Chapter 3

Materials and Methods

Rice beer is an age old drink of the tribal communities. Since time immemorial the ethnic communities produce the beer using the traditional know-how. It occupies an indispensable role in their socio-cultural life. Systematic study on the nutritional composition and microbiological aspects of the traditional rice beer has not been done in North East India except some sporadic studies in Sikkim and Nagaland. So, a pragmatic approach has been planned to study and gather knowledge and information about it that may help in future improvement of this inebriating social drink.

3.1 Collection of Starter Culture

Starter cultures used by different ethnic communities for making rice beer were collected from different places of Assam. Starter culture of Ahom community was collected from Namrup and Tinsukia; Bodo from Bagariguri and Udalguri; Karbi from Rajapathar and Bakulia, Karbi Anglong district; Mising from Dhakuakhana of Lakhimpur district and that of Tiwa (Lalung) from Barmanipur village of Morigaon district.

Starter cultures in the form of cakes (pitha) or balls (guti) were collected using sterile polythene bags. Each packet was labeled properly mentioning culture details and date of collection.

3.2 Analysis of Starter Culture

3.2.1 Determination of moisture Content

The moisture content of the cultures (pitha or guti) was determined by taking the initial weight just after the collection. Sample from each tribe was ground in to a fine powder and dried in an oven at 42° C till attaining a constant
weight. The difference in fresh and dry weight of culture was converted to percent moisture content.

3.2.2 Determination of pH

One starter culture cake/ball (pitha or guti) from each of the samples was ground in to a fine powder. An amount of 5 g of the powder was added to 25 ml of neutralized deionized water and then vortexed for 5 minutes. The pH of the suspension was measured and recorded using a Cyberscan 510 digital pH meter. Triplicate readings were taken for accuracy.

3.2.3 Determination of ethanol content in traditional rice beer

Ethanol content of the rice beer collected from different tribal communities as well as the one produced in the laboratory using the isolated yeast cultures was determined by using a GC-Mass spectrometer (Varian).

3.3 Isolation of microflora

3.3.1 Isolation of yeast and fungi

A starter culture cake/ball from each of the samples was ground in to a fine powder in a sterile environment. An amount of 5 g powder from each sample was homogenized in 25 ml of sterile distilled water. The suspension was serially diluted to $10^{-5}$ with sterile water. An aliquot of 100 μl from each of the dilutions was cultured on malt-yeast-glucose-peptone-agar (YM agar; Himedia, M424) plate. The plates were incubated at 25°C for 48 h. Yeast colonies appeared in the culture plates were divided in to 18 groups on the basis of their phenotypic characters. Pure cultures were established from the distinct single colonies by the conventional streaking method. Density of yeast cells was determined with the help of a colony counter (Lapiz, Medica Instrument Mfg. Co., Mumbai) and expresses in terms of colony forming unit (cfu) per g of fresh sample. The isolated strains were maintained on YM slants at -4°C and also in 20% glycerol at -20°C.

In the case of isolation of fungi after the dilution-plating on potato dextrose agar (PDA; Himedia, M096), the plates were incubated at 30°C for 72 h. The population of fungal colonies were calculated with the help of a colony
counter (Lapiz, Medica Instrument Mfg. Co., Mumbai) and expressed in terms of cfu (colony forming unit) per g of fresh sample. Pure culture of fungi were maintained at PDA slants and kept at -4°C.

3.4 Morphological characterization

3.4.1 Calibration of the microscope

Calibration factor for one ocular division of Leica ATC 2000 microscope for 40X magnification was determined following the formula described below: One division on ocular micrometer (mm) is equal to known distance between 2 line on stage micrometer divided by the no of division coinciding on ocular micrometer. Calibration factor was found to be 2.5 μm.

3.4.2 Morphological characterization of yeast

Colony and cell (microscopic) characters of the yeast strains were studied by growing them on YM agar plates and YM broth, respectively, for 48-72 h. To induce sporulation of the yeast strains, Corn Meal Agar (CMA) (Himedia, M146), Yeast Morphology Agar (YMA) (Himedia, M138) and V8 Juice Agar (VJA) (Himedia; M638) media were used following the procedure of Barnett et al. (2000). Microscopical observations in respect of cell shape, dimension, budding, presence or absence of hyphae or pseudohyphae, reproductive structures were recorded.

