MATERIALS AND METHODS
I. Screening for benzoate degrading microorganisms

The seawater and sediment samples were collected from a depth of 75-100 metres from various stations of Bombay High oil fields, during the cruise on board, Sagar Pachimi in mid November 1999 (Fig. 6). Water samples from the industrial effluent discharge sites were also collected and screened.

Water samples were plated on mineral medium (MM) (Appendix A.1) agar plates supplemented with 0.1% sodium-benzoate. For viable count on nutrient agar medium (Appendix A.2), the water samples were diluted tenfold in saline (0.85% NaCl). The soil sediments were suspended in saline at a ratio of 1:10 and incubated on Orbitek shaker for 1 h at 150 rpm. The suspension was allowed to settle and 0.1ml of clear supernatant was spread plated.

Plates were incubated at room temperature (28°C ± 2°C) for 24 to 48 h. The colonies were counted and the viable count was calculated.
II. Tests for identification of the bacterial strain P_2d

**Cultural, morphological and biochemical characters:** Colony characters of the culture, strain P_2d were studied by streaking the culture on nutrient agar and benzoate agar medium. Motility was studied by the hanging drop technique. Culture was stained by Gram's staining method (210) and observed under the light microscope. Standard methods were followed to study the biochemical characteristics of the culture (210).

**Rothera’s test:** Ring cleavage of aromatic degradation was studied by modified Rothera’s method (211). Strain P_2d cells grown in MM with different aromatic compounds, were centrifuged at 6000 rpm for 20 min at 4°C. Pellets were washed twice and resuspended in 0.05M phosphate buffer pH 7 to an absorbance of 4.0 at 540 nm. To 2 ml cell suspension, 0.5 ml of toluene and 3 ml of 13.3 mM catechol (final concentration = 5 mM) were added. After 3 min, the colour change was noted. Appearance of yellow colour indicates presence of meta ring cleavage enzymes. The mixture was further incubated on shaker
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for 12 h following which, 1 g of ammonium-sulphate was added and the solution was mixed well. 5 drops of freshly prepared 1% sodium-nitroprusside solution and 0.5 ml of liquor ammonia were then added slowly along the side of the tube and the colour change was recorded. Presence of ortho ring cleavage enzymes results in a purple ring formation.

III. Growth of *Pseudomonas mendocina P₂d* in liquid medium

(a) Culture grown for 24 h in the presence of benzoate was used as inoculum. MM (20 ml) containing 0.3% benzoate in the culture flasks was inoculated with 5% inoculum and growth was monitored as increase in absorbance at 600 nm on Elico colorimeter (filter no. 60). To check the effect of inhibitors, 2,2'bipyridyl, at concentration of 2 mM was added to benzoate medium during growth.

Viable count of the culture was routinely observed by serial dilution in saline and plating on nutrient agar medium.
Plates were incubated at room temperature and the colonies formed were counted.

(b) Activities of enzymes involved in benzoate metabolism:

**Preparation of cells:** The cells, P2d grown in benzoate medium (0.3%) for 24 h, were pelleted by centrifugation at 6000 rpm for 10 min. The pellet was suspended in phosphate buffer (0.05M, pH 7), vortexed and again centrifuged. The washed pellet was resuspended in the same buffer to an absorbance of 4 at 540 nm.

**Preparation of cell free extract (cfe)** (212, 63): Cells of strain P2d were suspended in phosphate buffer containing 10% acetone to absorbance of 4.0 (42). The cells were sonicated under cold conditions at 150 mA for 5 min with pulses of 30 sec in between. The suspension was centrifuged at 16,000 rpm for 1 h at 4°C. The supernatant was used for enzyme assays.

**Preparation of HMS** (212, 213): Cfe of *Pseudomonas cepacia* AC1100 cells, preheated at 53°C for 10 min with 10 mM catechol in 50 mM phosphate buffer was incubated till HMS formation.
stopped, as seen from absorbance at 375 nm. It was then extracted with diethyl-ether followed by acidification of aqueous layer. Again extracted with diethyl-ether and reextracted in phosphate buffer. Molarity calculated from absorbance value.

