CHAPTER 4:
MATERIALS AND METHODS
Materials and Methods

4.1 Materials

4.1.1 Chemicals

Different chemicals used for the present investigation are listed below.

Bacteriological Peptone (Himedia, India)

Beef extract (Himedia, India)

Yeast Extract (Himedia, India)

Sodium acetate (Merck Specialities Pvt. Ltd., India. AR grade)

Tri-ammonium citrate (Merck Specialities Pvt. Ltd., India. AR grade)

Lactose (LobaChemie Laboratory reagents and fine chemicals, India. AR grade)

Glucose (LobaChemie Laboratory reagents and fine chemicals, India. AR grade)

Manganese sulfate (Merck Specialities Pvt. Ltd., India. AR grade)

Magnesium sulfate (Merck Specialities Pvt. Ltd., India. AR grade)

Food grade inulin (Himedia, India)

TLC Silica gel G 60 Aluminium sheets 20 x 20 cm (Merck HX 816976, Germany)

Benzene (Ranbaxy, India. AR grade)

Acetic acid (Merck, India. AR grade)

Methanol (Merck Specialities Pvt. Ltd., India)

Resorcinol (Merck, India. AR grade)

De-ionized water (Millipore, Germany. AR grade)

Agar-agar (Merck Specialities Pvt. Ltd., India)

Di-potassium hydrogen phosphate (SRL, India. AR grade)
Hydrochloric acid (Merck Specialities Pvt. Ltd. India. AR grade) 3,5-dinitrosalicylic acid(DNS) (Merck Specialities Pvt. Ltd., India. AR grade)

Ethanol (Merck Specialities Pvt. Ltd., India. AR grade)

Sulphuric acid (Merck Specialities Pvt. Ltd., India. AR grade),

HPLC water (Merck, Germany)

Acetonitrile (Merck, Germany. AR grade)

Ammonium chloride (Merck Specialities Pvt. Ltd., India. AR grade)

Acetone (Merck Specialities Pvt. Ltd., India. AR grade)

Sodium hydroxide (Merck Specialities Pvt. Ltd., India. AR grade)

Sodium potassium tartrate (Merck Specialities Pvt. Ltd., India. AR grade)

Calcium chloride (0.27M) (Merck Specialities Pvt. Ltd., India. AR grade)

Sodium alginate (3%) (Merck Specialities Pvt. Ltd., India. AR grade)

Cysteine Hydrochloride (Merck, Germany. AR grade)

Benzene (Ranbaxy, India. AR grade)

Sodium chloride (Merck Specialities Pvt. Ltd., India. AR grade)

Tween 80 (Merck Specialities Pvt. Ltd., India. AR grade)

Potassium chloride (Merck Specialities Pvt. Ltd., India. AR grade)

Magnesium chloride (Merck Specialities Pvt. Ltd., India. AR grade)

Dipotassium hydrogen phosphate (Merck Specialities Pvt. Ltd., India. AR grade)

Silver Nitrate (SRL, India. AR grade)

4.1.2 Microorganisms

The bacterial strains used under the present research study were as follows:

*Pediococcus acidilactici* NCIM 2292 (ATCC 8042), has been procured from National Collection of Industrial Microorganisms, Pune, India.

*Escherichia coli* MTCC 443(ATCC 25922) has been collected from Microbial Type
Culture Centre,

*Pseudomonas aeruginosa* NCIM 5029 (ATCC 27853) has been procured from National Collection of Industrial Microorganisms, Pune, India.

*Staphylococcus aureus* NCIM 5021 (ATCC 25933) has been procured from National Collection of Industrial Microorganisms, Pune, India.

*Enterococcus faecalis* NCIM 5025 (ATCC 29212) has been procured from National Collection of Industrial Microorganisms, Pune, India.

*Salmonella abony* NCTC 6017 has been collected from National Centre for Type Culture

### 4.1.3 Microbial culture media

#### 4.1.3.1 Media composition of MRS broth

De-Man Rogossa Sharpe Broth (MRS) medium was prepared using the following composition.

