*) is a member of family Moraceae, Mulberry family (Neal 1948). It is a large tree in the banyan group of figs, growing to 30–40 m (rarely up to 60 m) tall, with a shout trunk up to 2-m diameter. It yields milky white latex also known as sap, which has been used in some cases to make rubber. Fresh latex contains a minimum of 90 % rubber hydrocarbon together with small amounts of proteins, resins, fatty acids, sugars, and minerals (Zyska 1981). The latex can be fractionated into three distinct zones by ultracentrifugation. The top fraction consists almost entirely of rubber, the middle fraction is the metabolic active aqueous phase of latex called C-serum, and the relatively heavy bottom fraction consists mainly of lutoids.

The rubber molecule is a high-molecular weight polymer consisting of isoprene units (C_5H_8) in the *cis*-configuration. It has been widely applied to many products, such as tires, gloves, balloons and balls for sports. According to previous reports, natural rubber

(NR)-degrading bacteria mostly belong to the group of *Actinomycetes* (Heisey and Papadatos 1995; Jendrossek et al. 1997; Linos et al. 1999; Rifaat and Yosery 2004; Banh et al. 2005). Recently, certain thermophilic bacteria were also reported to be rubber-degrading (Ibrahim et al. 2006). Degradation of NR latex by two Gram-negative bacteria viz. *Xanthomonas* spp. (Tsuchi and Takeda 1990) and *Pseudomonas aeruginosa* (Linos et al. 2000b) was also reported in previous works. However, there are only two reports on Gram-positive *Bacillus* (Cherian and Jayachandran 2009; Shah et al. 2009).

Biodegradation of NR latex is a rare event, and in this study, we report a thermophilic *Bacillus* strain as an efficient source for the degradation of NR latex. The sequencings of the gene coding for 16S rRNA gene and phylogenetic analyses were applied for the bacterium identification at the genus and specie levels.

Materials and methods

Sampling of crude latex

Latex from *Ficus elastica* grown in both farms of Faculties of Science and Agriculture, Assiut University was collected using tapping method (Wititsuwannakul and Wititsuwannakul 2001). It was obtained by injuring trunk of *F. elastica* and drawing by sterile disposable syringe under sterile conditions into sterile eppendorf tubes.

Fractionation of plant latex

Fresh latex was centrifuged at 17,000 rpm for 20 min at 4 °C in SR4000 Prolabo centrifuge (made in France). It was separated into a sticky top layer containing NR (Fig. 1). It was separated and washed three times by deionized water to remove impurities and dried at 30 °C for 24 h (Kang et al. 2000). A known weight (20 mg) was dissolved in 2 ml of tetrahydrofuran (THF), and its molecular weight was then determined by gel permeation chromatography (GPC) (Fig. 2).

Isolation and growth assessment

Microorganism was isolated on Mineral Salts Medium (MSM) [8.0 g K_2HPO_4, 1.0 g KH_2PO_4, 0.5 g $(NH_4)_2SO_4$, 0.2 g $MgSO_4 7H_2O$, 0.1 g NaCl, 0.1 g $Ca(NO3)_2$, 20 mg $CaCl_2$ $2H_2O$, 20 mg $FeSO_4$ $7H_2O$, 0.5 mg Na_2MoO_4 H_2O, and 0.5 mg $MnSO_4$ per liter of deionized water] containing 25–100 mg yeast extract and 20 g agar per liter (Heisey and Papadatos 1995). Pure NR was prepared and dissolved in Tetrahydrofuran (200 mg/20 ml THF), spotted on MSM agar media (each 50 µl/colony) and then left in an oven at 30 °C until THF evaporated, when the NR became white in appearance. Pure inocula of thermophilic bacterium was inoculated with tooth pick on the top of pure NR spot and then incubated for 15 days at 45 °C. The diameter of colony was measured at the end of the incubation period.