(i) Catechol 2,3-dioxygenase (40): The reaction mixture of the assay was taken in 3 ml silica cuvette and consisted of 2.7 ml of 0.05M phosphate buffer (pH 7) with 100 µl of cells or cfe and catechol (final concentration 10 mM). The increase in absorbance at 375 nm was monitored. Specific activity of the enzyme was calculated and expressed as units (U)/mg protein.

Enzyme unit = increase/decrease in absorbance per min or activity per min.

(ii) The reaction mixture for catechol 1,2-dioxygenase was the same as for catechol 2,3-dioxygenase. The absorbance was monitored at 260 nm for 5 min. Cells/cfe heated at 60°C for 10 min were also used.

(iii) HMS hydrolase (63): To 2.7 ml of 0.05 M phosphate buffer (pH 7), 2.5 µM HMS and 100 µl of cells/cfe were added
and fall in the absorbance was monitored at 375 nm for 5 min.

(iv) HMS dehydrogenase (63): 2.5 μM HMS, 0.1 μM NAD and 100 μls of cells/cfe were added to 2.7 ml of buffer and the absorbance checked at 375 nm.

(v) Tyrosinase assay (119): Strain P₂d grown in MM supplemented with 0.2% tyrosine for 48 h was pelleted and resuspended in 0.1 M sodium-phosphate buffer (pH 6), to get an absorbance of 4.0 at 540 nm. Substrate solution was prepared with DL-Dopa (0.8 mg/ml) in same buffer as above. Reaction mixture contained 2.9 ml of substrate solution taken in a 3 ml cuvette, incubated for 5 min at 30°C, followed by addition of 100 μl of cell suspension or cfe. The change in absorbance was read at 475 nm. The assay was carried out with culture grown in benzoate (0.3%), protocatechuate (0.1%) and glucose (0.2%) medium.

(vi) Catechol-oxidase assay (30): Strain P₂d cells were incubated at 35°C in the presence of catechol at a final concentration of 10 mM and the colour change was noted.
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Inhibition of enzyme activity was studied visually. Enzyme inhibitors namely sodium-metabisulphide, sodium-bisulfide, mercaptoethanol, cysteine, thiourea, ascorbic acid, potassium cyanide (KCN), sodium-azide, at 1 mM concentration were added and the reaction mixtures were incubated at 35°C for 10 min and the colour change was noted.

Similarly the effect of these inhibitors was studied by incorporating the above inhibitors in growth medium supplemented with 0.3% benzoate and 0.2% tyrosine. Appropriate controls were kept for the experiment.

(vii) Protocatechuate 2,3-dioxygenase (43): Reaction mixture contained 2.8 ml of 0.05 M phosphate buffer (pH 7) and 50 µl of 0.1% protocatechuate solution. The reaction was started by adding 100 µl of cells/cfe and absorbance was monitored at 350 nm for 5 min. The assay was carried out with culture grown in protocatechuate (0.1%), benzoate (0.3%), glucose (0.2%), tyrosine (0.2%), 3-hydroxybenzoate (0.1%) and p-hydroxybenzoate (0.1%).
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Benzoate cells induced in the presence of salicylate, gentisate, and protocatechuate were also assayed.

(viii) Protocatechuate 3,4-dioxygenase (35): The reaction mixture was same (vii). Activity was monitored at 290 nm.

(ix) Protocatechuate 4,5-dioxygenase (46): Reaction mixture was same as (vii) and the absorbance was read at 410 nm.

(x) Gentisate 1,2-dioxygenase (214, 53): Cells grown in the presence of various aromatic compounds were pelleted and adjusted to absorbance of 4.0. Phosphate buffer (0.05M) pH 7.4 was taken in 3 ml cuvette with 1.6 mM gentisate. Cells/cfe (100 μl) were added to the reaction mixture and activity was monitored at 330 nm. Gentisate 1,2-dioxygenase was also assayed by measuring oxygen uptake using 10 μl of 0.1 % stock substrate in 1.6 ml reaction mixture.

IV. Extraction of transformation products of benzoate metabolism

Culture supernatant (20 ml) or reaction mixtures (20 ml) containing cells in buffer incubated with benzoate or related
compounds, were extracted with 10 ml diethyl-ether. Organic layer was separated and concentrated with nitrogen gas. The aqueous phase was acidified to pH 2 with 6 N HCl and centrifuged at 6000 rpm for 10 min. The supernatant was extracted with 10 ml of diethyl-ether and the ether layer was concentrated by passing nitrogen gas and analyzed by TLC.