3.4.3 Characterization of Fungi

The colony morphology of the fungal strains was examined after growing cultures on (PDA) for 72 h. Cellular observations were carried out under 10 x 40X magnification of a compound microscope following staining the specimen with lactophenol cotton blue. The fungal isolates were studied using the taxonomic keys described by Hesseltine (1991) and Lee and Fujio (1999). These were photographed with a manually operated camera (Cosina C1s) mounted over trinocular microscope (Leica ATC 2000).

The starch hydrolyzing activity of fungal strains was assessed for preliminary screening by growing them in 2% Starch Agar (SA) (Himedia,
plates for 48 h at 25°C. Plates were then flooded with 1:5 Lugol’s iodine solution to check for the zone of hydrolysis.

3.5 Microscopic observation of vegetative cells

3.5.1 Non-filamentous vegetative cells

An aliquot of a freshly growing culture (1 day-old) was inoculated in a 100 ml Erlenmeyer flask containing 30 ml of YM broth and then incubated at 25°C with continuous shaking (250 rpm). After 24 h cell mass was concentrated by centrifuging 1.5 ml culture for 1 min at 8000 x g. Yeast cells were stained with methylene blue and observed under the microscope (Leica ATC 2000) at 10X x 40X and 10X x 100X magnification.

Observed cells were photographed with a (Cosina C1s) manually operated camera. Photonegatives were developed and printed. Photopositives were scanned with a HP Scanjet 3670 scanner.

3.5.2 Filamentous vegetative cells

A piece of sterile filter paper was placed in to a sterile Petri dish and a sterile glass rod was put over it. Two sterile microscopic slides were dipped separately in to warm potato dextrose agar (PDA) media kept in a wide and deep tube. The slides were drained and replaced over the glass rod support in the Petri dish. These slides were lightly inoculated with actively growing culture along the length and a sterile coverslip was put over a portion of the inoculated agar. The same were incubated at 25°C. After wiping the agar from the back of each slide they were observed under microscope every alternate day to assess the presence of filamentous growth.

3.5.3 Microscopic observation for ballistoconidia

Yeast from a growing young culture (1 day-old) was inoculated in a Petri dish containing Corn Meal Agar (CMA) medium in straight lines laid out at right angles. Each inoculated Petri dish was inverted over another Petri dish containing the same medium and a sterile microscopic slide placed over the media. The two Petri dishes were tied together all round the circumference and incubated at 20°C for 3 weeks. The lower Petri dish was observed periodically to
see the presence of colony formed from the discharged ballistoconidia of the upper Petri dish. The slide was observed under the microscope to locate the presence of ballistoconidia.

3.5.4 Microscopic observation for ascospores

An actively growing young yeast culture (1 day-old) was inoculated in to a Petri dish containing ascosporulation-medium V8 agar (Himedia) and (CMA) (Himedia, M146), and incubated at 25°C for 3 days. Aliquots from this culture were observed up to 6 weeks under the microscope at 10X x 40X and 10X x 100X magnification for the presence of ascospores.

3.6 Biochemical characterization

3.6.1 Aerobic growth response in different carbon sources

Aerobic growth test or assimilation test was done to examine the ability of the yeast species to use organic compounds as the sole source of carbon. The test was done in test tubes of 180 mm x 16 mm size containing yeast nitrogen base and 50 mM of the test substrates. Nitrogen base with D-glucose was the positive control and without carbon source as negative control. The yeast strain, grown overnight on YM agar, was suspended in nitrogen base to get ca. 25x10⁶ cells ml⁻¹. An aliquot of 100 μl of this suspension was inoculated in a test tube containing 10 ml of YNB and test substrate and incubated at 25°C in continuous shaking (250 rpm) condition. The growth of culture tube was measured spectrometrically at every alternate day up to 1 week and at weekly interval there after up to 4 weeks.