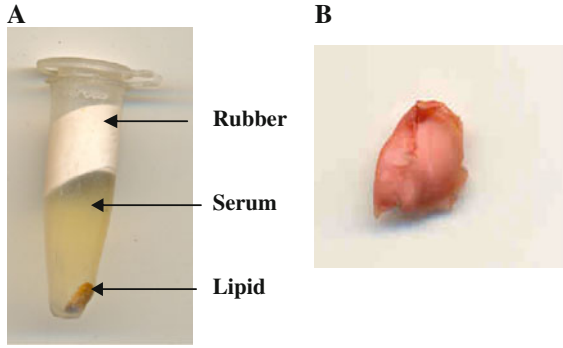

Fig. 1 Fractionation of latex (**a**), natural rubber of *F. elastica* after preparation (**b**)

Assessment of biodegradation ability

Natural rubber (dried) was cut into pieces (20 mg each), and washed three times with ethyl alcohol, and then left to evaporate at 30 °C for 15 min. The NR-degrading bacterial strain was cultivated in sterile 100 ml Erlenmeyer flask containing 20 mg sterile piece of natural rubber/25 ml sterile MSM broth. The inoculated flasks were incubated in a shaking incubator at 150 rpm for 15 and 30 days at 45 °C.

After each incubation period, the rubber pieces and microorganism were separated by centrifuging at 10,000 rpm for 15 min. Microorganism (pellet) was subjected for protein determination while natural rubber pieces (top layer) were collected, washed with deionized water, dried at 30 °C for 24 h and then subjected for analytic methods (molecular weight, viscosity and Schiff reagent) as well as scanning electron microscopy (SEM) photography as follows:

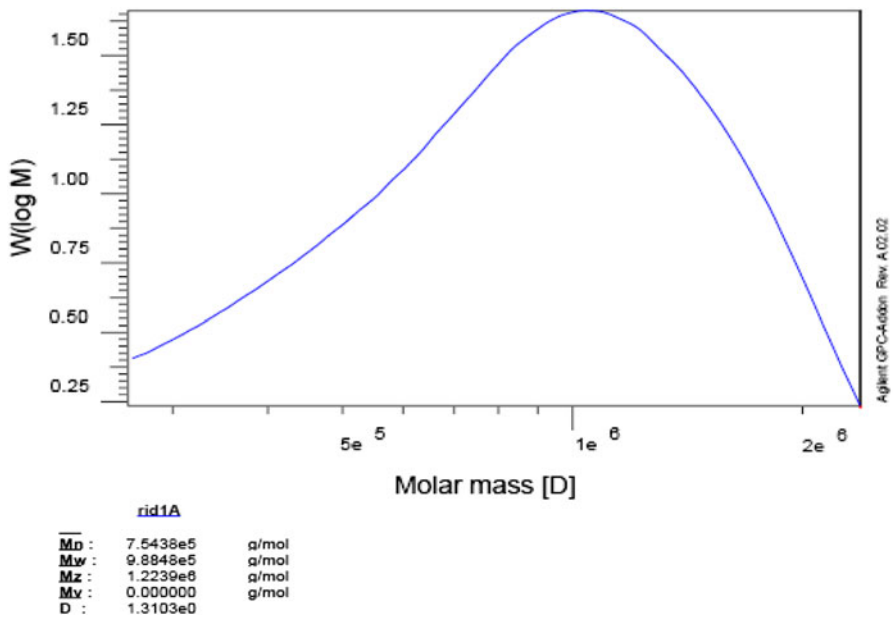

Fig. 2 Identification of natural rubber latex *of Ficus elastic*a by GPC

Protein determination

The total protein was determined according to the method of Lowery et al. (1951).

Viscosity determination

A known weight of NR was dissolved in 15 ml THF and the inherent viscosity of NR solutions at 29 °C was determined using an Ubbelohde suspended level viscometer. The inherent viscosity of NR was calculated according to the method of Aly et al. (2009) as follows:

$$\eta_{\text{rel}} = \frac{\eta}{\eta o} \quad \text{Relative Viscosity}$$

$$\eta_{\text{sp}} = \frac{\eta - \eta o}{\eta o} = \eta_{\text{rel}} - 1 \quad \text{Specific viscosity}$$

$$\eta_{\text{inh}} = \frac{\text{In}\eta_{\text{rel}}}{c} \quad \text{Inherent viscosity.}$$

Inherent Viscosity (η_{inh})

Definition The quotient of the natural logarithm of relative viscosity and the concentration.

