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RESEARCH ARTICLE

Studies on Isolation, Purification and Molecular Identification of Pectinase Producing Bacteria

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Abstract

Pectin is a structural polysaccharide present in primary cell wall and middle lamella of fruits and vegetables. Pectin or pectic substances are heterogeneous group of high molecular weight, complex, acidic structural polysaccharides with a backbone of galacturonic acid residues linked by α-(1-4) linkages. Pectins show widespread commercial uses. Pectinases are a group of enzymes that breakdown pectins. Because of the potential and wide applications of pectinases, there is a need to highlight recent developments on several aspects related to their production. However, aspects regarding the most common microorganisms and processes for hydrolytic depolymerising pectinase (PGase) production have not been considered until now. The present study was undertaken to isolate bacteria from soil samples that produce pectinase enzyme using a selective medium after enrichment and to identify the bacterial isolates based on biochemical and molecular characterization by sequencing the 16S rRNA coding gene and based on the 16s rRNA sequence, the culture was identified as *Bacillus subtilis.*

Introduction

Pectin is a structural polysaccharide present in primary cell wall and middle lamella of fruits and vegetables. Pectin or pectic substances are heterogeneous group of high molecular weight, complex, acidic structural polysaccharides with a backbone of galacturonic acid residues linked by α-(1-4) linkages (Kapoor *et al.*, 2000; Kashyap *et al*., 2001; Singh *et al*., 1999; Kapoor *et al*., 2000; Naidu and Panda, 1998; Hoondal *et al.*, 2002). Pectins show widespread commercial use, especially in the textile industry (Henriksson *et al*., 1990), in the food industry (Liu *et al*., 2006), in the pharmaceutical industry (Chambin *et al*., 2006), they have cholesterol-lowering effect (Fernandez *et al.*, 1994) and they are used in making biodegradable films (Hoagland and Parris, 1996). Despite these applications, pectins are similar to cellulose and hemicelluloses, common waste materials that can be converted to soluble sugars, ethanol (Doran *et al*., 2000), and biogas (Hutnan *et al*., 2000). Pectic substances are basically classified into four main types based on the type of modifications of the backbone chain. They are, Protopectin, Pectic acid, Pectinic acid and Pectin (Kashyap *et al*., 2001).

Pectinases are a group of enzymes that breakdown pectins. Pectolysis is one of the most important processes for plant as it plays a role in cell elongation, growth and fruit ripening. Microbial pectolysis is important in plant pathogenesis, symbiosis and decomposition of plant deposits (Lang and Dornenberg, 2000). The main source of the microorganisms that produce pectinolytic enzymes are yeast, bacteria and large varieties of fungi, insects, nematodes and protozoas (Luh *et al*., 1951; Whitaker, 1991). These enzymes are inducible, produced only when needed and they contribute to the natural carbon cycle. Microbial pectinases account for 10-25% of the global food and industrial enzyme sales (Singh *et al.*, 1999) and their market is increasing day by day (Stutzenberger, 1992). These are used extensively for fruit juice clarification, juice extraction, manufacture of pectin free starch, refinement of vegetable fibers, degumming of natural fibers, waste-water treatment, curing of coffee, cocoa

and tobacco and as an analytical tool in the assessment of plant products (Singh *et al.*, 1999; Alkorta *et al*., 1998). Pectinolytic Enzymes are also applied in fruit juices, jams, jellies, sauces, ketchups, flavoured syrups, desserts and other food industries (Pilnik and Rombouts, 1985; Whitaker, 1984; Girdharilal *et al.*, 1998). In Dairy Industries, these are used to prevent the formation of casein clumps. In wine industry pectinases are mainly used for decreasing astringency by solubilizing anthocyanins without leaching out procyadin polyphenols and pectinases also increase pigmentation by extracting more anthocyanins (Tucker and Woods, 1991). In Textile Industries, pectinases are used for the lower discharge of waste chemicals and to improve safety of working conditions for textile workers and the quality of the fabric. The most upcoming application of pectinolytic enzymes use in the degumming of plant fibers (Bruhlmann *et al*., 1994; Cao *et al*., 1992; Henriksson *et al*., 1999; Kapoor *et al*., 2001). Pectinases are also used in retting of plant fibers, pretreatment of pectic waste waters (Horikoshi, 1990; Tanabe *et al.*, 1988), coffee and tea fermentation (Carr, 1985), paper and pulp industry (Bajpai, 1999; Kirk and Jefferies, 1996), in poultry feed (Hoondal *et al*., 2000), in oil extraction (Scott, 1978) and in medical field.

