2.0 MATERIALS AND METHODS

2.1 Screening of bioadhesive producing bacteria

2.1.1 Primary screening based on mucoidal nature of the colony

For this variety of samples from different ecological niches were used (as enlisted in Table 3A). Bacterial isolates producing exopolysaccharide (EPS) were selected on the basis of mucoid phenotype of colonies, grown on a high C:N ratio medium containing (g/l): Sucrose, 40; K$_2$HPO$_4$, 0.5; MgSO$_4$.7H$_2$O, 0.2; CaCl$_2$.2H$_2$O, 0.1, and NaCl, 0.1 with or without yeast extract (0.1 g/l) supplementation. As nitrogen source either KNO$_3$ (1 g/l) or monosodium glutamate (0.2 g/l) was added. pH of the medium was adjusted to 7.2 ±0.2 and sterilized by autoclaving at 10 psi for 20 minutes. The culture plates were incubated at 30 ±1 °C for 48 h and bacterial isolates producing copious amount of EPS were selected and preserved in the form of agar slopes at 4 °C.

2.1.2 Secondary screening based on adhesive property

Initially using whole cell biomass from culture plate, “paper peel test” was carried out to assess the adhesive nature of the isolates and isolates showing comparatively better adhesive nature were cultivated in above mentioned liquid culture medium, EPS was recovered from culture filtrate by acetone precipitation, dried in oven (50 °C) and aqueous 10 % (w/v) EPS was used for a test. Using printing paper (60 gsm), pieces having dimension of 6 x 5 cm were cut and EPS solution was applied evenly using brush over 5 cm$^2$ area of paper leaving un-gummed flap of one cm size. Such pieces with applied adhesive were pasted firmly on a large piece of same paper and kept for 1 h at a temperature of 30 ±1 °C and relative humidity of 50 ±5 %. Using un-gummed flap, adhered paper pieces were pulled apart to assess the adhesive nature of different isolates (Bureau of Indian Standard, 1989).
2.2 Study on EPS-BM adhesive

2.2.1 Cultivation of the organism and production of EPS-BM adhesive at shake flask level

The isolate was grown by inoculating 10 % (v/v) of 0.4 O.D$_{600nm}$ into 50 ml medium (pH 7.2 ±0.2) containing (g/l): Sucrose, 40; KNO$_3$, 1; K$_2$HPO$_4$, 0.5; MgSO$_4$.7H$_2$O, 0.2; CaCl$_2$.2H$_2$O, 0.1; NaCl, 0.1; and yeast extract, 0.1 in 250 ml Erlenmeyer flask, on a rotary shaker (180 rpm) at 30 ±1 °C for 72 h. Biomass from the culture broth was separated by centrifugation (at 10,000 rpm for 30 minutes) at 4 °C, the EPS was precipitated from the supernatant using 3 volumes of chilled acetone, re-dissolved in distilled water, precipitation step was repeated, precipitates were dried at 50 °C to a constant weight, ground to powder form and used for adhesion studies.

2.2.2 Characterization of EPS-BM adhesive

2.2.2.1 Gross chemical analysis of EPS-BM adhesive

Thrice precipitated EPS solution was dialysed against distilled water for 18 h freeze-dried and 1 % (w/v) aqueous solution was used for chemical analysis.

2.2.2.1.1 Estimation of total sugars

This was estimated by following phenol-sulphuric acid method of Dubois et al. (1956). To 1 ml of sample, 1 ml of 5 % phenol was added and mixed. Using a fast flowing pipette, 5 ml of concentrated H$_2$SO$_4$ was added directing the stream of acid onto the surface of the liquid and shaking the tube simultaneously. The tubes were allowed to stand for 10 minutes, cooled to 30 ±1 °C and the absorbance was measured at 488 nm. Glucose was used as the reference sugar in the range of 20 to 100 μg.

2.2.2.1.2 Estimation of uronic acid

This was carried out using the method as described by Bitter and Muir (1962). To 1 ml of sample, 5 ml of borate reagent (0.025 M sodium tetraborate in concentrated sulfuric acid) was added, mixed, heated in a boiling water bath for 10
minutes and cooled to room temperature. 0.2 ml of carbazole reagent [0.125 % (w/v) in ethanol] was then added and the mixture was again heated for 15 minutes in a boiling water bath, cooled and the absorbance was measured at 530 nm. Glucuronic acid (5 to 100 μg) was used as the standard.