For column chromatography, the wine-red culture supernatant from 2 L culture broth, was lyophilised to dryness using LABCONCO lyophiliser. The dried powder was then extracted with methanol. The methanol extract was concentrated and used for column chromatography.

V. Analytical techniques

a. UV-Visible spectrophotometry: UV-Visible scans of the culture supernatants and other samples were done using UV-1601 Shimadzu spectrophotometer.

b. Thin layer chromatography (TLC): The slurry, prepared by mixing 6 to 8 g of Silica Gel G (Acme's) in 15 ml of distilled water, was poured on glass plates and drawn into thin layers. The plates were air-dried and then activated at 110°C for 30
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min. Samples were spotted on the activated plates using thin glass capillaries. After drying the spots, plates were developed in solvent chambers, saturated overnight. Solvent was allowed to run upto 3/4th of the plate, the solvent front was marked and coloured spots, if any, were noted. The plates were then placed in iodine chamber. Rf values were calculated using the formula:

\[ \text{Rf} = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent}} \]

Various solvent systems were developed and used for separation of transformation products:

<table>
<thead>
<tr>
<th>Solvent system</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>I benzene: methanol: acetic acid</td>
<td>45:10:1</td>
</tr>
<tr>
<td>II benzene: methanol</td>
<td>10:45</td>
</tr>
<tr>
<td>III benzene: methanol: ethyl-acetate</td>
<td>40:60:40</td>
</tr>
<tr>
<td>IV benzene: acetone</td>
<td>90:10</td>
</tr>
<tr>
<td>V toluene: chloroform: acetone</td>
<td>40:25:35</td>
</tr>
<tr>
<td>VI hexane: ethyl-acetate</td>
<td>85:15</td>
</tr>
<tr>
<td>VII benzene: chloroform: xylene</td>
<td>40:40:20</td>
</tr>
<tr>
<td>VIII benzene: ethyl-acetate: acetic acid</td>
<td>75:24:1, 24:75:1, 50:50:1, 40:60:1</td>
</tr>
<tr>
<td>IX chloroform: ether</td>
<td>66:33</td>
</tr>
</tbody>
</table>
c. **Column chromatography:** A 30×2 mm glass column was washed, cleaned and dried. Slurry of Sephadex LH-20 (Sigma) was prepared by mixing 8 g of the gel in 20 ml of methanol. Column was packed by adding the slurry slowly with the help of a glass rod. The packed column was equilibrated by washing with methanol, twice. Sample (0.5 ml) was loaded onto the column using a Pasteur pipette and eluted using methanol as mobile phase. Aliquots were collected as 1 ml fractions in clean test-tubes and the fractions with similar TLC patterns were pooled together.

d. **High Performance Liquid Chromatography (HPLC):**

Samples were analyzed on Spectra-Physics HPLC system, using a reverse phase C_{18} Nucleosil column. The system was initially cleaned using methanol before the run. The HPLC pump was programmed to give 100% of mobile phase at the rate of 1 ml per min. The UV-visible detector was switched on and allowed to stabilize. The integrator was programmed to record the data.
During standardization, the mobile phase, flow-rate, wavelength of detector and amount of sample injected were varied. Results are tabulated in Appendix B. The column was initialized with mobile phase under isocratic conditions. The samples were filtered using Millipore 0.45 μm filters and 10 μl were injected in the injection port and turned to ‘inject’ position while simultaneously starting the recording on the integrator. After the run time was over, the injection port was brought back to ‘load’ position for the next injection. Results were recorded as peaks on the chromatogram. From the data, the retention time for the peaks was obtained.