Because of the potential and wide applications of pectinases, there is a need to highlight recent developments on several aspects related to their production. Microbial production of pectic transeliminases was reviewed earlier (Gummadi and Kumar, 2005). However, aspects regarding the most common microorganisms and processes for hydrolytic depolymerising pectinase (PGase) production have not been considered until now. The aim of this review is to present an overview of the pectinase activity obtained by *Bacillus subtilis* as well as the strategies used to obtain higher activities.

The present study was undertaken with the following objectives:

To isolate bacteria from soil samples that produce pectinase enzyme using a selective medium after enrichment.

To identify the bacterial isolates based on biochemical and molecular characterization by sequencing the 16S rRNA coding gene.

Materials and Methods:

Isolation of Bacterial Strains

Soil is collected from different villages of Guntur District (Duggirala and Burripalem) from a depth of 1-15 inches to isolate desired bacteria.

Enrichment of the soil sample

100 g of the soil sample was taken and to this 1g of the pectin was added and incubated for 30 days for enrichment.

Enrichment Broth for Isolation of Pectinolytic Bacteria

Pectin (1%)-1g; Yeast extract-0.1g; Peptone-0.5g; $CaCO_3$-0.2g; NaCl-0.2g; Distilled Water-100ml. The enrichment broth was inoculated with the enriched Soil sample and placed on the rotatary shaker at 200 rpm for 10 days.

Serial dilution technique

10 – fold serial dilution (10^{-1} to 10^{-8}) of the enriched soil samples were prepared. 1ml portion of the dilution (10^{-4} to 10^{-6}) was inoculated into 100 ml of the selective broth (Vincent's mineral salts broth at pH 7).

Selective Media

A selective medium i.e. Vincent's medium (Vincent, 1970) was employed here that allows only the desired bacteria to grow which utilizes pectin as the sole carbon source. The bacteria was inoculated and grown in Vincent's mineral salts broth at pH 7 (Culture broth).

Vincent's Agar Medium

The colonies obtained from the Vincent's agar medium were isolated and maintained at 37^0C.

Identification of the Bacteria

The isolated bacteria were identified by different staining techniques (Simple and Gram's), biochemical and molecular techniques.

Biochemical Tests

The strain isolated from soils of different villages of Guntur District (Duggirala and Burripalem) were identified by conventional biochemical tests in accordance with Bergey's Manual of Systematic Bacteriology (Sneath, 1986; Holding and Colle, 1971; Buchanan and Gibbons, 1974; Taiwo and Oso, 2004). They are Indole production test, Methyl red and Voges proskauer tests, Gelatin Hydrolysis (Production of Gelatinase), Starch Hydrolysis, Oxidase Production, Catalase Activity, Citrate utilization test, Nitrate reduction test, Caesin Hydrolysis.

Identification of the Isolated Bacteria by Sequencing of the Amplified 16S rRNA Gene

The most powerful tool to identify the unknown bacteria is to sequence the DNA coding for 16s

rRNA, since the 16s rRNA is encoded by the gene in the chromosome of the bacteria. So the gene coding for the 16s rRNA is amplified using the Polymerase Chain Reaction (Mullis, 1990), and the amplified product has been subjected to sequencing and the sequence obtained has been compared with the sequence obtained from the Nucleotide Database of NCBI.

Genomic DNA Isolation of the bacterial Isolates

Genomic DNA was isolated from *Bacillus* according to the following procedure. 50 mL LB broth was inoculated with a single bacterial colony and grown to an OD 600 of 0.5–1.0. Cells were collected by centrifugation at 5000 rpm, 4°C, for 10 min. The genomic DNA of the Bacteria was isolated by using the Bacterial Genomic DNA isolation kit. Then the quantity and quality of the DNA is estimated.