2.2.2.1.3 Estimation of pyruvate

Pyruvyl content in EPS-BM adhesive was estimated by the method as described by Slonekar and Orentas (1962). Various aliquots of sample (2 ml each) were hydrolysed with 1 N HCl for 3 h, and incubated with 1 ml of 2,4-dinitrophenyl hydrazine reagent (0.5 % w/v in 2 N HCl) for 5 minutes. The reaction mixture was extracted with 5 ml of ethyl acetate and the aqueous layer was discarded. The ethyl acetate fraction was further extracted with 5 ml of 10 % (w/v) Na₂CO₃ and the concentration of pyruvic acid was determined by measuring the absorbance at 375 nm. Pyruvic acid was used as a standard in concentration range of 0.01 to 0.05 μg/ml.

2.2.2.1.4 Estimation of acetyl content

The estimation of O-acetyl groups in the EPS-BM adhesive was carried out as described by Hestrin (1949), using acetylcholine (0.1 to 1 mg) as a standard. To 1 ml aliquot of hydrolyzed polysaccharide sample, 2 ml of freshly prepared alkaline hydroxyamine reagent [hydroxylemine-HCl, 2 M and NaOH 3.5 N, 1:1] was added. The pH of the reaction mixture was adjusted to 1.2 ±0.2 using 6 N HCl, 1 ml of FeCl₃ solution was added and the absorbance was read at 540 nm.

2.2.2.1.5 Estimation of protein content

The presence of protein in EPS-BM adhesive was analysed by using the method of Bradford et al. (1976). To 0.1 ml of sample, 1 ml of Coomassie Brilliant Blue G-250 reagent (10 mg/100 ml, made in a mixture of water, absolute ethanol and O-phosphoric acid in the ratio of 8:1:1) was added. After incubation for 2 minutes, the absorbance was noted at 595 nm. Bovine serum albumin was used as standard in concentration range of 5 to 100 μg.
2.2.2.1.6  Estimation of phosphate

Phosphate, associated/complexed with EPS-BM adhesive was estimated by the method as described by Fiske-SubbaRow (1925). One ml aliquot of polysaccharide samples were treated with 1 ml of 5 N H$_2$SO$_4$ followed by the treatment with 1 ml of 2.5 % Ammonium molybdate solution. After mixing thoroughly, 0.1 ml of reducing ANSA (0.2 g 1-amino-2-naphthol-4-sulfonic acid, 1.2 g Na$_2$HSO$_3$ and 1.2 g of Na$_2$SO$_3$ in 100 ml distilled water) reagent was added and the total volume of the tube was made up to 10 ml with distilled water. The reaction mixture was further incubated for 10 minutes at 30 ±1 °C and the absorbance was measured at 660nm. Phosphate (KH$_2$PO$_4$) was used as a standard in concentration range of 0.1 to 1 μM.

2.2.2.1.7  Determination of monomeric composition of EPS-BM adhesive

For this purpose EPS hydrolysate was used. EPS-BM adhesive was hydrolysed with 2 M tri-fluoro acetic acid (TFA) at 100 °C for 2 h and TFA was evaroted in vacuo, freeze-dried and used for analysis.

2.2.2.1.7.1  Paper chromatographic analysis

The monosaccharide composition of the hydrolysed polymer was initially determined by paper chromatography using Whatman filter paper-one and butanol/pyridine/water (6:4:3 by volume) as mobile phase. The developed chromatograms were dipped into silver nitrate reagent for one minute, dried, and were sprayed with ethanolic 0.5 N NaOH solution to visualize the spots. Silver nitrate reagent was prepared as described by Trevelyan et al. (1950).

