The Pharma Innovation Journal 2018; 7(8): 604-609

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277- 7695
ISSN (P): 2349-8242
NAAS Rating: 5.03
TPI 2018; 7(8): 604-609

www.thepharmajournal.com
Received: 28-06-2018
Accepted: 30-07-2018

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Enzyme activity and genetical characterisation of *Bacillus amyloliquefaciens* from textile effluents

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Abstract
Textile effluent is one of the well-known sources of pollution, which usually contain several chemicals, which contaminate the receiving water resources. In Tirupur, the textile factories discharge millions litres of untreated effluents into the drains that eventually empty into Noyyal river. Textile effluents contain carcinogenic aromatic amines, dyes, organic and inorganic materials, bacteria. Bacterial strain was isolated from textile effluents. *Bacillus amyloliquefaciens* was identified based on the microscopic observations and biochemical characterization. Biochemical characteristics revealed the isolated organism as *Bacillus amyloliquefaciens*. The identification was confirmed by 16S rRNA sequencing. They were identified as *Bacillus amyloliquefaciens.* The sequences were deposited in GENBANK. The accession number was KU041530. The present research was conducted to isolation, enzyme activity and confirmation of *Bacillus amyloliquefaciens*.

Keywords: Textile effluents, Noyyal river, *Bacillus amyloliquefaciens,* blast

1. Introduction
The textile industry is one of the industries that generate a high volume of waste water. Approximately 10,000 pigments and different dyes were manufactured worldwide with a total annual market of more than 7×10^5 metric tones per year (Ahluwalia and Goyal, 2007) [1]. This textile dye effluents causes serious environmental issues by absorbing light in receiving water reservoirs such as ponds, lakes, rivers and streams etc., and ultimately interfering with aquatic biological processes (Moore and Ramamoorthy, 1984) [2]. The traditional textile finishing factory consumes about 100 liters of water to process about 1 Kg of textile material. The recent science and technologies like the reuse of microbial or enzymatical treatment of textile dyeing effluents could help reducing this enormous water pollution [3]. These effluents contain considerable amount of suspended solids, additives, detergents, surfactants, carcinogenic amines, formaldehyde, heavy metals, and dyes that poses serious environmental threats to receiving water bodies [4].

There are many microorganisms which grow in textile waste water. A wide variety of microorganisms such as bacteria, yeast, algae, protozoa, and fungi are found in waters receiving industrial effluents. The river bed of Noyyal across Tirupur may be a source of microbes having potential to degrade pollution load of textile dye effluent (Arunprasad and Bhaskara Rao 2010) [5]. *Bacillus* spp. was widely distributed in the natural environments. Their unique nature lies in their ability to inhabit a variety of extreme and contaminated environments. *Bacillus amyloliquefaciens* was a gram positive soil bacteria closely related to the species *Baciullus subtilis*. The two species share many homologous genes and appear so similar it is not possible to visually separate the two species [6]. Their tolerance to stresses are attributed to external shield, which is the cell membrane and internal enzymatic system, besides its spore coat which protects it against physical and chemical agents (Popham *et al.*, 1995; Nicholson *et al.*, 2000; Riesenman and Nicholson, 2000) [7-9].

Alpha amylase is a hydrolytic enzyme and in nowadays, interest in its microbial production has increased dramatically due to its widely spread used in food, textile, baking and detergent industries (Asgher *et al.,* 2007) [10]. Preliminary characterization of the bacterial isolate regarding its morphological, biochemical characteristics' and decolourisation activity as well as the molecular level identity gives valuable information with regard to the further application of strain for different purposes [11]. Bacteria are commonly identified by 16S rDNA sequencing. Recently, Singh *et al.,* (2013) [12] used this gene for identification and characterization of a strain of *B. amyloliquefaciens*. The conventional biochemical tests used for identification of bacteria are time consuming, laborious and are not always conclusive.

The ribosomal operons mainly 16S rRNA has proven to be a stable and specific molecular marker for the identification of bacteria. The copy number of 16S rDNA genes may fluctuate from 1 to 15 among different bacterial genomes. 16S rRNA sequencing analysis is widely used, (Bansal, Meyer, 2002) [13] and more useful in phylogenetic analysis compared to 16S-23S rRNA sequencing (Song *et al.,* 2004) [14] and also due to its rapidity, reliability, simplicity and reproducibility (Lane *et al.,* 1985; Patel 2001; Easter 2003) [15-17]. Therefore, 16S rRNA is a significant target to the molecular level identification. The upstream region of 16S rRNA is well known to be highly conserved in species to species, so this region could also be used for the verification of the thermodynamic stability on the basis of conserved secondary structures of RNA. Thus the present study is mainly focused on the isolation, biochemical characterization and molecular identification of bacteria from the textile effluents through 16S rRNA based molecular technique.