Agarose Gel Electrophoresiss

To 0.8 g of agarose 100 ml of 1X TBE buffer was added and it was heated in a microwave oven till all agarose gets melted up, then taken into gel casting unit for polymerization. After polymerization, the comb was removed and the gel was placed in an electrophoretic tank consisting of 1X TBE buffer. About 2 µl of the isolated genomic DNA was mixed with 2 µl of the gel loading dye (Bromophenol blue) and it was loaded in 0.8 % agarose gel. The gel was then electrophoresed at 90 volts for about 30 minutes and it was observed in a gel documentation system.

Spectrophotometry

The basic principle is that the DNA absorbs the ultra violet light between 250 to 270 nm owing to the spectral characteristics of the four bases. With the help of the absorbance, the concentration of the DNA can be calculated. About 2 µl of the DNA was added to 500 µl of the distilled water, and was taken in a 0.5 quartz cuvette. After calibration with distilled water the samples were read at 260 nm and 280 nm respectively. The absorbance obtained was then used for calculating the quality and quantity respectively.

Calculating the purity and yield

One absorbance unit at 260 nm of the double stranded DNA is equal to 50 µg/ml of the double stranded DNA. One absorbance unit at 260 nm of the single stranded DNA is equal to 40 µg/ml of the single stranded DNA.

- Total A_{260} Units = (A_{260}) x dilution factor
- Concentration (µg/ml) = total A_{260} units x (50 µg/ml)
- Yield (µg) = Volume x Concentration
- Pure DNA exhibits an absorbance ratio (A_{260}/A_{280}) of 1.8 to 2.0

  If the DNA exhibits an absorbance ratio (A_{260}/A_{280}) of less than 1.7, the sample is contaminated by protein.

Amplification of the 16s rRNA Gene of Bacterial Chromosome

The polymerase chain reaction is an enzyme catalyzed biochemical reaction in which small amount of the specific DNA sequences are amplified into large amounts of linear double stranded DNA (Mullis, 1990). PCR is used to amplify the DNA sequence in between two known sequences. About 30 cycles of the DNA amplification was performed that resulted in a very large amplification of the DNA. The amplified DNA was subjected to Agarose gel electrophoresis along with the marker DNA (DNA Ladder) and basing on the size of the amplified 16S rRNA (DNA) fragment, it was confirmed that the 16S rRNA gene was amplified. The DNA band of the amplified product was cut from gel, eluted and subjected for sequencing. The sequence so obtained was compared with the reported results present in the public databases (NCBI) and the sequence of the unknown bacteria was determined.

Scanning Electron Microscopy of *Bacillus subtilis*

Scanning Electron microscopic studies was carried out by taking 24 hrs old cultures of *Bacillus subtilis* and fixed in 6% buffered glutaraldehyde followed by post fixation in osmium tetroxide and then dehydrated in increasing concentration of ethyl alcohol. The samples were mounted on copper stubs with double sided adhesive tape, coated with gold polaron, AU/PD sputter-coater and scanned in SEM (Jeol JSM 5600, Japan) and photographed.

Production of Pectinase

For production of pectinase, 2.0 ml of the seed was taken and inoculated in to 250 ml Erlenmayer flasks containing 50 ml sterile productive medium. The inoculated flasks were incubated at 37°C for 96 hours. At the end of the fermentation cycle, 5.0 ml of the fermented broth was aseptically removed and centrifuged at 2000 rpm for 10 minutes. The clear supernatant containing the enzyme was used in the enzyme assay. Assay of Pectinase was done by DNS method (Miller, 1959). The activity of the enzyme was expressed in µmol / ml / min, by the following formula,

The OD of test was subtracted from the OD of unknown

= OD of Test – OD of Unknown = to the OD of the Colour intensity of the liberated products.

Results & Discussion:

Collection of soil sample

Different soil samples were collected from agricultural lands at Duggirala and Burripalem in Guntur (Dt), A. P. in sterilized polythene bags.