3.6.2 Aerobic growth response in different nitrogen sources

To examine the ability of the yeast strains to use nitrogen compounds for aerobic growth 5mM of the test substrate was taken in 10 ml of yeast carbon base. This was inoculated with an aliquot of 100 μl from a suspension of 25x10⁶ cells ml⁻¹. The pH of the media was adjusted to 6.5 as toxic nitrous acid may form at pH values below 6. The inoculated tubes were incubated at 25°C in continuous shaking condition. The growth of the culture tube was measured
spectrometrically at every alternate day up to 1 week and at weekly interval thereafter.

3.6.3 Determination of the ability to use sugar anaerobically

In a 50 ml-test tube 15 ml yeast extract medium at a concentration of 0.5% (w/v) was taken along with 50 mM of the test sugar. The test sugars were sterilized by autoclaving. One small test tube with the same medium was put in to the large one in inverted position and sterilized. No sugar was added to the negative control. Tubes were inoculated with 100 µl of actively growing yeast suspension of ca. 10⁷ cells ml⁻¹ and incubated at 25°C for 7 days with regular shaking to sediment the yeast growing on the upper part of each tube and examined for the appearance of bubbles of gas. Emission of CO₂ replaces the media inside the inner tube and was regarded as the positive.

3.6.4 Vitamin requirement by yeasts

To determine the requirement of vitamins, yeast strains were cultivated initially in medium having no vitamins. Culture medium was taken in a series of test tubes, each with one vitamin and the yeast strains were tested for its growth. Vitamins added to each of the media are biotin, folic acid, myo-inositol, p-aminobenzoic acid, nicotinic acid, calcium pantothenate, pyridoxine hydrochloride, riboflavin and thiamin hydrochloride.

3.6.5 Production of extracellular starch like compounds

One drop of Lugol’s iodine solution was added to each yeast culture tube having the liquid culture medium, which showed positive results in the presence of a sugar. The positive result was highlighted with appearance of blue/purple/green colour.

Preparation of Lugol’s iodine

Iodine 5 gm and 10 gm of potassium iodide were dissolved in 10 ml of distilled water and the volume was made up to 100 ml. The solution was diluted with distilled water in the ratio of 1:5 for subsequent use.

3.6.6 Growth at high osmotic pressure

To assess the osmotic tolerance, yeast strains were grown in yeast extract agar medium containing 50 and 60 % (w/v) D-glucose and 10 and 16 % (w/v)
Sodium chloride. Slants of yeast extract agar were prepared and were inoculated with actively growing (1 day-old) yeast strains and incubated at 25°C for growth. Observations were recorded up to 4 weeks of culture.

3.6.7 Growth in cycloheximide containing medium

Test tubes (180 mm x 16 mm size) containing yeast nitrogen and 50 mM of D-glucose were supplemented with filter sterilized cycloheximide solution to result the concentration of 0.1% and 0.01% (w/v). Yeast strains, grown overnight on YM agar medium was suspended in nitrogen base to get ca. 25x10⁶ cells ml⁻¹. An aliquot of 100 µl of this suspension was inoculated in a test tube and incubated at 25°C in continuous shaking (250 rpm) condition. The growth of the yeast strains in cultures tube was measured spectrophotometrically at every alternate day up to 7 days and thereafter at weekly interval up to 4 weeks.

3.6.8 Test for urea hydrolysis by urease enzyme

The test was carried out to determine the activity of urease enzyme secreted by the yeast strains. Urea broth was dispensed in aliquot of 0.5 ml in to test tubes and was kept in deep freeze for 6 weeks. A loopful of cells from an actively growing 1 day-old culture was suspended in to it and incubated at 37°C. Tubes were observed at every half an hour interval up to 4 h for change to red colour. Appearance of red colour indicate the presence of urease activity.

3.6.9 Diazonium blue B (DBB) test

A positive response to DBB is indicative of the presence of basidiomycetous yeast. A 10 days-old culture of yeast strains on YM agar was held at 55°C for 5 h and then flooded with DBB reagent. As and when the culture turned dark red within 2 min at room temperature, it was regarded as positive.