Inherent viscosity (η_{inh}) is expressed in the following equation: Where c is the concentration and η_{rel} is the relative viscosity (unit less ratio).

Molecular weight determination by gel permeation chromatography (GPC)

A known weight of standard (Fig. 1) and the reset of NR after growing of organism were washed, dried, dissolved in 2 ml THF, and passed through a 0.45-μm pore size filter. GPC of the model Agilent technologies 1100, Germany (present in National Research Center, Doki, Cairo) was used. THF was used as the eluent with flow rate 1 ml/min. Polymethylmethacrylate and polystyrene standards were used to calibrate the columns using the refractive index detector 10^4A°, Colum PL gel Micrometer 100; 10,000; 100,000. The GPC apparatus was run under the following conditions: flow rate = 2.000 ml/min, injection volume = 100.000 μl, and sample concentration = 1.000 g/L. The values of molecular weight were computed by means of a computer program.

Detection of aldehyde or keto group by Schiff's reagent

Reagent was applied to detect aldehyde or ketone groups in polyisoprene degradation products. 10 ml of fuchsin reagent was added to the sample (50 μL of the NR dissolved in *n*-hexane) as described by Linos et al. (2000a). The composition of the fuchsin reagent was prepared as follows: 2 g of fuchsin was dissolved in

50 ml of glacial acetic acid plus 10 g of $Na_2S_2O_5$ plus 100 ml of 0.1 N HCl + 50 ml of H_2O (Ehrlich et al. 1948). Positive results are indicated by purple color formed at room temperature after 30 min.

Scanning electron microscopy

The morphological change during growth of the organism on NR was also assessed by SEM. The inoculated samples as well as the control were fixed in glutaraldehyde (5 %) overnight, and then dried at 50 °C. The samples were mounted on metal stubs and coated with gold and palladium (Jeol JFC1100E Iosputtering Device). Micrographs were taken by means of a Joel JSM-4500 LV electron microscope operating at 15 kV in Electron Microscopy Unit, Assiut University.

Bacterial genomic DNA extraction

The extraction of total bacterial genomic DNA was performed according to the procedures described by Hesham et al. (2006). In brief, Cells from 50-ml cultures were harvested by centrifugation, and the pellets were re-suspended by adding 400 µl breaking buffer (1% SDS, 100 mM NaCl, 10 mM Tris with pH at 8.0, and 1 mM EDTA with pH at 8.0) and 600 µl of phenol–chloroform (v/v = 25:24). The cells were then homogenized by vortexing and incubated at 60 °C for 10 min. After centrifugation, the supernatant was extracted once with 1 volume of phenol–chloroform–isoamyl alcohol (v/v/v = 25:24:1, pH = 8.0) and once with 1 volume of chloroform–isoamyl alcohol (v/v = 24:1, pH = 8.0), and the extracts were mixed. Nucleic acids were precipitated by adding 0.1 volume of sodium acetate (pH = 5.2) and 0.6 volume of isopropanol to the supernatant. The mixture was incubated in ice for 30 min and centrifuged (15,000 g for 20 min at 4 °C) to recover the precipitated nucleic acids. The pellets were washed with 70 % ice-cold ethanol and centrifuged (15,000 g for 20 min at 4 °C). The pellets were dissolved in 60 µl TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0).

Amplification of the 16S RNA gene

Molecular genetics identification of the bacterial isolate was performed by amplification of 16S rRNA gene with bacterial universal primers 27F (5-AGA GTTTGATCCTGGCTCAG-3) and 1492R (5-CGGC TACCTTGTTACGACTT-3) (Lane 1991) generating a PCR product corresponding to nucleotide positions 27–1,492 of the *Escherichia coli* 16S rDNA sequence. PCR reaction was carried out in a final volume of 50 µl containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, each dNTP at a concentration of 0.2 mM, 1.25 IU of *Taq* polymerase, each primer at a concentration of 0.2 mM, and 1 µl of the DNA template PCR was performed according to the following program: 5 min denaturation at 95 °C, followed by 36 cycles of 1 min denaturation at 94 °C; 1 min annealing at 55 °C; 1.5 min extension at 72 °C; and a final extension step of 7 min at 72 °C. 5 µl of the amplified mixture was then analyzed using 1.5 % 0.5× TBE agarose gel electrophoresis. The gel was stained with ethidium bromide, visualized under UV light, and photographed.