2.2.2.1.7.2  High performance liquid chromatography (HPLC) analysis

The monosaccharide composition of the hydrolyzed polymer was also analyzed by using Waters HPLC model 2410 with Sugar-Pak™ I column and Millennium 32 software. Water containing EDTA (40 mg/l) and CaCl$_2$ (15 mg/l) was used as a mobile phase at a flow rate of 0.4 ml/min, internal oven temperature 40 °C and the peaks were detected using refractive index detector. 30 μl of either standards (20 mg/ml) or hydrolysate (20 mg/ml) was injected. Authentic monosaccharide standards were also run to determine the identity of the peaks.
2.2.2.2 Fourier transform-infrared (FT-IR) analysis

The pellets for analysis were obtained by grinding a mixture of 1.2 mg of purified EPS-BM adhesive with 150 mg of dry KBr powder followed by pressing the mixture into a mold (Yanping et al., 2010). The FT-IR spectrum was recorded in the region of 4000–2500 cm\(^{-1}\), at a resolution of 1 cm\(^{-1}\) by using Perkin-Elmer FT-IR spectrometer (Germany).

2.2.2.3 Determination of ionic nature

The ionic nature of the EPS-BM adhesive was determined by measuring its efficiency of binding to anion (Dowex 1) and cation (Dowex 50) exchange resins as described by Ashtaputre and Shah (1995). Resins (5 g each) were activated by treating with either NaOH (1 N) or HCl (1 N), washed with water and then equilibrated with buffers of 10X strength (100 mM) i.e. Tris-HCl (pH 8.9) for Dowex 50 and acetate buffer (pH 5.0) for Dowex 1. Columns (1x6 cm) were packed with the resins and aqueous solution of EPS-BM adhesive (0.5 mg/ml) was loaded on to each of the columns. EPS-BM adhesive was then eluted with the respective buffers (10 mM), and the fractions (0.5 ml each) collected were subjected to total sugar estimation using phenol-sulphuric acid method. The bound polymer was quantitated using a calibration curve.

2.2.2.4 Molecular weight determination

The molecular weight of the EPS-BM adhesive was determined by gel permeation chromatography performed on sepharose-4B column. Samples (250 µg) were loaded onto the column and eluted with 10 mM phosphate buffer (pH 7.2 ±0.05) at a flow rate of 0.5 ml/min. Fractions (2 ml) were collected and subjected to total sugar estimation by the phenol-sulphuric acid method. Dextrans of different molecular weight ranging from 9 x 10^3 to 2x10^6 Da were used as standards.

2.2.3 Evaluation of the adhesive property

Specimens such as wood, metals and plastics were used individually and in combinations for evaluation of adhesive property. Specimen dimensions were 30×2.5×0.3 cm for wood (Shorea robusta) and 30×2.5×0.1 (metals) /0.2 cm (plastics). Surfaces of adherends were roughed using sand paper, wiped with ethanol and an aqueous adhesive (10 % w/v, having pH 7) was applied (13.33
mg/cm²) to 2.5x3 cm corner area of one of the adherend and the second adherend overlapped that area. Both adherends were clamped tightly together for 1 h and were pressed under 2.5 kg weights for further 18 h to allow the adhesive to set up. Finally bonded specimens were cured for 7 days at 30 °C ±1 and 50 ±5 % relative humidity. Lap shear strength was determined using Universal Testing Machine (UTM) model Alfred J. Amsler and Co. Schaffhouse (Switzerland) 223/445 and reported in terms of MPa (1MPa = 10.197 Kg/cm²). Above studies were an adaptation of ‘American Standard for testing Material’ ASTM D905-03 (2003) (for wood as well as other similar materials) and D1002-10 (2010) (metal to metal). Fevicol (48 to 50 % w/v solids), a commercial wood cum multipurpose adhesive, was used as a positive control for wood specimen. Each individual determination was carried out in triplicate and each complete experiment was carried out twice.

2.2.3.1 Effect of environmental factors (temperature, pH and salinity) on adhesive strength

Curing at 4 °C, 30 °C (Control- relative humidity 50 ±5 %) and 50 °C was carried out for seven days to study the effect of temperature on shear strength of bonded specimen.

An Adhesive solution was prepared in distilled water priorly adjusted to pH 4, 7 (control) and 8 and was used to see the effect of pH on shear strength.

Adhesive used contained none (control). 5 % or 20 % (w/v) NaCl to follow the effect of salinity on shear strength.