Table 4.1 Composition of modified De-Man Rogossa Sharpe Broth medium (Basis: 1L)

<table>
<thead>
<tr>
<th>Serial No.</th>
<th>Components</th>
<th>Quantity, g</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Peptone</td>
<td>10.00</td>
</tr>
<tr>
<td>2.</td>
<td>Yeast extract</td>
<td>5.00</td>
</tr>
<tr>
<td>3.</td>
<td>Beef extract</td>
<td>10.00</td>
</tr>
<tr>
<td>4.</td>
<td>Dipotassium hydrogen phosphate</td>
<td>2.00</td>
</tr>
<tr>
<td>5.</td>
<td>Sodium acetate</td>
<td>5.00</td>
</tr>
<tr>
<td>6.</td>
<td>Tri-ammonium citrate</td>
<td>2.00</td>
</tr>
<tr>
<td>7.</td>
<td>Manganese sulphate</td>
<td>0.05</td>
</tr>
<tr>
<td>8.</td>
<td>Magnesium sulphate</td>
<td>0.05</td>
</tr>
<tr>
<td>9.</td>
<td>d-glucose</td>
<td>10-40.00</td>
</tr>
</tbody>
</table>
4.1.4 Equipment

The following equipments were used in all the experiments under the present research study.

BOD-Shaker incubator (G.B. Enterprises, Kolkata, India) (Figure 4.1)

Hot Air Oven (G.B. Enterprises, Kolkata, India)

Autoclave (G.B. Enterprises, Kolkata, India)

Laminar Air Flow (G.B. Enterprises, Kolkata, India) (Figure 4.1)

Heating Mantle (G.B. Enterprises, Kolkata, India)

Refrigerator (Whirlpool; Corona Deluxe, India)

Freezer -20ºC (Blue Star; model C8F320, India)

Weighing machine (Sartorius BS124S, Germany)

Ultra centrifuge (Superspin R-V/FM, Plasto Crafts, India)

Magnetic Stirrer (Remi; 5MLH, India)

Lyophilizer (Lyophilization Systems, Inc., India)

Rota vapour (Buchi Labor Technik AG R3, Switzerland)

4.1.4.1 Analytical Instruments

The following analytical instruments were used in the present research work

4.1.4.1.1 Spectrophotometer (Varian UV-visible spectrophotometer Cary 50 Bio)

4.1.4.1.2 Fourier Transform Infrared Spectrometer (FT-IR) (Shimadzu FTIR Spectroscope 8400) (Figure 4.1)

4.1.4.1.3 HPTLC- CAMAD; Applicator –LINOMAT 5; Scanner 3; Reprostar 3

4.1.4.1.4 High performance liquid chromatography (HPLC) (Shimadzu Corporation Reverse Phase with RI detector) (Model no: CBM-Ro A) (Figure 4.1)

4.1.4.1.5 Field Emission Scanning Electron Microscope (FESEM) (JSM 6700A, Jeol Ltd., Japan)

4.1.4.1.6 Transmission Electron Microscope (TEM) (Zeiss Leo 906E)
4.1.4.1.7 X-ray diffraction (XRD) (Rikagu-RadB) (Figure 4.1)

4.1.4.1.8 Cold Centrifuge (C-24, Remi)

4.1.4.1.9 Microscope (Optica XDS 2, Italy) (Figure 4.1)

Figure 4.1: Photographs of instruments
4.2 Methods

4.2.1 Extraction of Inulin

The prebiotic native sources, namely, garlic, wheat, oat, and dalia were washed properly with distilled water. In case of garlic, peeling of outer skin was done manually and the core portion was taken for the preparation of paste. 100 g garlic cloves were cut into small pieces and were blended with Mixer-Grinder after addition of 150 mL distilled water. The protocol for examination of inulin from chicory as reported by Mavumengwana [1], was followed with some modification to extract inulin from garlic, wheat, dalia and oat. The flow sheet showing different operations in the protocol is shown in Figure 4.2.