e. Gas chromatography (GC): Gas chromatography was carried out as per the method of Bhosle, et. al. (215). 5 mg of lyophilized sample was hydrolyzed with 2 ml of 2N HCl by flushing with nitrogen gas. The mixture was heated on a sand-bath for 3 h, after which the hydrolysed samples were filtered. To the supernatant, 100 μl of Inositol was added as internal standard. Sample was dried in Perfit Rotovapor bath under vacuum for 5 min. Sample was dissolved in 1 ml of distilled water and passed through Dowex resin column, then
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vacuum dried for 5 min and kept in dessicator, overnight. The sample was incubated at 45°C for 30 min after adding 1 ml of 0.1 M Na₂CO₃ to it. Then, 0.5 ml of Na₂HBr₄ (20 mg/ml) was added and the sample incubated in the dark for 2-3 h. To destroy Na₂HBr₄, glacial acetic acid was added drop-wise till no more effervescence was seen. The sample was vacuum dried and 4 ml of methanol was added to the sample thrice, vacuum dried and then kept in vacuum dessicator for 3 h. 1 ml of 99% acetic-anhydride and 1 ml of 99% pyridine were added to the sample and incubated overnight at room temperature. Then the sample was vacuum dried and kept overnight in the desiccator. It was washed with 1 ml of distilled water and extracted thrice with 4 ml of 99% methylene-chloride. To the lower layer, collected in a clean test-tube, anhydrous Na₂SO₄ powder was added and then filtered through the same. Sample was collected in a flask, vacuum dried out, extracted with diethyl-ether and then flushed with nitrogen gas. Sample volume was made to 100 µl with methylene-chloride before injection. 0.4 µl of sample was injected. A capillary Gas Chromatography system of Chrompack Model CP 9002 with capillary column CP Sil 88 (25m, id =0.32mm, df=0.12) and
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flame ionization detector was used at oven temperature of 70°C.

The sample was analyzed for 40 min. Standard sugars and methylene chloride as blank were also run for 40 min.

f. Light microscopy of stained samples:

   *EPS staining (216)*: Smears of culture were prepared on clean slides by routine method and air-dried. The smear was flooded with saturated congo-red stain for 2 min and washed. Carbol-fuchsin was then added for 2 min. The slide was washed and air dried.

   *Capsule staining*: was done by using Manewal’s stain (Appendix C.4). A drop of congo-red was mixed with loopful of culture and a smear was made using another slide. The smear was flooded with Manewal’s stain. The air-dried slides were observed under 100x objective using Leitz phase contrast microscope.

g. Scanning electron microscopy (SEM): Adopted from Specimen Preparation Methods from SEM, JEOL application note.
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The culture was centrifuged at 5000 rpm for 5 min and the supernatant discarded. The pellet was dispersed in 0.05M phosphate buffer (pH 7) and smeared onto the stub. Smear on the stub was fixed in 2 ml of 2.5% glutaraldehyde fixative (pH 7.2-7.4), overnight at R.T.. The stub was placed in 0.05M phosphate buffer and then in 30% acetone. It was allowed to stand for 10 min. The dehydration procedure was repeated likewise with 50%, 70% and 90% acetone for 10 min, each and finally in 100% acetone for 30 min. The stub was then put in the critical point drying device wherein the acetone gets replaced by liquid carbon-dioxide at high pressure. This was evaporated by raising the temperature to 45°C and liquid carbon-dioxide gets converted to gaseous carbon-dioxide and escapes. The process takes 1 h. The stub was placed on the sputter coater (spi-module) specimen holder, after drying. The position of the stage is set such that the specimen is approximately 50 mm from the bottom of the sputter head. After sputtering the specimen with 10-15 nm thin film of gold, the stub was placed onto the electron microscope sample chamber and observed with JEOL- 5800 LV SEM.
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h. Infra-red spectroscopy: Samples were mixed with KBr pellets and applied to window of FTIR 8101A Shimadzu-Fourier transform infra-red spectrophotometer.

i. Oxygen analysis: The rate of oxygen uptake was analyzed by Gibson Oxy 5/6 analyser. The instrument was stabilized at 8 V. Paper speed was maintained at 0.1 mm/sec. Scale was adjusted to ‘zero’ using zero suppression key and to 100, using the sens key. Phosphate buffer 0.05 M pH 7, was aerated for 5 min prior to use and 1.6 ml was added in the reaction chamber. Magnet speed was adjusted at 6 units. Once the baseline stabilized, 100 µl of cells, adjusted to an absorbance of 4.0 at 540 nm, were added into the reaction mixture. The endogenous oxygen uptake was recorded for 5 min, followed by addition of 10 µl of substrate into the same mixture. The uptake rate was recorded again for 5 min. The chamber was cleaned, washed twice with distilled water and 1.6 ml of buffer was added for the next reading. This procedure was repeated twice with each substrate and the mean of oxygen consumed was calculated (Appendix D).
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j. Polyacrymide gel electrophoresis (217): Sample preparation for whole cell protein profile: Cell pellet obtained from 25 ml of culture broth was resuspended to an optical density of 4.0 at 540 nm. 100 µl of cells were treated with 50 µl of sample buffer [Appendix C.1 (viii)] and 100 µl of cells were used for protein estimation by Folin-Lowry’s method (218). Cells with sample buffer were boiled for 10 min in a boiling water-bath. 30 µl of loading dye solution was added to the treated sample prior to electrophoresis.