Enrichment of the Soil sample

All the collected soil samples were subjected to enrichment with pectin powder and incubated for 4 to 5 weeks at 37°C (Márcia *et al*., 1999; Zeni *et al.*, 2010). This enriched soil sample was again enriched by adding one gram of the enriched soil sample to the broth containing 1% pectin, 0.1% Yeast Extract, 0.5% of peptone, 0.2% $CaCO_3$, and 0.2% of NaCl (Wen-Jing *et al.*, 2005) and kept on a temperature regulated shaking incubator for 4 to 5 weeks at 200 rpm/min at 37°C and at pH 7. The enriched broth was serially diluted using sterile molecular grade water up to 10^{-7}. (Milli Q - Millipore water System) (Chellapandi and Himanshu, 2008).

Isolation of Pectinase producing Bacteria Using Selective Media

1ml of the enriched diluted sample was inoculated into 250 ml of sterilized conical flask (Borosil) containing 100 ml of the Vincent's medium (broth) at pH 7 and kept in a temperature controlled rotating shaking incubator at 200rpm/min, where the temperature was maintained at 37°C for 7 days, 1ml of the culture broth was inoculated into Vincent's medium (Culture broth).

Serial Dilution of the Culture Broth

Like the above procedure, the dilutions were carried out up to 10^{-7}. 0.5 ml of the culture broth from the dilutions 10^{-4} and 10^{-5} was then inoculated on to Vincent's agar medium (Reda *et al.,* 2008), which was a selective medium (Chellapandi and Himanshu, 2008) and the inoculated plates were incubated at 37°C. Then, Simple and Gram's Staining procedures were followed for the identification of the morphology of the bacteria (Nasrin *et al*., 2008). The isolate 'A' appeared as Gram positive bacilli (Table 1).

SEM (Scanning Electron Microscopy)

The preliminary study on morphology of the bacterium suggested that the bacterium is from *Bacillus* species. The bacterium was rod shaped and had a positive gram stain reaction. SEM analysis had revealed that the bacterium was rod shaped and size of the bacterium was about 2 -3 µm (Fig 1).

Biochemical characterization

The isolate was considered to be the member of the group *Bacillus* based on the staining procedures and the biochemical results (Catia *et al.,* 2008; Kasing *et al.,* 2000). The isolate 'A' was negative for Indole production, Methyl Red test, Citrate utilization and Catalase test and the organism was positive to Caesin Hydrolysis, Gelatin, Nitrate reduction, Starch hydrolysis, Voges-Proskauer and Oxidase Tests (Table 1); (Plate 1 & 2).

Molecular Characterization Based on 16s RNA Sequence

The DNA isolated from the desired pectinase producing bacteria suspected to be *Bacillus subtilis.* When checked for purity it exhibited an absorbance ratio of 1.823 and 1.9 respectively (A_{260}/A_{280} ratio 1.8 to 2.0 to be pure), which can be concluded that the DNA isolated from the source was pure and the same DNA sample, when run on an agarose gel also confirmed to be pure as the band of DNA was single and distinct and no traces of contaminants were found when observed under the UV transilluminator (Fig 2).

The genomic DNA of the organism was isolated and gene (DNA) coding for the 16s rRNA was amplified by Polymerase chain reaction, yielded a DNA band of 800 base pairs for *Bacillus subtilis* (Fig 3).

Sequencing of the 16S rRNA gene of *Bacillus subtilis*

In order to characterize the strain, the nucleotide sequences of the 16S rDNA of the strain were determined. Phylogenetic tree was constructed by the neighbour-joining (N-J) method based on the 16S rDNA sequences. The 16S rRNA gene from the genomic DNA of the *Bacillus* (based on the Biochemical and Staining properties) was enzymatically amplified by Taq DNA polymerase by using a universal eubacterial primer set, (Forward Primer) 5' -GAGTTTGATCCTGGCTCAG-3' [positions 9–27 (*Escherichia coli* 16S rDNA numbering)] and (Reverse primer) 5'-AGAAA GGAGG TGATC CAGCC-3' [positions 1542–1525 (*E. coli* 16S rDNA numbering)] were used, according to method described by William *et al.,* 1991. After purifying the PCR product with DNA purification kit, the resulting PCR product was sequenced commercially. The obtained sequenced was blasted in NCBI data base, and phylogenetic analysis of the bacteria *Bacillus* was carried out.