![Figure 4.2: Process of inulin extraction from natural prebiotic sources](image)

According to the protocol, suspensions of the aqueous pastes of different samples were prepared. The volume of distilled water used for the preparation of suspension was 750 mL for all samples except wheat, for which 900 mL water was required. The suspension was heated up to 70°C and was subsequently screened. Heating up to 70°C had been recommended by previous workers [1, 2], to de-activate the inulinase, which might have otherwise led to the conversion of inulin to fructose. The solid cake from the filter cloth was re-suspended several times in distilled water and was recycled to the filtration unit. Finally the filtrate was treated with calcium hydroxide to raise the pH up to 8.0. The pH was readjusted to 7.0 by using 0.8 M HCl. After the adjustment of pH, filtrate was frozen at -22°C for 3h and was thawed. The supernatant was
evaporated under vacuum to evaporate water and to obtain crude inulin powder. The crude inulin powder obtained from garlic, wheat, dalia and oat were analysed quantitatively and qualitatively using HPLC. Quantity of inulin in the extracts of prebiotic samples was also determined using DNS method. Chemical bonds present in native inulin powder were assayed using FTIR spectroscopy.

4.2.2 Determination of Inulin in Natural Prebiotics

Inulin in different natural prebiotics was qualitatively and quantitatively determined using following methods.

4.2.2.1 Characterization of Extracted Inulin Powder

4.2.2.1.1 FESEM (Field Emission Scanning Electron Microscopy)

The FESEM experiment was carried out at Indian Association for the Cultivation of Science, Kolkata. The objective of this study was to establish the morphological characterization of the bacterial strains under investigation.

4.2.2.1.2 FTIR (Fourier Transformed Infrared Spectroscopy) Analysis

FTIR analysis was carried out in-house using KBr plate. The intention of the study was to verify qualitatively the presence of inulin in the selected raw materials by comparing the chemical bonds of inulin extracts of natural prebiotic materials with those of pure inulin.

4.2.2.1.3 TLC (Thin Layer Chromatography)

1 mL of standard Inulin solutions of three different concentrations (5g/L, 10g/L, and 20g/L) were hydrolysed using 4 mL of 5% oxalic acid and heated in boiling water bath for 30 minutes [3].

Same protocol was followed for hydrolysis of isolated natural prebiotics test samples having concentration 20 g/L.

Approximately 10 μL of each hydrolysed sample was applied to spot on the aluminium sheet (TLC Silica gel G 60 Aluminium sheets) by a micropipette (make: Biohit proline 0.5 to 10 μL) and was allowed to stand for ~ 3 h in the n-butanol: acetic acid: methanol solvent (4:2:2) [4, 5] mixture in a closed chamber. The mobile phase
(solvent) was at the bottom of the tank. The mobile phase gradually moved upward carrying the spotted samples. Once the solvent front moved ~ 2/3 of the sheet, the sheet was removed; the solvent front was marked immediately and dried with a portable dryer. The sheet was sprayed with spraying reagent which had been prepared by mixing 0.2% orcinol in sulphuric acid and was incubated in hot air oven for 15 minutes at 70°C. Dark brown spots appeared for different samples. R_f values (Relative Fronts of the solute and the solvent) were calculated using the following equation.

\[ R_f = \frac{\text{dis tan cemoved by the solute}}{\text{dis tan cemoved by the solvent}} \] .........(I)

Sample spot present on TLC paper at the same position as obtained for pure food grade inulin, was scraped out and collected in a sample vial containing 10 mL distilled water each and OD values of supernatants were determined with the spectrophotometer at 540 nm, to quantify the concentration of inulin. Standard curve obtained using OD values of supernatant of solution of TLC spots corresponding to pure inulin solutions of different concentrations were used to determine inulin concentration in the natural prebiotic extracts.

4.2.2.1.4 HPLC (High Performance Liquid Chromatography)

HPLC experiments with pure inulin solution and extracted inulin were carried out using Column: C-18 Phenomenex (250 x 4.6 mm x 5μ) at Indian Institute of Chemical Biology, Kolkata.