16S rRNA gene sequences and phylogenetic analysis

To verify the presence of appropriately sized amplicons, the PCR product was subjected to electrophoresis in 1 % agarose gel according to the standard methods. Product of the correct size was purified with a TakaR agarose Gel DNA Purification Kit Ver.2.0 and sequenced in both directions using an ABI 3730 automated sequencer. The bacterial 16S rDNA sequences obtained were then aligned with known 16S rDNA sequences in Genbank database using the basic local alignment search tool (BLAST) at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/), and percent homology scores were generated to identify bacteria. A phylogenetic tree was constructed by means of MEGA version 4.0 using a neighbor-joining algorithm, and the Jukes-Cantor distance estimation method with bootstrap analyses for 1,000 replicates was performed.

Nucleotide sequence accession number

The 16S ribosomal DNA sequence of strain ASU7 reported in this study has been deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases under Accession No. JN128610.

Results and discussions

Biodegradation ability of the thermophilic *Bacillus* strain ASU7 after 15 and 30 days of incubation

The strain ASU7 could grow on both latex agar and liquid media with higher concentration of rubber latex, and it was selected as the most efficient strain for the degradation of NR. The organism was later identified as *Bacillus amyloliquefaciens* based on its morphological and biochemical properties using Bergey's manual of systematic bacteriology (Sneath 1986). The identity was confirmed by 16S rRNA gene sequencing and phylogenetic analysis.

The Protein content for the isolate grown on NR increased significantly from 3.77 mg/g cell dry wt after 15 days to 5.39 mg/g cell dry wt after 30 days. On the other hand, rubber viscosity decreased significantly from 6.3 dl/g after 15 days of incubation with *Bacillus* ASU7 to 4.1 dl/g after 30 days. Regarding molecular weight of separated NR, it decreased from 7.2×10^5 after 15 days to 4.9×10^5 g/mol after 30 days (degradation rate of the *Bacillus* strain after 30 days comparable with the control = 43.7 % (Table 1).

Detection of aldehyde or keto group resulting from degradation of natural rubber by Schiff's reagent

The 30-day-old-treated natural rubber samples with the thermophilic *Bacillus* strain was tested using Schiff's reagent for the releasing aldehyde or keto group compounds. These samples gave positive results with Schiff's reagent indicated by formation of purple color on releasing aldehyde or keto group compound due to NR degradation. These results are in agreement with those of Tsuchii et al. (1996), Linos et al. (2000a), and Rose et al. (2005) who pointed out that the purple color produced by the Schiff's reagent was evidence that isoprene oligomers containing aldehyde groups have been produced and accumulated during the microbial degradation.

Scanning electron microscopy

The 15- and 30-day-old-treated natural rubber samples with the thermophilic *Bacillus* strain was photographed using SEM (Fig. 3). These SEM microphotographs clearly indicate the growth as well as the colonization of the *Bacillus* strain on the NR surface. In this respect, the colonization and/or growth on the rubber surface shown by SEM micrographs have been observed for *Pseudomonas aeruginosa* (Linos et al. 2000b); *Streptomyces* sp. (Gallert 2000); *Amycolatopsis*, *Streptomyces* and *Nocardia* strains (Heisey and Papadatos 1995); *Streptomyces lividans* and *Streptomyces* sp. (Rose et al. 2005), and *Aspergillus* sp. and *Pseudomonas* sp. (Roy et al. 2006).