The above sequence was compared with the known sequences in the public databases in NCBI and the

BLAST results which were given in the form of a phylogenetic tree. Based on the 16s rRNA sequences, the bacterial isolate was confirmed as *Bacillus subtilis*. This work was carried out based on the conclusions given by Abdelnasser Salah Sheble Ibrahim and Ahmed I El-diwany, 2007. The phylogenetic analysis of the strain was confirmed using its 16S rDNA sequence. The results from the present study were in agreement with their observation. In order to characterize the strain, the nucleotide sequence of the 16S rDNA of the strain was determined. There was 97% sequence homology with the sequences available in the database (Catia and Orlando, 2008).

Assembled sequence of PCR Product of *Bacillus subtilis*

>Sample_01

TTTATCGGAGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGG
ACAGATGGGAGCTTGCTCCCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAG
ACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGGTTGTTTGAACCGCATGGTTCAAACATAAAA
GGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCA
AGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCT
ACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATG
AAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTGCCGTTCGAATAGGGCGGTACCTTGA
CGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTT
GTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAA
CCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGT
GAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGA
GCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTG
TTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAA
GACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACG
CGAAGAACCTTACCAGGTCTTGACATCCTCTGACAATCCTAGAGATAGGACGTCCCCTTCGGGGGCAGAG
TGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAA
CCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGG
TGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACAGAACAAA
GGGCAGCGAAACCGCGAGGTTAAGCCAATCCCACAAATCTGTTCTCAGTTCGGATCGCAGTCTGCAACTC
GACTGCGTGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTG
TACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCTTTTAGGAGCCAG
CCGCCGAAGGTGGGACAGATGATTGGGGTGAAGTCGTAACAAGGTAGCCGTATCGGAAGGTGCGGCTGGA
TCACCTCCTTTCT

The Isolate was subjected to 16S rRNA PCR amplification using universal primers. The PCR product obtained was subjected to further analysis by using RDP – Ribosomal Project for analysis. Based on the 16s r RNA sequence the culture was identified as *Bacillus subtilis*.

Enzyme Assay

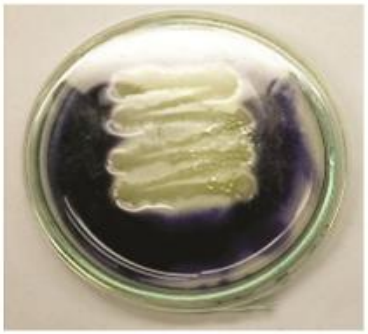
Enzyme assay was based on the determination of reducing sugars produced as a result of enzymatic hydrolysis of pectin by dinitrosalicylic acid reagent (DNS) method (Miller, 1959).

Table 1. Microscopic examination and biochemical reaction of bacterial, Population from isolated soil of Duggirala & Burripalem, Guntur, A.P.

Microscopic and Biochemical Examinations of an Isolate	Isolate A
1. Simple staining	Rod shaped
2. Gram staining	Gram +ve rod bacilli
3. Motility Test	Motile
1. Indole production	Negative
2. Methyl Red test	Negative
3.Voges-Proskauer	Positive
4.Citrate utilization Test	Negative
5. Catalase Test	Negative
6. Nitrate reduction test	Positive
7. Starch Hydrolysis	Positive
8. Caesin Hydrolysis	Positive
9. Gelatin Test	Positive
10.Oxidase Test	Positive