4.2.2.1.5 DNS (3-5 Di-nitro salicylic Acid method)

1g of natural inulin powder of each prebiotic sample was taken in 5 mL distilled water and 5 mL solution of 4:1 acetonitrile: water was added to it. This solution was kept for a sufficient time to allow complete precipitation. When the precipitate settled at the bottom, the aqueous phase was drained and solid residue was repeatedly washed with distilled water. When the volume of the slurry reduced to 4 mL, washing was stopped and the 4 mL slurry was divided into two parts. One part was taken directly for fructose determination using DNS (3, 5- Di-nitro salicylic Acid) method and another part was hydrolysed with 5% oxalic acid following the same protocol used in case of TLC analysis and further analysed using DNS [6]. Since extracted inulin powder contains inherently associated sugar molecules, DNS analysis of extracted inulin
powder would provide the concentration of sugars associated with inulin powder. On the other hand after hydrolysis of inulin when the sample is subjected to DNS analysis, concentration of sugar obtained from the breakdown of inulin structure as well as the original ones associated with inulin will be determined. The difference of the reducing sugar content between the hydrolysed product and the parent inulin powder would be the fructose obtained due to hydrolysis of the inulin. The concentration of pure inulin in the extracted inulin powder was obtained by the simple molar ratio relationship given by Mavumengwana [1].

\[
[I] = 162X \frac{[F]}{180} \text{.........................(2)}
\]

Where \([I]\) = concentration of inulin, g/L and \([F]\) = concentration of fructose obtained from inulin, g/L.

4.2.3 Optimization study

In the present investigation, response surface methodology (RSM), a statistical optimization process, was used to evaluate the relative significance of several interactive independent variables on the target response variable. The RSM involves three steps, namely design an experiment, response surface modelling through multivariable regression analysis and optimization. The objective of such statistical analysis was to determine the functional relationship between the dependent variable, namely specific growth rate of \(P. \text{acidilactici}\) (response, \(Y\)) and the three independent variables, viz. concentration of glucose and inulin and the pH of the medium. These independent variables were designated as \(X_i\). The optimum condition for the growth of \(P. \text{acidilactici}\) was determined by Box–Behnken Design (BBD) technique. The reason for selecting BBD on the face of the other alternative central composite design (CCD) in designing experiments is that the BBD requires only a fraction of trials for a threelevel factorial as present in quadratic model. According to this design, the following equation is used to determine the total number of experiments

\[
N = k (1)^2 + k + cp \text{-----------------------------(2)}
\]

where \(k\) is the factor number and \(cp\) is the number of replicate of the central point. The
RSM uses the quantitative data generated by the BBD to solve the multivariate equation for the purpose of optimizing the response variable. It has already been reported by several investigators [7, 8, 9, 10] that second-order polynomial equation relating response and independent variables gives satisfactory accuracy. Thus in the present investigation, the following second-order polynomial has been selected.

\[ y = b_0 + \sum_{i=1}^{3} b_i X_i + \sum_{i=1}^{3} b_{ii} X_i^2 + \sum_{j=1}^{i} \sum_{j=i+1}^{3} b_{ij} X_i X_j \]

where \( y \) is the response variable, \( X_i, X_j, \ldots \) are the input variables, \( X_i, X_i^2, X_i X_j \) represent the linear, square and interactive relationships with coefficients \( b_i, b_{ii} \) and \( b_{ij} \), respectively. The software Design Expert 8.1® (Stat-Ease Inc., Minneapolis, USA) was used for experimental design, regression and graphical analysis of the predicted and experimental data. Using Eq. (2), the total number of experiments was determined to be 17. Response surface modelling in terms of coded factors for the range of operating parameters, pH (6–8), concentration of glucose (10–30 g/L), concentration of inulin (10–30 g/L).