Tsuchi and Takeda (1990) explained the degradation of rubber as a two-step reaction. They found that the crude enzyme isolated from *Xanthomonas* spp. could degrade the isoprene chain mainly into two fractions. In the first step, the original polymer with very high molecular weight was degraded into polymers with medium molecular weights. In the second step, the polymers with medium molecular weight were again degraded to form polymers with low molecular weight. Therefore, the wide molecular weight distribution of the degraded fraction in the

Table 1 Degradation of *Ficus elastica* rubber fraction by the thermophilic *Bacillus* strain through determination protein content (mg/g dry wt), viscosity (dl/g), and molecular weigh (g/mol) after shaking, incubation for 15, and 30 days at 45 °C

	Protein content mg/g dry wt		Viscosity measurements dl/g		Molecular weight g/mol $\times 10^5$	
Time (days)	15	30	15	30	15	30
Control (rubber only)			8.02 ± 0		8.7	
Rubber + *Bacillus* strain ASU7	3.77* ± 0.01	5.39* ± 0	6.3* ± 0.10	4.1* ± (48.87)	7.2	4.9 (43.68)

Figures in the table are mean of three replicates ± standard deviation

Values in brackets represent % percentage of degrading natural rubber by microorganisms in case of viscosity and molecular weight after 30 days

*Values Significant at PC 0.05 level

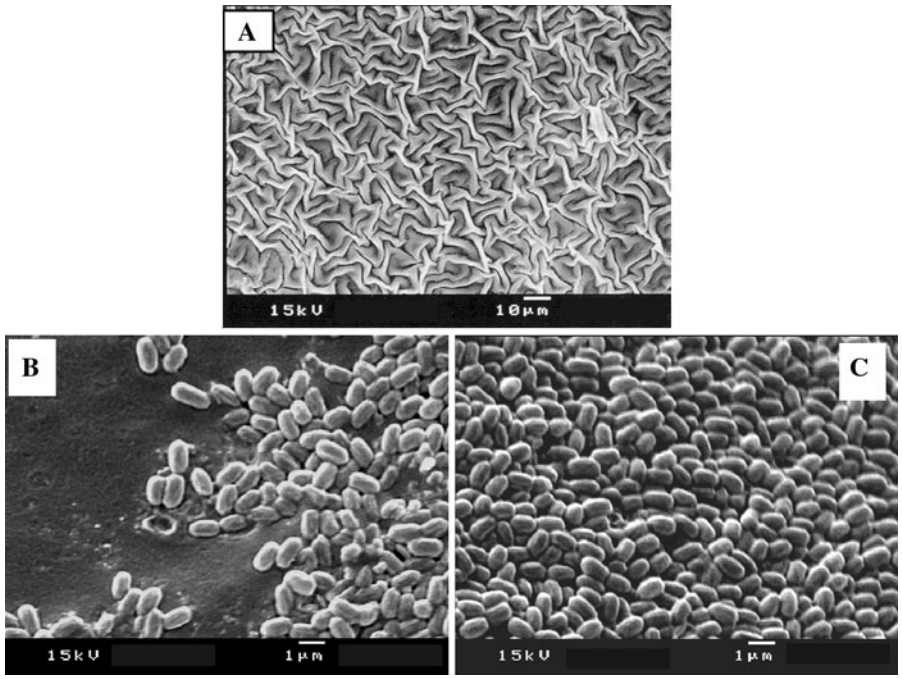

Fig. 3 Scanning electron micrographs (SEMs) of the un-inoculated rubber (**a**), and degradation of rubber fig latex (as revealed by the inoculation of 25 ml mineral salt medium amended with 20 mg rubber with *Bacillus* strain ASU7 and grown under 150 rpm by shaking at 45 °C conditions for 15 days (**b**) and 30 days (**c**)

MS suggested the random scissions of the original polymer in endwise form (Tsuchi and Takeda 1990). The mechanism involved in the degradation of NR may be the oxidative cleavage which is very well reflected in the reduction of double bond character as well as in the presence of aldehydes. Previous studies on NR biodegradation with various microorganisms indicated that during rubber degradation, oxidative cleavage of the double bond in the poly *cis*1, 4 isoprene backbone occurred as the first step (Rose and Steinbuchel 2005). The presence of aldehydes and ketones have been reported among the degradation products in several studies (Linos et al. 2000a, b; Berekaa et al. 2000; Rose et al. 2004).