Preparation of Gels: A modified method of Laemmli (7) was followed. The glass plates after washing and drying were wiped with acetone and clamped together with spacers in place. The assembly was sealed from inside using molten 1% agar and allowed to solidify. All the solutions for separating gel (Appendix C.1) were taken in a clean beaker and mixed well. Ammonium-per-sulphate and TEMED were added, together, just before pouring the gel. The mixture was added in between the 2 plates using a 10 ml pipette, upto 3/4th of the space. Distilled water was added above the separating gel to get a uniform
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surface. Water was drained off after the gel had solidified.

Similarly, stacking gel was prepared and added over the separating gel. A comb was introduced into the stacking gel and it was allowed to set. For denatured protein separations, 0.2% of sodium-dodecyl-sulphate (SDS) was added to separating and stacking gels during preparation. The lower spacer and comb were removed and the assembly was placed in electrophoretic chamber. Samples were loaded in the wells with the help of a syringe. Tank buffer [Appendix C.1 (vii)] was added to upper and lower tanks. Electrodes were connected to powerpack and the gel was run at 120 V till the tracking dye reached the bottom of gel. Plates were separated carefully after removing the spacers and the gel was put in staining solution.

Staining of gels: Protein staining was carried out using Coomassie- Brilliant Blue R-250 stain [Appendix C.2 (i)]. Gel was placed in this staining solution for 6-8 h. It was then destained in Destain solution I [Appendix C.2 (ii)] for 1 h and in solution II [Appendix C.2 (ii)] till clear bands appeared.

Polysaccharide staining was done by silver staining method (92). After electrophoresis, the gel was put in fixative solution
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[Appendix C.3 (i)] for 2 h with intermittent shaking. The fixative was drained out and gel was washed with deionised water for 1 h. It was soaked in dithiothreitol solution [Appendix C.3 (ii)] for 2 h and then the solution was poured out. Silver-nitrate solution [Appendix C.3 (iii)] was added for 1½ h and gel was again washed with deionised water, twice. Addition of formaldehyde solution [Appendix C.3 (iv)] resulted in appearance of dark bands within 5 min duration. Citric acid (10 ml) [Appendix C.3 (v)] was added immediately to stop the reaction and avoid the whole gel from turning dark. Gel was washed with deionised water and stored in the same.

**k. Melting point determination:** 10 mg of compound was added into a melting point capillary of 1 mm diameter. The capillary was introduced in the capillary holder of Centrofix melting point instrument and a thermometer was introduced in the thermometer holder. Temperature was allowed to rise at the rate of 3 degrees per min and the temperature where the sample melts completely was noted.
VI. Qualitative and quantitative tests for transformation products

a. Precipitation of catechol (219): This method was used for detection and quantitation of catechol, during growth of *Pseudomonas mendocina* P₂d in benzoate medium. Lead-acetate solution, 0.3 ml of (20% w/v) was added to 1 ml of culture supernatant. Presence of catechol gave a white precipitate, which was centrifuged at 1000 rpm for 5 min and the wet weight was noted.

b. Colorimetric estimation of Catechol (105): 1 ml of 10% sodium-molybdate solution was added to 1 ml of culture supernatant, followed by addition of 0.5 ml of 0.5 N HCl and 1 ml of 0.5% sodium-nitrite solution. Presence of catechol gives a yellow colour to the solution. Addition of 1 ml of 0.5 N NaOH forms cherry-red colour. Absorbance was read at 510 nm on Shimadzu UV-1601 spectrophotometer (Fig. a).

c. Spot tests for quinones: Red culture supernatant was subjected to the following spot tests for quinone detection.