4.2.4 Cell growth study

4.2.4.1 Preparation of starter culture

Adaptation of the strain to a medium containing high concentration of glucose (50 g/L) and inulin (50 g/L) was performed by three times repetitive sub-culturing. The pre-culture process was conducted in an incubator at 37 °C using 250-mL Erlenmeyer flasks for 1 day, based on sufficient growth. The cell from the last adaptation experiment was stored for use in further experiments [11].

4.2.4.2 Batch experiments

To study the growth kinetics of \( P. \ acidilactici \) on substrates, glucose and inulin, three separate series of batch experiments, one using glucose as sole carbon source, one using inulin as carbon source and another using both glucose and inulin as carbon source were conducted. In the series of experiments, in the absence of inulin, four 500-mL Erlenmeyer flasks each containing 250 mL MMRS media containing different concentrations of glucose (10–40 g/L) were used. Similarly, MMRS media for series two were prepared by using different concentrations of inulin (ranging from
0.5 to 60 g/L) in place of glucose. Another set of experiment was also carried out using MMRS media containing both glucose and inulin. At each fixed concentration of glucose (10 g/L or 20 or 30 or 40 g/L), concentration of inulin was varied from 10 to 40 g/L. For the determination of optimum growth condition, MMRS media of 17 different compositions, suggested by Box–Behnken model of response surface methodology (RSM), were used. The initial pH of the medium was set at 7 for both the series. For all experiments, 1% (v/v) inoculum was added. Ten millilitres of paraffin oil was used immediately after the inoculation of the *P. acidilactici* to maintain the anaerobic condition for both sets of experiments with and without inulin. Each flask was closed with perforated cork through which a narrow glass tube was inserted inside the solution for the purpose of withdrawing the sample under anaerobic and aseptic condition at 2-h interval. The incubation temperature was 37 °C [11].

4.2.4.3 Determination of microbial biomass concentration

The concentration of biomass was determined using spectrophotometric method. A standard plot was prepared by plotting optical density of cellular solution at 600 nm against the known concentrations determined using dry cell weight method. For dry cell weight technique, 15 mL of cellular solution was centrifuged at 10,000 gyrations, at 4 °C for 15 min. The cell precipitate so formed was separated, washed and transferred into a pre-weighed aluminium cup. This was dried at 50 °C overnight in the hot air oven, and the cup was then cooled and weighed further. Weight of the cellular mass was determined by difference. The concentration of biomass was determined by dividing the dry weight of cell precipitate by the volume of sample taken (15 mL).

4.2.5 Preparation of crude extract of garlic, basil leaf and betel leaf

4.2.5.1 Extract of garlic

100 g of core portion of garlic cloves were taken after peeling of outer skin manually and washed several times with de-ionized water. Cloves were then cut into very small pieces and crushed into a smooth paste by using mortar and pestle. This paste was used for further study.
4.2.5.2 Extract of basil and betel leaves

100g each of basil and betel leaves were washed several times with de-ionized water, dried and then placed into blender. 100 mL de-ionized water was added to it and the whole mass was blended. The slurry material was then filtered through Whatman No.1 filter paper to get the prebiotic extract. This stock solution was used as a source of prebiotic present in basil and betel leaf [12].

4.2.6 Extraction of the prebiotic from psyllium husk (Isabgol)

Arabinoxylan was extracted from Isabgol following the protocol reported by Saghir et al [13]. 50 g psyllium seed husk was soaked in sterilize distilled water overnight (seed husk: water 1:50, w/v) to form the mucilage. Aqueous NaOH solution (2.5%) was added to the mixture to adjust the pH to 12 for the hydrolysis of the fibres [14]. The husk fibres were separated from the gel by applying vacuum filtration after stirring for 2–3 minutes. Concentrated acetic acid was added to the filtrate mixture to coagulate the sample at pH 3. The gel was then washed for several times over a period of 3–4 days with distilled water until the pH becomes constant at pH 3. The sample was then freeze dried. The photograph of husk free arabinoxylan gel prepared is shown in Figure 4.3.