Materials and Methods

Sample collection and Isolation of Bacteria

The textile effluent samples were collected from textile effluent plant Tirupur, Tamil Nadu India. The samples were collected in a sterile plastic container and transported to laboratory for bacteriological analysis. Bacterial isolates were screened on Nutrient Agar (NA) plates by the standard pour plate method. Plates were incubated at 37°C/24h and a total of one hundred and forty four isolates were obtained, from that one isolate was selected and used for further studies. The isolated bacteria were identified based on colony characteristics, gram staining methods and by various biochemical tests as given by Bergey's (1984) Manual of Determinative Bacteriology.

Screening and Identification of Amylase Producing Bacteria

A total of thirty bacteria were isolated from soil and screened for amylase production on nutrient agar plate containing (%) soluble starch 2.0, yeast extract 0.3, peptone 0.5, NaCl 0.5, agar 2.0, pH 7.0 at 37°C. Amylolytic isolates were selected by flooding the agar plates with Lugol solution [18]. Isolates were grown in nutrient broth containing 2% starch, selected on the basis of higher ratio of clear zone to colony size. The level of amylase was detected in culture filtrate. The bacterium showing maximum amylase production was analyzed and sequenced by 16S rRNA analysis. The selected isolate was identified as *Bacillus* sp. Strain PM1 (accession No.KU041530) and maintained by sub culturing after every fifteenth day.

Enzyme Production

The nutrient broth medium for enzyme production comprised (%): soluble starch 2.0, yeast extract 0.3, peptone 0.5, NaCl 0.5, pH 7.0. The medium (25 ml) was inoculated with 1 ml of the inoculums with an optical density of 0.6 at 600 nm and incubated at 37ºC for 96 h. Samples were withdrawn after a regular interval of 24 h and centrifuged at 10,000 rpm for 15 min in a Remi centrifuge (CPR-24, Rajendra Electric Motor Industries, Mumbai, India). Cell free supernatant was used as the crude enzyme. All the experiments were carried out in triplicates and results presented are the mean of three values. The standard deviation was within 5%.

Enzyme Assay

The amylase activity was assayed by adding 0.3 ml of culture filtrate, 0.7 ml of 0.5% starch solution in 2 ml of 50 mM glycine-NaOH buffer, pH 8.0 and incubating at 60ºC for 20 min [19]. The total amount of reducing sugars in the reaction mixture was determined by method of Miller, 1959 [20]. Absorbance was measured at 575 nm using a spectrophotometer (Systronics 106, Systronics India Limited, India). Maltose (1 mg/ml) was used as reference standard. Enzyme activity was expressed in units (µM of maltose released/ml/minute).

DNA isolation

Five ml of overnight culture were washed and suspended in TE buffer, PH 8.0. Genomic DNA was isolated as per the method of Schmalenberger [21].

PCR conditions

All the reaction mixtures contained 1X PCR buffer (10 mM Tris HCl, pH-9.0, 50 mM KCl and 0.01% gelatin); 100 mM concentration of each dNTPs and 0.75 unit of Taq polymerase. The final concentration of $MgCl_2$ was adjusted to 1.5 mM in PCR-Ribotypng. In PCR-ribotyping $MgCl_2$ concentration was adjusted to 3 mM. Each primer concentration was 20 pmol for PCR-Ribotypng. PCR-ribotyping was carried out according to the method of Franciosa. The primers for the amplification of DNA spacer regions between the 16S-5S genes were F (50 -TTG TAC ACA CCG CCC GTC A-30) and R (50 -GCT TAA CTT CCG TGT TCG GTA TGG G-30). The amplification was carried out by after initial denaturation at 94 1C for 2 min, followed by 35 cycles at 94 1C for 1 min, 35 1C for 1 min and 72 1C for 2.5 min, with a ram time of 2 min between 35 and 72 1C; a final extension was performed at 72 1C for 5 min. Agarose gel electrophoresis.