Preparation of Diazonium blue B (DBB) reagent

Diazonium blue B salt @ 1 mg/ml was added to cold 0.1 M tris-HCl buffer (pH 7) and mixed thoroughly. The reagent was kept ice cold and used within few min of the preparation before it discolours.
3.6.10 Composition of various media

i) YM (malt-yeast-glucose-peptone-agar)
Dried yeast extracts 3.0 g
Dried malt extracts 3.0 g
Mycological Peptone 5.0 g
D-glucose 10.0 g
Water 1000 ml
Unadjusted pH ~5.5
Agar 20% was added for solid medium

ii) CMA (corn meal agar)
Yellow corn meal 12.5 g was stirred with 300 ml of water at 60°C for 1 h. Filtrate was diluted to 300 ml and 3.8 g agar was added to it. The medium was sterilized by autoclaving at 120°C for 15 min.

iii) V-8 juice agar
This medium contains a mixture of juices from several vegetables and baker's yeast. In a vessel 14 g agar was dissolved in 340 ml of water. In another vessel 350 ml of V-8 (Campbell Camden, N. J. USA) juice was well mixed with 5 g of compressed yeast previously dispersed in 10 ml of distilled water. The content was heated for 10 min in steam and the pH was adjusted to 6.8 at 20°C. The content of both vessel were mixed and autoclaved.

iv) PDA (potato dextrose agar)
Potatoes were washed thoroughly, peeled and finely grated. 100 g of these potato was soaked overnight in 300 ml of water in a refrigerator, filtered through muslin cloth. The filtrate was autoclaved for 1 h at 120°C. Then 230 ml of the autoclaved extract was added to 730 ml of distilled water, 20 g of D-glucose and 20 g of agar. The medium was sterilized at 120°C for 15 min.

v) Chemically defined media
a) (YMA) Yeast Morphology Agar
The medium contains all ingredients as listed below and agar 2% (w/v).
b) YNB (Yeast Nitrogen Base)

It contains all ingredients listed below: except 5 g (NH₄)₂SO₄ and no L-Asparagine or no D-glucose.

c) YCB (Yeast Carbon Base)

It contains ingredients as listed below; without major source of nitrogen, but with 1 mg L-histidine, 2 mg DL-methionine and 2 mg DL-tryptophan.

d) Vitamin free medium

The medium contains all ingredients of chemically defined media listed below, except 5 g (NH₄)₂SO₄, no L-Asparagine and no growth factors:

Table 3.1 List of ingredients of Chemically defined media

<table>
<thead>
<tr>
<th>Source</th>
<th>Compound</th>
<th>Quantity</th>
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<tr>
<td>Nitrogen sources</td>
<td>(NH₄)₂SO₄</td>
<td>3.5 g</td>
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<tr>
<td></td>
<td>L-Asparagine</td>
<td>1.5 g</td>
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<tr>
<td>Carbon source</td>
<td>D-glucose</td>
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<tr>
<td>Amino acids</td>
<td>L-Histidine</td>
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<tr>
<td></td>
<td>DL-Methionine</td>
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<tr>
<td></td>
<td>DL-Tryptophan</td>
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<tr>
<td>Growth factors</td>
<td>Biotin</td>
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<tr>
<td></td>
<td>Folic acid</td>
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<tr>
<td></td>
<td><em>Myo-inositol</em></td>
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<tr>
<td></td>
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<tr>
<td></td>
<td>Ca pantothenate</td>
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<tr>
<td></td>
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<tr>
<td></td>
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<td></td>
<td>Na₂MoO₄.2H₂O</td>
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<tr>
<td></td>
<td>ZnSO₄.7H₂O</td>
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3.6.11 Identification of the yeast strains based on biochemical and morphological tests

Yeast strains were identified based on the identification keys as described by Barnett et al. (2000). The physiological and biochemical characterization of the yeast strains were also carried out following the procedures and keys described by Deak (1986), Teramoto et al. (2001) and Tamang and Sarkar (1995).

3.7 Molecular genetic characterization

3.7.1 Yeast genomic DNA isolation

Burst and Grab protocol described by Harju et al. (2004) with certain modification, was used for the isolation of genomic DNA of the yeast strains.