2.2.4 Yield improvement of EPS-BM adhesive

2.2.4.1 Box-Behnken design

For media optimization, “Fractional Factorial Design” approach (Box-Behnken design) was used and an attempt was made to standardize the concentrations of main medium components i.e. carbon, nitrogen and phosphate. Factorial design involving three factors at three levels was created using Statgraphics plus 3.1 software. Each factor was assigned a low (-), middle and a high (+) value. The values of the variables were as given in the table 2.1. Accordingly fifteen medium
flasks were prepared along with the other nutrients of the growth medium and were processed as described previously in the section (2.2.1). Two responses i.e. EPS yield (g/l) and biomass (g/l) were determined after 72 h. The order of experiments was fully randomized to provide protection against the effects of lurking variables.

**Table 2.1 Levels of independent process variables used for Box-Behnken design**

<table>
<thead>
<tr>
<th>Test</th>
<th>Sucrose (g/l)</th>
<th>KNO₃ (g/l)</th>
<th>K₂HPO₄ (g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50</td>
<td>1</td>
<td>0.25</td>
</tr>
<tr>
<td>2</td>
<td>50</td>
<td>1</td>
<td>0.75</td>
</tr>
<tr>
<td>3</td>
<td>30</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>4</td>
<td>40</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>5</td>
<td>50</td>
<td>1.5</td>
<td>0.5</td>
</tr>
<tr>
<td>6</td>
<td>40</td>
<td>1.5</td>
<td>0.25</td>
</tr>
<tr>
<td>7</td>
<td>40</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>8</td>
<td>30</td>
<td>1</td>
<td>0.75</td>
</tr>
<tr>
<td>9</td>
<td>40</td>
<td>0.5</td>
<td>0.25</td>
</tr>
<tr>
<td>10</td>
<td>40</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>11</td>
<td>30</td>
<td>1.5</td>
<td>0.5</td>
</tr>
<tr>
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<td>1.5</td>
<td>0.75</td>
</tr>
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<td>0.25</td>
</tr>
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<td>0.5</td>
<td>0.75</td>
</tr>
<tr>
<td>15</td>
<td>50</td>
<td>0.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>
2.2.4.1.1 Growth measurement

Biomass from the culture broth was separated by centrifugation at 10,000 rpm for 30 minutes; cell pellet was washed twice, transferred to pre-weighed aluminium foil and dried at 50 °C to a constant weight. When required, dry weight was also determined by estimating the whole cell protein of cell aliquote and was converted to dry weight using standard relationship established between whole cell protein and dry weight.

2.2.4.1.2 Estimation of EPS

The EPS was recovered from cell free supernatant by precipitation using 3 volumes of chilled acetone, re-dissolved in distilled water, precipitation step was repeated, and precipitates were dried in oven (50 °C) for 2 h to a constant weight.

2.2.4.2 Effect of maintenance of pH and supplementation of phosphate on EPS production and growth

The culture was cultivated as described previously in the section (2.2.1) in the several 250 ml flasks, each containing 50 ml buffered (0.02 to 0.1 M, pH 7.2 ±0.2 phosphate/tris buffer) and also non-buffered but phosphate supplemented (0.02 to 0.1 M K$_2$HPO$_4$, pH 7.2 ±0.2) medium for 72 h. The control medium was non-buffered and contained 0.003 M K$_2$HPO$_4$ only. Culture flasks were harvested after incubation period; biomass and EPS yield were estimated gravimetrically as described above (section-2.2.4.1.1 and 2.2.4.1.2).

2.2.4.3 Effect of aeration on EPS production

This was observed by making variation in volume of broth/flask volume. The culture was cultivated as described previously in the section 2.2.1 in the several 250 ml Erlenmeyer flasks containing 20, 40, 50 (control), 60 and 100 ml growth medium for 72 h. Culture flasks were harvested after incubation period of 72 h, biomass and EPS production were estimated gravimetrically as described above (section-2.2.4.1.1 and 2.2.4.1.2).
2.3 Characterization of strain ADE-0-1

2.3.1 Morphological, cultural and biochemical characterization

The strain was primarily characterized by morphological and cultural features and biochemical tests and identified following Bergey’s Manual of Systematic Bacteriology (Sneath, 1986).

2.3.2 Phylogenetic characterization

“Bacterial Identification Service” based on partial 16S rDNA gene analysis, provided by Bangalore Genei, Bangalore, India; was used for confirmation of identification of the strain.