Agarose gel electrophoresis

The PCR products (10 ml) were separated by electrophoresis on a 1.5% agarose gel containing ethidium bromide (0.5 mg/ml) at 100 V for 1 h. in 1X TBE (Tris-Boric acid-EDTA) buffer. The gel images were digitized through UV gel image acquisition camera (Kodak, Japan).

16S r RNA sequencing analysis

The PCR amplified DNA was sequenced in ABI 3730 DNA Analyzer (Applied Biosystems). The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems). Sequence alignment required editing of the obtained sequences were carried out using Geneious Pro v5.

Phylogenetic analysis

The 16s rDNA sequences of *B.a* isolates aligned with the sequences of similar species which were retrieved from the Gene bank database. The sequences were converted in to FASTA format. The retrieved sequences were fed with ClustalW for multiple sequence alignment to observe sequence homology. Evolutionary tree was inferred by using the neighbour-joining method [22]. Dendrogram was constructed by neighbour-joining method using PHYLIP software package.

Results and Discussion

Table 1: Morphological characterization

S. No	Morphological test	*B. amyloliquefaciens*
1	Gram staining	Gram Positive
2	Shape	Rods

Table 2: Biochemical characterization

S. No	Biochemical tests	*B. amyloliquefaciens*
1	Indole Test	+
2	Citrate Utilization Test	+
3	Vogues Proskaeur Test	-
4	Amylase Test	+

+-Positive, – Negative.

Table 3: Enzymatic Activity of *B. amyloliquefaciens*

S. No	Bacteria	Diameter of Zone(mm)	Starch Degrade%	Bacterial Ability
1	*B.a* 1	7	10	W+
2	*B.a* 2	9	12	W+
3	*B.a* 3	27	53	H+
4	*B.a* 4	22	55	H+
5	*B.a* 5	13	21	M+
6	*B.a* 6	30	60	H+
7	*B.a* 7	21	20	M+

B. a 1 - *B. a* 7-Isolates of *B. amyloliquefaciens;* mm – millimeter of clear hole zone;
W+ - Weak; M+ - Moderate; H+ - High.

Morphological and biochemical characterization

In the present study morphological features were observed for the isolates grown on Nutrient agar medium (Figure 1). Strains were rod shaped in nature with the help of microscope. Bacteria were Gram positive which accepted the gram stains (Table 1). The genus taxonomy is first based on the cell morphology, presence of flagella and assay of the gram-positive type of the cell wall.

In the present investigation bacterial strains showed positive for Indole test, Citrate utilization test, Amylase test and negative for Vogues Proskaeur test. The results were represented in the Table 2.

Among 30 bacterial isolates, seven isolates were amylolytic and could be classified into three categories (high, moderate and weak) according to the degree of starch hydrolysis. Table 3 illustrates their percentage distribution which is expressed as diameter of clear zone (mm). Thirty-one amylolytic isolates could be also classified into two types according to their optimum growth temperature. Ten isolates were mesophilic whereas, twenty-one isolates grew at 50–60ºC better than 30 ºC. Amylase secretion is a peculiar character of *B. amyloliquefaciens* (Table 3).