1. Actively growing (20-24 h) 5ml culture (YM broth) was taken in a microcentrifuge tube and pelleted by centrifuged at 13,500 rpm at room temperature.
2. Added lysis buffer 300 µl (25 TritonX-100, 1% SDS, 100 mM NaCl, 10 mM tris-HCl (pH 8) and 1mM EDTA (pH 8) in to the pellet.
3. Submerged the tube in to a liquid nitrogen bath for 2 min and transfer to a hot water bath of 95 °C for 1 min. Repeat the cycle and vortexed for 30 sec.
4. Added 300 µl chloroform and vortexed for 2 min.
5. Centrifuged at room temperature for 10 min at 13500 rpm.
6. Transfer upper aqueous layer to another microcentrifuge tube containing 500 µl ice cold 100% ethanol.
7. Incubated at room temperature for 5 min.
8. Centrifuged at room temperature for 15 min at 13500 rpm. Removed supernatant.

9. Washed the pellet with 0.5 ml of 70% ethanol. Spin down by centrifuging at room temperature for 15 min at 13500 rpm. Removed supernatant.

10. Air-dry the pellet at room temperature.

11. Resuspended the pellet in 50 μl TE buffer.

12. Added 3 μl of RNase A (10mg/ml) and incubated at 37°C for 30 min and stored at 4°C.

3.7.2 DNA quantification and purity test by UV spectrophotometry

1. An aliquot of 5 μl of DNA sample was put in to a quartz cuvette and made up the volume to 3 ml.

2. The cuvette was placed in to the UV- spectrophotometer (Beckman DU® 530 Life Science UV/VIS spectrophotometer) and absorption was measured at 260 nm and at 280 nm along with the ratio of OD_{260} and OD_{280}.

3. DNA concentration was calculated by using the relationship of soluble standard DNA being 1 O.D. at 260 nm = 50 μg/ml.

4. The ratio between the absorption data at 260 nm and 280 nm was calculated to check the purity of the isolated DNA. A good DNA preparation exhibits a ratio value (ratio) in between 1.75 and 2.0.

3.7.3 Electrophoresis of yeast genomic DNA

1. Agarose 0.8% solution was prepared in a 250 ml conical flask by adding 49 ml of distilled water, 1ml of TAE buffer (1x) and 0.4g of agarose powder.

2. The solution was put in a microwave oven for 1-3 min until the agarose was fully dissolved. Prior to the solidification of the gel, 2 μl of ethidium bromide (final concentration of 0.5 μg ml^{-1}) was added to the solution.

3. The pre-cleaned gel tray was cello-taped in both ends and the comb was placed in the tray approximately 2.5cm from the end.
4. The gel solution was poured in to the tray to a depth of approximately 5 mm and then allowed to solidify for about 30 min at room temperature.

5. After the solidification of the gel, the comb and cello-tapes were removed. The gel was then put in the electrophoresis tank and then covered with about 250 ml of 1x TAE buffer or until the wells of the gels were submerged.

6. From each digestion mixture, DNA sample (5 µl) was taken and mixed with 5 µl of bromophenol blue. All samples were then loaded into wells of the gel.

7. Marker DNA (3 µl λ Hind III digested DNA) was loaded on the extreme left side of the gel or on both sides of the gel. The gel was electrophoresed at 150V for 1 h until the dye marker migrated to two third distance of the gel.

8. After electrophoresis, the DNA was visualized in a trans-illuminator and documented by taking photographs in a Gel Doc system (BIO RAD Gel Doc 1000).

3.7.4 Reagents, chemical and solution

10 M TrisHCl (pH 8.0)

Tris base 121.1 g was dissolved in 80 ml of distilled water (dH₂O). The pH was adjusted to 8.8 by adding concentrated HCl. The solution was allowed to cool to room temperature. The volume was adjusted to 1 l. The solution was dispensed in aliquots, sterilized by autoclaving and stored at room temperature.

5 M NaCl

NaCl 292.2 g was dissolved in 800 ml of water and adjusted the volume to 1 l. Dispensed into aliquots and sterilized by autoclaving.

10 % SDS

Electrophoresis grade SDS 10 g was dissolved in 900 ml of distilled water, heated to 68 °C for complete dissolving. The pH was adjusted to 7.2 by adding a few drops of concentrated HCl. The volume was adjusted to 1 l and dispensed into aliquots.
10 % (v/v) TritonX-100

TritonX-100 10 ml was dissolved in 90 ml of distilled water. The solution was mixed and sterilized by Millipore filter paper, stored in dark condition at room temperature.