For this, i) isolation of genomic DNA, ii) amplification of ~1.5 Kb 16S rDNA fragment using the "universal" forward primer 27f (5’-AGA GTT TGA TCC TGG CTC AG-3’) and reverse primer, 1541r (5’-AAG GAG GTG ATC CAG CCG CA-3’) and high-fidelity PCR polymerase (Macrae et al., 2000), iii) bi-directional sequencing of PCR product using forward and reverse primers, iv) alignment and analysis of sequence data for finding the closest homologues for strain were carried out.

2.4 Study on biofilm

The culture was cultivated, using the medium and conditions as described previously (in section-2.2.1), in the several (fourteen) 250 ml flask each containing 50 ml medium with two glass slides (7.5X2.5X0.2 cm). Two flasks were harvested at regular interval of time and slides/samples were used for measurement of biomass, biofilm density, EPS production, cell surface polysaccharide and pH change. Samples for cell surface hydrophobicity and cell surface charge were obtained from similar type of separately cultivated culture flask.

2.4.1 Measurement of biofilm density

Biofilm developed on glass slides were quantified using “crystal violet staining procedure” as described by O’Toole et al. (1999). At regular interval of time, slides were removed from culture broth, loosely attached cells from the slides were removed by washing thrice with 2 M phosphate buffer saline (PBS-pH 7.4), slides were stained with crystal violet for 2 minutes, washed twice with 0.2 M
PBS (pH 7.4). Slides were exposed to absolute ethanol for 15 minutes and absorbance of eluted dye was measured spectrophotometrically at 595 nm.

2.4.2 Measurement of cell surface hydrophobicity

Relative bacterial cell’s surface hydrophobicity measurement procedure, developed by Rosenberg (1980), was followed for this purpose. Cells were separated by centrifugation, washed and resuspended in sterile distilled water to 0.4 O.D_{600nm}. Such 3 ml suspension was added to 3 ml hexadecane, mixed on a vortex mixer for 1 minute, incubated at 30°C for 10 minutes and then vigorously mixed again on a vortex mixer for 2 minutes. After settling, the absorbance of the aqueous phase was measured at 600nm after standing at ambient temperature for 20 minutes. The percentage of hydrophobicity (% h) was determined from the absorbance of the bacterial suspension (A_i) and the absorbance of the aqueous phase after mixing with hexadecane (A_d) using the formula:

\[
% h = \frac{(A_i - A_d)}{A_i} \times 100
\]

2.4.3 Measurement of electrochemical potential on cell surface

This was determined as described by Denyer et al. (1993). The culture was cultivated in the several 250 ml flask each containing 50 ml liquid growth medium and flasks were harvested at regular interval of time. The cell pellet was washed and resuspended in 1 ml of phosphate buffer (0.1 M, pH 7); the samples were adjusted to 0.7 O.D_{600nm}. The electrochemical potential of the cells harvested at different time intervals, was measured with an electrometer (keithley instruments, Model 6514 system electrometer). The mean value of six measurements were calculated and used for correlation with attachment.

2.4.4 Measurement of cell surface polysaccharides (CPS)

This was carried out as described by Zhang et al. (1999). Samples (culture broth) were removed at regular interval of time, centrifuged and cell pellet was resuspended in 1:1 (vol/vol) mixture of 0.01 M KCl and 2 % EDTA. The EDTA-cell suspension was incubated at 4 °C for 30 minutes, followed by centrifugation at 10,000 rpm for 30 minutes at 4 °C. Released carbohydrate content of the supernatants was determined by phenol-sulphuric acid method.
2.4.5 Microscopic examination of biofilm

2.4.5.1 Light microscopy

The culture was cultivated into 250 ml Erlenmeyer flask containing 50 ml growth medium with glass slides (two per flask) on a rotary shaker (180 rpm) at 30 ±1 °C for 72 h. Glass slides were removed at regular interval of time, washed thrice with 2M Phosphate Buffer Saline (PBS-pH 7.4) to remove planktonic cells/loosely adhered cells, stained by 1 % crystal violet for 2 minutes and washed twice with 0.2 M (PBS-pH 7.4) and observed under light microscope (Accumax UB200i) at 1000 X magnification.