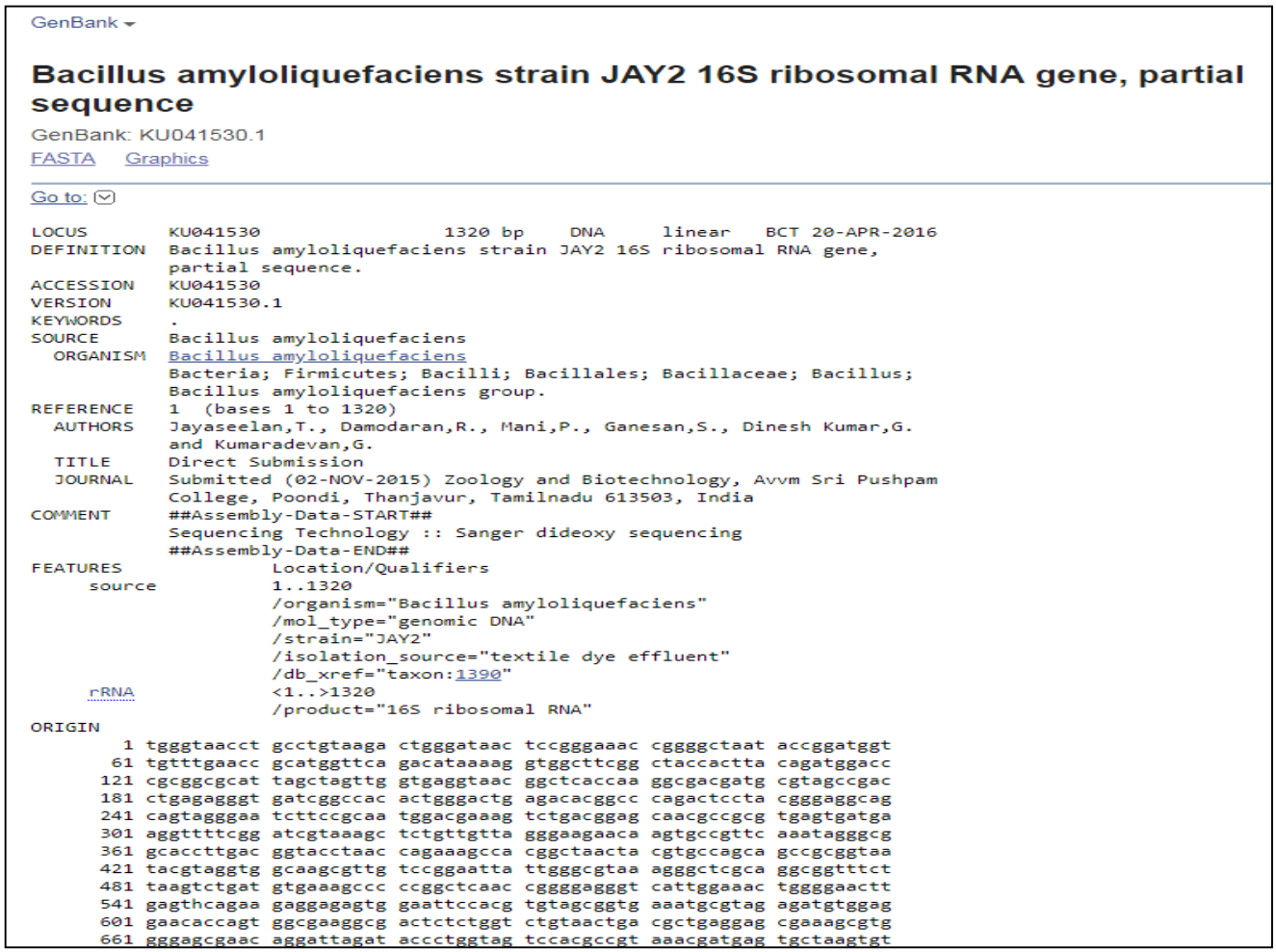
VaseeKaran *et al.,* (2010) [23] reported that the highest ratios of starch degradation ranged from 3.4 to 4 for tested isolates, while Alkando and Ibrahim (2011) [24] indicated that the ratio of starch degradation by *B. licheniformis* was 1.5 compared to the other tested bacterial species.

Thermophilic microorganisms were capable of producing thermostable enzyme reported by Rasooli *et al.,* (2008) [25]. These capabilities may be due to their molecular modifications at cellular and subcellular. Vaseekaran *et al.,* (2010) [23] and Panda *et al.,* (2013) [26] isolated three strains from soil receiving bakery waste produced the highest α-amylase activity at 90 ºC. Jogezai *et al.,* (2011) [27] observed that amylases activity was maximal at 40ºC for *B. subtilis*.

Fig 1: *B. amyloliquefaciens* culture on Petri disc

16S rRNA sequencing

After the determination of colony colour, the morphological, physiological biochemical and enzymatic characteristics, the isolate selected by the PCR analysis was approximately identified as *B. amyloliquefaciens* and it was confirmed by the 16S rDNA sequencing with help of universal bacterial primers, 16s-UP-F and 16s-UP-R. The universal primers were used for the amplification of 16S rRNA ribosomal smaller subunit as this plays an important role in the protein synthesis. The small subunit of ribosomal DNA is highly conserved in nature and can be used for molecular identification of bacteria and remain same in the process of evolution. Here, Clarridge *et al.,* (2004) used the 16S rRNA fragment for molecular level characterization of isolate AV25 (*Aeromonas veronii*). The comparison of the bacterial 16S rRNA gene sequence has emerged as a preferred genetic technique (Clarridge, 2004) [28]. Sequence of the *B. amyloliquefaciens* isolate showed partial 16S rDNA sequences, consisting of 1320 nucleotides which was submitted to the Gene bank (National Center for Biotechnology Information, USA) and an Accession Number (KU041530) was obtained (Figure 2). Phylogenetic tree was deduced from species of *B. amyloliquefaciens* using Neighbour-joining method (Figure 3). Bacteria have 1320 nucleotides were compare with already well known all bacterial genomes. The sequences showed 100% similarity with the already established the species *B. amyloliquefaciens*. From this study, the isolate was confirmed as the isolate of the species *B. amyloliquefaciens.*