Molecular identification and phylogenetic analysis using 16S rRNA gene sequence comparison

Although conventional methods are still widely used in bacterial identification, recently the development of molecular genetics techniques based on sequence analysis of the 16S rRNA have become the "golden index" in the field of bacterial identification (Shen and Feng 2004; Ma et al. 2008; Rani et al. 2008). Comparisons of 16S rRNA sequences are among the most powerful tools for the classification of microorganisms (Wu et al. 2006). The current classification of species within the genus *Bacillus* and related genera is well established and is based on molecular genetics approaches (Xu and Côte′ 2003). Tolba et al. (2007) demonstrated that when the use of molecular identification methods is justified, the employment of partial 16S rDNA PCR and sequencing provides a valuable and reliable method of identification of *Bacillus* sp. Moreover, several *Bacillus* sp. were reclassified based on the alignment of their 16S rRNA sequences (Catia et al. 2008; Liu et al. 2010; Sihem et al.2011; Maity et al. 2011). Therefore, the genomic DNA was extracted from the isolated bacterial strain ASU7, and universal primers 27F and 1492R were used for the amplification and sequencing of the 16S rRNA gene fragment. An almost complete sequence ($\sim$1.5 kb) of 16S rRNA gene was obtained for the *Bacillus* strain ASU7 and compared with the sequences of 16S rRNA regions in GenBank database by means of BLAST search. Results show that the 16S rRNA sequence of the isolated strain was highly homologous to *B. amyloliquefaciens*, with 99 % sequence similarity. To

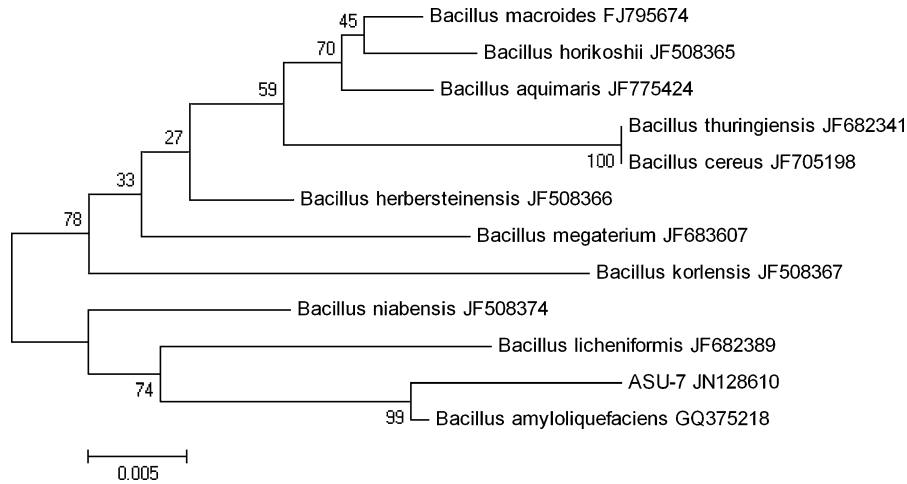

Fig. 4 Phylogenetic relationship between the *Bacillus* strain ASU7 and other 16S rDNA sequences of published strains. In the phylogenetic tree, ASU7 and *B. amyloliquefaciens* were clustered together as one clade

confirm the position of the strain ASU7 in phylogeny, a number of sequences representative some *Bacillus* sp. were selected from Genbank database for the construction of a phylogenetic tree. As shown in Fig. 4, the phylogenetic tree indicated that strain ASU7 and *B. amyloliquefaciens* shared one clade cluster. Therefore, the strain ASU7 was identified as *B. amyloliquefaciens*. Recently, *B. amyloliquefaciens* was identified based on 16S rRNA gene sequencing and phylogenetic analysis (Wei et al. 2011; He et al. 2012).

Conclusion

Bacillus amyloliquefaciens ASU7 strain was able to degrade natural rubber effectively. It used the hydrocarbon of NR as the sole source of carbon and energy. It was able to produce degradation products with low molecular weights. The results of increase in protein content, the decrease of viscosity and molecular weight, the detection of growth by SEM, and the production of keto group by Schiff reagent strongly confirmed the degradation of NR by this strain.

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