i) To 5 ml of supernatant, 6 N HCl was added dropwise and the colour change was observed. The solution was then
made alkaline with 10 N NaOH and again the colour change was noted.

ii) 0.1 N NaOH was added dropwise to 5ml of red supernatant and the colour change was observed.

iii) Sodium-dithionite (2%) was added to the red supernatant, mixed well and allowed to stand for 4-6 h. The colour change was noted and the solution was kept on shaker to check the effect of aeration.

iv) A pinch of sodium-borohydride was added to the red supernatant and the colour change was observed.

v) 0.5 ml of 2% potassium-iodide on acidification was added to 5 ml of supernatant and benzoate agar medium plate was flooded with the same. The observations were noted.

d. Derivatization of quinone using aniline (105):

2,5-dianilino-o-benzoquinone derivative: 1% aniline was added to a solution containing 25 mM catechol and culture pellet of Pseudomonas mendocina P2d. The reaction mixture was incubated on the shaker at 150 rpm for 3 h. The precipitate formed at the end of the incubation period was filtered and the
residue was dissolved in diethyl-ether. Ether was allowed to evaporate and the dried sample was reextracted in petroleum ether (40°-60°C). The extract was dried to powder and weighed.

VII. Standard estimation methods

a) Protein estimation by Folin-Lowry’s method: The protein concentration in samples was quantitated by Folin Lowry’s method (218) (Fig. b).

b) Sugar estimation by phenol sulphuric acid method: Samples were analyzed for their sugar content by the phenol-sulphuric acid method (220). To 1 ml of aqueous sample containing polysaccharides, 1 ml of 5% aqueous phenol was added. The tubes were placed in ice and 5 ml of concentrated sulphuric acid was added quickly into the tubes. Tubes were incubated in ice for 10 min. After allowing the tubes to come to R. T., absorbance was measured at 480 nm. Standard curve was plotted using glucose (0-100 μg/ml) as standard (Fig. c).

c) Tyrosine estimation by Arnow’s method (221): The tyrosine estimation method is based on Millon’s reaction. To 1 ml solution, 1 ml mercuric-sulphate reagent [Appendix C.5 (i)] was
added. The mixture was mixed well and kept in boiling water-bath for 10 min. After cooling the mixture, 1 ml nitrite reagent [Appendix C.5 (ii)] and 1 ml D/W was added to it. The turbid solution formed was centrifuged at 6000 rpm for 5 min and the clear red solution read at 540 nm. Standard curve is shown in Fig. d.

d) Dopa estimation by Arnow’s method (221): One ml test solution was acidified with 1 ml 0.5 N HCl and mixed well. To this, 1 ml sodium-molybdate reagent [Appendix C.5 (iii)] was added which gives yellow colour. Addition of 1 ml of 1 N NaOH gave red colour. The solution was diluted with 1 ml D/W and read at 540 nm. Standard curve is shown in Fig. e.

VIII. (a) Extraction of EPS from Pseudomonas mendocina

P₂d cells:

EPS extraction was carried by the method described by Adhikary (170). Cells grown in benzoate medium were incubated on ice for 2 h, followed by centrifugation at 6000 rpm for 10 min. The viscous pellet was suspended in 10 ml of deionised water and kept on magnetic stirrer, overnight. Cells were separated from the solution by centrifugation at
6000 rpm for 10 min. EPS was precipitated from the supernatant by adding 3 volumes of cold ethanol and incubating at 4°C for 3-4 h. The white precipitate of the EPS was weighed.

(b) Determination of emulsifying activity of EPS:

EPS extracted from the cells incubated on ice was used to find the emulsifying activity (222). The EPS (100 μl) was dissolved in 0.5 ml deionised water by heating intermittently and the volume was then made to 2 ml. The absorbance was adjusted to 0.1 at 540 nm using Elico colorimeter (filter no.54). Hexadecane (0.5 ml) was added to the above solution and vortexed for 1 min. The 0 min absorbance (A₀) was noted and the suspension allowed to stand for 30 min (Aₜ). The absorbance was read after the incubation period and further kept for another 30 min. Percent emulsifying activity was calculated using the formula,

\[
\% \text{ Emulsifying} = \frac{A_t - A_0}{A_0} \times 100
\]