GenBank

Bacillus amyloliquefaciens strain JAY2 16S ribosomal RNA gene, partial sequence

GenBank: KU041530.1

FASTA Graphics

Go to:

```
LOCUS       KU041530               1320 bp    DNA     linear   BCT 20-APR-2016
DEFINITION  Bacillus amyloliquefaciens strain JAY2 16S ribosomal RNA gene,
            partial sequence.
ACCESSION   KU041530
VERSION     KU041530.1
KEYWORDS    .
SOURCE      Bacillus amyloliquefaciens
  ORGANISM  Bacillus amyloliquefaciens
            Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus;
            Bacillus amyloliquefaciens group.
REFERENCE   1  (bases 1 to 1320)
  AUTHORS   Jayaseelan,T., Damodaran,R., Mani,P., Ganesan,S., Dinesh Kumar,G.
            and Kumaradevan,G.
  TITLE     Direct Submission
  JOURNAL   Submitted (02-NOV-2015) Zoology and Biotechnology, Avvm Sri Pushpam
            College, Poondi, Thanjavur, Tamilnadu 613503, India
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            Sequencing Technology :: Sanger dideoxy sequencing
            ##Assembly-Data-END##
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                     /product="16S ribosomal RNA"
ORIGIN
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       61 tgtttgaacc gcatggttca gacataaaag gtggcttcgg ctaccactta cagatggacc
      121 cgcggcgcat tagctagttg gtgaggtaac ggctcaccaa ggcgacgatg cgtagccgac
      181 ctgagagggt gatcggccac actgggactg agacacggcc cagactccta cgggaggcag
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      361 gcaccttgac ggtacctaac cagaaagcca cggctaacta cgtgccagca gccgcggtaa
      421 tacgtaggtg gcaagcgttg tccggaatta ttgggcgtaa agggctcgca ggcggtttct
      481 taagtctgat gtgaaagccc ccggctcaac cggggagggt cattggaaac tggggaactt
      541 gagthcagaa gaggagagtg gaattccacg tgtagcggtg aaatgcgtag agatgtggag
      601 gaacaccagt ggcgaaggcg actctctggt ctgtaactga cgctgaggag cgaaagcgtg
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```

Fig 2: *B. amyloliquefaciens* sequences in NCBI

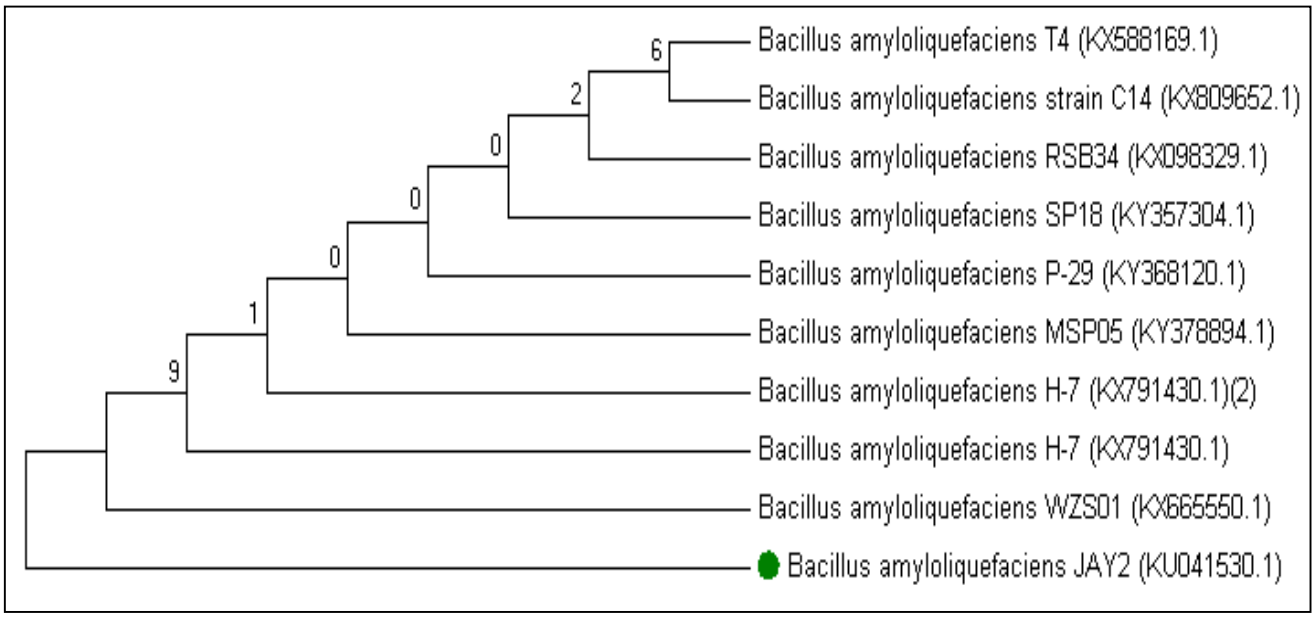

Fig 3: Phylogentic tree of *B. amyloliquefaciens*

The BLAST search demonstrated that these isolates were closely related to B. amyloliquefaciens, with sequence similarity of more than 99% to the 16S rRNA gene of B. amyloliquefaciens, and this was sufficient to indicate that these eight isolates belong to the same species. *Bacillus amyloliquefaciens* is closely related to *Bacillus subtilis* and *Bacillus licheniformis*, and separation of these organisms solely on the basis of classical tests is not possible [29]. The amplified product was purified to remove the excess primer for sequencing and it was sequenced using the automated DNA sequencer. Sequence of the bacterial isolate showed partial 16S rDNA sequences, consisting of 1320 nucleotides which were submitted to the Gene bank (National Center for Biotechnology Information, USA) and an Accession Number