2.4.5.2 Scanning electron microscopy

The culture was cultivated with glass slides as described above (section- 2.4.5.1). Glass slides were removed, rinsed thrice with sterile 2 M Phosphate Buffer Saline (pH 7.4), biofilms were first fixed using glutaraldehyde (2.5 % in PBS buffer) for 1 h, rinsed with PBS for 15 minutes and then using osmium tetroxide (1 % in PBS buffer) for 1 h, dehydrated by treating with ethanol, coated with gold and viewed at 2000X magnification under scanning electron microscope (JSM-5610LU, Hitachi, Japan).

2.4.5.3 Confocal Laser Scanning Microscopy (CLSM)

Culture was cultivated with glass slides as described above in the section (2.4.5.1). Glass slides were removed, rinsed thrice with sterile 2 M PBS (pH 7.4), stained with nucleic acid stain (SYTO 9) (Invitrogen) according to manufacturer’s instructions and observed at 400 X magnification under Zeiss (LSM 510 Meta) confocal laser scanning microscope (CSLM). Three independent slides and three to seven fields from each slide were randomly chosen to acquire images. Raw images were processed by Zeiss software ZEN2010.

2.4.6 Kinetics of biofilm forming ability in buffered and non-buffered medium

The culture was cultivated in the phosphate buffered (0.02 M, pH 7) and non-buffered (Control- only K₂HPO₄ at 0.003 M concentration) media as described
previously in the section (2.4.5.1). Glass slides were removed and biofilm density was estimated by “crystal violet staining procedure” as described previously (Section-2.4.1). The pH was measured through pH meter.

2.4.7 Kinetics of production of extracellular EPS-depolymerase

The culture was cultivated as described previously in the section (2.2.1) in the several 250 ml flask, each containing 50 ml medium, for 96 h. Culture flasks were harvested at regular interval of time and proteins present in culture filtrate were precipitated by 100 % (w/v) ammonium sulphate saturation, dissolved in water and used as a source of enzyme. Enzyme was assayed using 1 % (w/v) EPS as a substrate and acetate buffer (0.2 M, pH 5.6), Phosphate buffer (0.2 M pH 7) and tris-buffer (0.2 M pH 8.5). Released sugars were estimated by following di-nitro salicylic acid (DNS) method as described by Miller (1959).

2.4.7.1 Kinetics of removal of preformed biofilm cells on treatment with EPS-depolymerase

Proteins present in the culture filtrate, obtained after 72 h of growth period, were precipitated by 100 % (w/v) ammonium sulfate saturation, dissolved in acetate buffer (0.2 M, pH 5.6) and used as a source of enzyme EPS-depolymerase. Biofilms developed on glass slides, withdrawn at 24 h growth period, were treated with the enzyme for different period of time (0-5 h) at 30 ±1 °C. Biofilm density on enzyme treated slides and control slides was estimated by “crystal violet staining procedure”. From separate set of experiment, such slides were also observed by light microscopy at 1000 X magnification.

2.4.7.2 Assay of EPS-depolymerase activity

EPS-BM adhesive produced as described previously in the section (2.2.1) was used as a substrate for depolymerase. The reaction mixture consisted of 0.5 ml of 1 % EPS, 0.5 ml of acetate buffer (0.2 M, pH 5.6), an aliquote of enzyme preparation and distilled water to make up the total volume 2 ml. After 30 minutes of incubation at 30 °C, liberated reducing sugars were estimated, by following dinitrosalicylic acid (DNS) method as described by Miller (1959).
2.4.7.2.1 Estimation of reducing sugars

Reducing sugars were estimated by the method of Miller (1959). To 3 ml of sample in test tubes, 1 ml of DNS reagent was added and the test tubes were then placed in boiling water bath for 5 minutes, cooled to room temperature and the absorbance at 540 nm was measured. Glucose (up to 250 μg) was used as standard. DNS reagent was prepared by mixing solutions, (A) 300 g sodium potassium tartarate in 500 ml distilled water, and (B) 10 g of 3, 5-dinitro salicylic acid in 200 ml of 2 M sodium hydroxide. The final volume of the solution was made up to 1 litre with distilled water.

2.5 Chemicals

All chemicals used were of analytical grade and were obtained locally. Dextran molecular weights of different range and authentic sugars were obtained from Hi-Media, Mumbai, India. Dowex-1 and Dowex-50 ion exchange resins were obtained from Sigma chemicals Co., USA.

2.6 Reproducibility

Each individual determination was carried out in triplicate and each complete experiment was carried out twice.
2.7 References