![Figure 4.3: Photograph of husk free arabinoxylan gel](image)

4.2.6.1 Solubilisation of arabinoxylan

Solubilisation of arabinoxylan extract was performed using the protocol suggested by Anson et al [15]. 0.5 g of the freeze dried arabinoxylan was suspended in 40 mL of distilled water and kept in water bath for 2 h. 60ml of colonic juice was added to it.
This mixture was again heated in water bath at 100°C for half an hour. The mixture obtained after heating was cooled down and used directly as prebiotic source for further experiments.

4.2.6.2 Assay of arabinoxylan

Arabinoxylan extract and its hydrolysate obtained after hydrolysis following the protocol recommended by Craeyveldt et al [14] were analyzed using, HPTLC and TLC respectively. The chromatograms were compared with those of standard arabinose and xylose. FTIR analysis of arabinoxylan extract was also performed to identify the chemical bonds present in it.

4.2.7 Characterization of Extracted arabinoxylan

4.2.7.1 FTIR (Fourier Transformed Infrared Spectroscopy) Analysis

FTIR test was done to identify the functional groups present in the Psyllium husk.

4.2.7.2 TLC analysis

A qualitative analysis using TLC was carried out to determine the presence of arabinoxylan in isolated arabinoxylan from Psyllium husk. Isolated arabinoxylan was hydrolysed to arabinose and xylose (as stated earlier) followed by TLC. Development of TLC plate has been shown in Figure 4.4.

![Figure 4.4: Photograph showing TLC](image)
4.2.7.3 HPTLC analysis

Isolated arabinoxylan was hydrolysed to obtain arabinose and xylose and qualitatively analysed using HPTLC. Comparison of hydrolysed arabinose and xylose were compared with that of standard arabinose and xylose in terms of $R_f$ values.

4.2.8 Determination of prebiotic score

The prebiotic activity score was determined using the following procedure given by Huebner et al. The prebiotic activity score was then computed using the following formula [16]

\[
\text{Prebiotic activity score} = \left( \log \frac{N_{24}}{N_0} \right)_{\text{prebiotic}} - \left( \log \frac{N_{24}}{N_0} \right)_{\text{glucose}} - \left( \log \frac{N_{24}}{N_0} \right)_{\text{probiotic}} - \left( \log \frac{N_{24}}{N_0} \right)_{\text{E.coli}}
\]

Huebner et al.

4.2.9 Determination of antimicrobial activity of *Pediococcus acidilactici* in terms of zone of inhibition

During cell growth in presence of garlic extract 1mL of sample at different intervals of time was taken out in an eppendorf tube and centrifuged at 10000 rpm for 10 minutes. After centrifugation the supernatant was used for antimicrobial study. Small discs (diameter 0.5 cm) of filter paper was prepared and loaded with 0.1 mL of supernatant obtained after the centrifugation of the respective sample. Each disc treated with supernatant of the samples withdrawn at different time intervals was impregnated onto the MMRS agar plate spreaded with *E.coli* culture. These plates were then kept for incubation at 37°C for 24 h and the zone of inhibition was measured in each plate.
4.2.10 Basil-Silver and Betel-silver nanoparticles preparation

Basil leaves extract was prepared according to the protocol suggested by Yilmaz et al. [12] Crushed shadow–dried basil leaf was taken by sieving with 200 µm mesh openings to obtain leaf biomass. 0.1 g of leave biomass is added to 50 mL De-ionized water and stirred for 1 h in a magnetic stirrer at room temperature. This was then filtered through Whatman 1 filter paper. Supernatant was centrifuged at 10000 rpm for 30 minutes at 4°C. After centrifugation, equal volume of 0.1 mM silver nitrate solution was immediately added to this clear basil leaf extract. One part of each conjugate was taken in separate previously sterilized amber coloured bottle to form basil-silver nanoparticles (basil-AgNPs1) and the other part was divided into two more parts to synthesize the nanoparticles by varying two different parameters. In one case, the conjugate was heated at 70°C temperature for 30 minutes to form basil-AgNPs2 and for the other case; pH of the conjugate mixture was set to 10 by adding sodium hydroxide (basil-AgNPs3). These conjugates were taken in previously sterilized amber coloured bottles and kept in dark for 24 h for complete reduction of basil-silver nanoparticles (Besil-AgNPs). The whole process was carried out aseptically in a laminar air flow chamber using sterilized apparatus. The same protocol was followed to synthesize two betel-silver nanoparticles varying different parameters to form betel-AgNPs1 and betel-AgNP2.