(KU041530) was obtained. Next, Phylogenetic tree was deduced from species of *B. amyloliquefaciens* using Neighbour-joining method.

Although different studies have identified groups of bacteria for which 16S rRNA gene sequences are not discriminative enough, no study has attempted to solve this problem in a systematic way. The molecular approach using 16S rDNA sequence analysis especially with genus containing very closely related species like Bacillus (Berthier and Ehrlich 1998, Almeida and Araujo 2013) [30-31]. Genes that have no obvious evolutionary relatedness might have short segments that are similar to one another but with overall low percentage of similar nucleotide sequences (Brown 2002) [32]. BLAST results of 1320 nucleotides showed 100% similarity with *B. amyloliqufaciens*. In this study revealed that out 1320 base pairs were compare with already identified *B. amyloliquefaciens.* Gene sequences were 100% similar to that of *B. amyloliquefaciens.*

Conclusion

The effluents contain highly media to grow and spread microbial population. A correct taxonomic classification of the isolated bacteria to a genus or species required the execution of basic morphological and biochemical identification tests. Several bacterial isolates were isolated from Tirupur textile effluents and were capable to grow and produce amylases. Among these isolates was found to produce the highest amylases activity on productive medium supplemented with 1% starch at 50ºC for 48 h using shake flasks as a batch culture. This isolate was identified as *B. amyloliquefaciens* according to phenotypic tests and was confirmed by 16S rRNA gene sequencing. These techniques will be useful for species identification of bacteria and should be applicable in the studies of epidemiology, diagnosis, virulence and molecular taxonomy. The molecular approaches used in this study were successfully able to identify all of the isolates as *B. amyloliquefaciens.* In later, this *B. amyloliquefaciens* strain will be used for the Decolouration process of Textile Dye effluents. Further the 16S rRNA gene sequences will construct new drugs and treatment of diseases.

Acknowledgement

The author is very grateful to the University Grant Commission (UGC), Government of India, New Delhi, for providing financial assistance in the form of Rajiv Gandhi National Fellowship which buttressed me to carry out my research work successfully. The author is also very grateful to the Secretary and Correspondent and the Principal of A.V.V.M. Sri Pushpam College (Autonomous), Poondi-613 503, Thanjavur (Dt.) for providing the excellent infrastructure and necessary facilities to carry out my research work successfully.

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