0.5 M EDTA

Na$_2$EDTA.2H$_2$O 186.1 g was dissolved in 800 ml of distilled water and stirred vigorously. The pH was adjusted to 8.0 with 10 M NaOH. The volume was adjusted to 1 l, sterilized by autoclaving and stored at room temperature.

5X TBE buffer

Tris 5.4 g, boric acid 2.75 g and 0.5 M EDTA 2 ml (pH 8.8) were mixed in distilled water and make up the volume to 100 ml. The solution was stored at room temperature.

Bromophenol blue (loading dye)

(6 X loading dye, 20 ml)

Glycerol (autoclaved) 10 ml was added to 5 ml of 1 X TBE. To the solution, 50 mg of bromophenol blue was added, the volume adjusted to 20 ml and stored at 4°C

Ethidium bromide (10 mg/ml)

Ethidium bromide 100 mg was dissolved in 10 ml sterile distilled water, mixed properly and then stored at 4°C in darkness.

3.7.5 Random Amplified Polymorphic DNA (RAPD) analysis

RAPD-PCR was done according to the method described by Succi et al. (2003) and Torriani et al. (1999) with some modification. The PCR reaction mixture (25 μl) was prepared as follows:

1. 10 mM Tris-HCl (pH 8.3)
2. 200 μM DNTP mixture
3. 1.5 mM MgCl$_2$
4. 20 ng primer
5. 80 ng DNA sample for Primer M13, 20 ng for Primer 21 and Primer RF2
6. 2 U Taq DNA polymerase
7. 2.5 μl 10 X PCR buffer without MgCl$_2$. 

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Amplifications were performed in a mastercycler gradient, GeneAmp PCR System 9700 (Applied Biosystem) using the following primers and amplifications:

**Primer M13** (5'-GAG GGT GGC GGT TCT- 3')

**Primer 21** (5'-GCT CGT CGC T- 3')

- Initial denaturation at 94°C for 2 min
- 40 cycles at 94°C for 1 min
- Annealing at 45°C for 20 sec
- Extension at 72°C for 2 min
- Final extension at 72°C for 5 min

Amplification products were separated by gel electrophoresis on 1.4 % (w/v) agarose gel in 0.5 X TBE buffer.

RAPD-PCR a profile for both the primer were combined together for each of the strain and dendrogram was constructed that allowed classification of the yeast strains based on their similarity level.

### 3.7.6 Restriction Fragment Length Polymorphism (RFLP)

PCR-RFLP analysis of the internally transcribed ribosomal spacer region and the 5.8S rDNA was done according to the method described by Naumova et al. (2003) with some modifications. The PCR reaction mixture (50 µl) was prepared as follows:

1. 100 ng genomic DNA
2. 0.2 mM of each DNTP
3. 50 pM of each primer ITS1 and ITS4
4. 5 µl 10 X PCR buffer with 15mM MgCl₂
5. 2.5 U Taq DNA polymerase

Amplifications were performed in a mastercycler gradient GeneAmp PCR System 9700 (Applied Biosystem) using the following primers and amplifications:

**Primer ITS1** (5'-TCC GTA GGT GAA CCT GCG G- 3')

**Primer ITS4** (5'-CCT CCG CTT ATT GAT ATG C- 3')
Initial denaturation at 94°C for 3 min
30 cycles at 94°C for 2 min
Annealing at 60°C for 1 min
Extension at 72°C for 1 min
Final extension at 72°C for 5 min

Amplification products were separated by gel electrophoresis in 1.2% (w/v) agarose gel at 65 V in 0.5 X TBE buffer.

RFLP assay was carried out with Hinfl, HpaII and HaeIII restriction endonucleases purchased from Bangalore Gennie, India. Amplified DNA (10 μl) was digested with 2 units (U) restriction endonuclease at 37°C overnight. The restriction digests were visualized by electrophoresis in 2.5 % (w/v) agarose gel at 65 V in 0.5 X TBE buffer for 2 h. Similarity among various profiles resulting from the digestion with eadonucleases and separation by gel electrophoresis was determined by Jaccard coefficient and also by clustering of strains which was accomplished by using Unweight Pair Group Method with arithmetic average (UPGMA).