Plate 1. Starch Hydrolysis

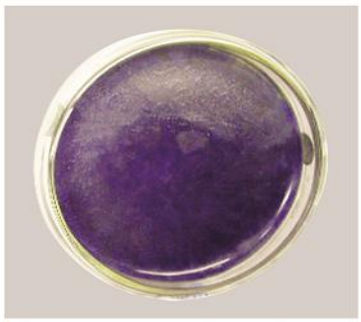
Positive

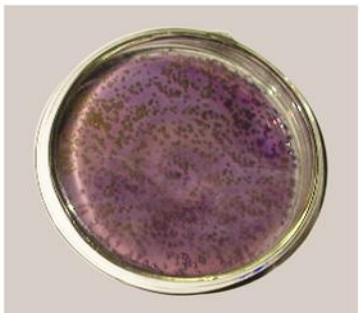


Negative

Plate 2. Oxidase Test

Positive





Negative

Fig 1. SEM of *Bacillus subtilis*

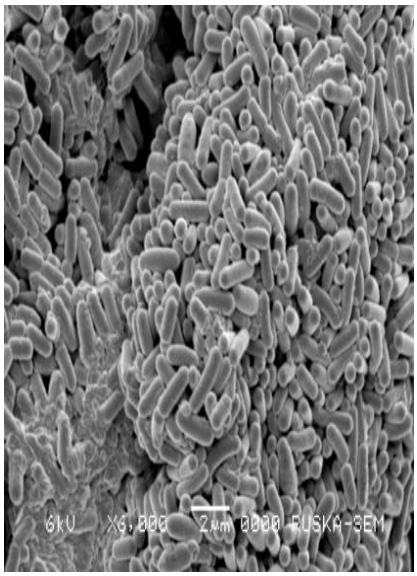
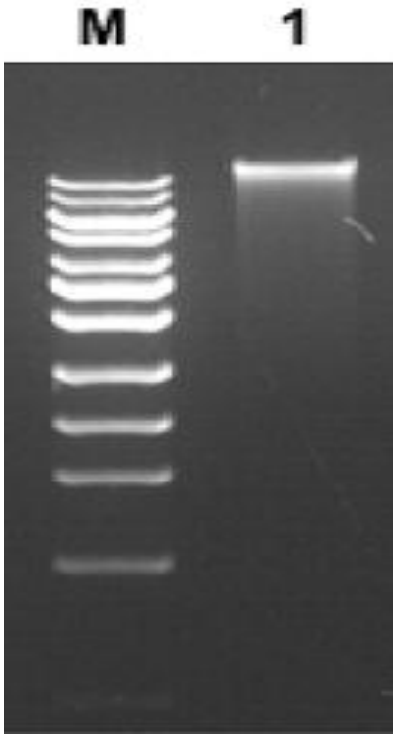


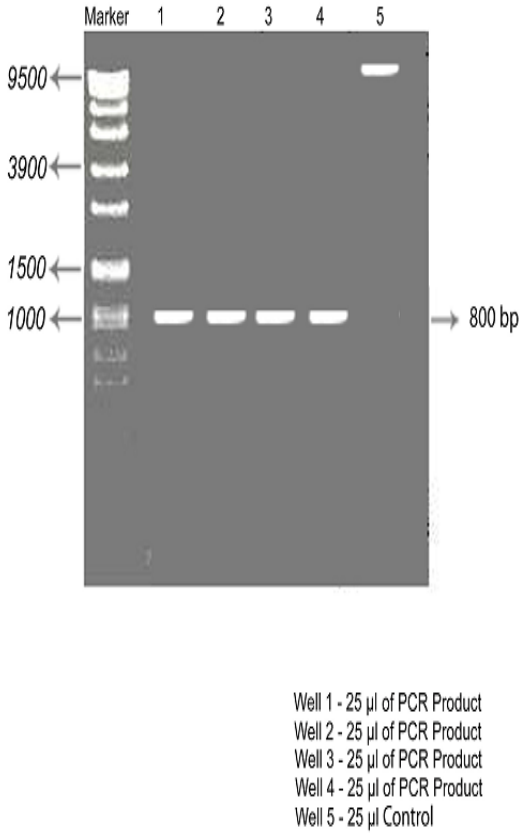
Fig 2. Isolated genomic DNA of *Bacillus subtilis*



M – DNA Marker

Well 1 – Genomic DNA of *Bacillus subtilis*

Fig 3. Amplified 16S r RNA of *Bacillus subtilis*



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