Basil and betel-silver nanoparticles solutions so formed were then subject to UV-visible spectrophotometry in the range of 200-800 nm. The solution was then centrifuged at 10000 rpm for 30 minutes at 4°C to precipitate the nanoparticles. The precipitated nanoparticles were washed with de-ionized water several times and centrifuged twice at 10000 rpm for 15 minutes. The precipitated particles were freeze dried and the samples were used for further studies.

4.2.10.1 FTIR analysis of prebiotic nanoparticles

FTIR analysis was carried out to identify the chemical bonds present in the biosynthesised prebiotic-nano particles.
4.2.10.2 TEM analysis

Transmission electron microscope (TEM) analysis was carried out at Indian association for the cultivation of science, Kolkata to determine the sizes and shapes of the particles by dropping the precipitated phase on a formvar-coated copper grid.

4.2.10.3 XRD analysis

X-ray diffraction (XRD) pattern of the drop-coated films on a microscope lame was recorded by a Rikagu-RadB diffractometer equipped with a graphite monochromator and a CuKα source. The experiment was carried out at Indian Institute of Chemical Biology, Kolkata.

4.2.10.4 Cell growth study in presence of biosynthesized nanoparticles

4.2.10.4.1 Preparation of starter culture

Adaptation of the strain to a medium was performed by three times repetitive sub culturing. The pre-culture process was conducted in an incubator at 37°C using 250 mL Erlenmeyer flasks for 1 day, when appreciable growth was obtained. The cell from the last adaptation experiment was stored for use in further experiments [11].

4.2.10.4.2 Batch Experiments

To study the prebiotic effect of biosynthesized nanoparticles with basil and betel leaf extracts on the growth kinetics of *Pediococcus acidilactici* two different sets in four separate series of batch experiment viz., i) in presence of Basil-AgNPs1 and BetelAgNPs1 ii) in presence of Basil-AgNPs2 and Betel-AgNPs2 iii) in presence of BasilAgNPs3 iv) in presence of crude leaf extracts of basil and betel were conducted. In one series of experiments, four 500 mL Erlenmeyer flasks each containing 250 mL MMRS media containing basil-AgNPs1, 2, 3, 4 were used. Another set of experiment was also carried out using MMRS media containing and betel-AgNPs1 and 2. For all experiments, 1% (v/v) inoculum was added. 10 mL paraffin oil was used immediately after the inoculation of the *Pediococcus acidilactici* to maintain the anaerobic condition for both sets of experiments with and without inulin. Each flask was closed with perforated cork through which a narrow glass tube was inserted inside the solution for the purpose of withdrawing the sample under anaerobic and aseptic condition at 2 h interval. The incubation temperature was 37°C [11].
4.2.10.4.3 Determination of Microbial biomass concentration

The microbial biomass concentration was determined using dry cell weight technique following the method described under section 4.2.4.3.

4.2.10.4.4 Determination of cell count by spread plate method

A known amount of freshly prepared seed culture was directly loaded on a Neubauer Haemocytometer counting chamber and covered with a cover slip, followed by direct count under microscope and total numbers of cells were calculated from the data.

Reference


[14] Craeyveld VV, Jan A. Delcour, Christophe M. Courtin, Extractability and chemical and enzymic degradation of psyllium (Plantago ovata Forsk) seed husk arabinoxylans, Laboratory of Food Chemistry and Biochemistry, Leuven Food Science and Nutrition research Centre (LFoRCE), Katholieke Universiteit Leuven, Kasteelpark Arenberg 20 bus 2463, B-3001 Leuven, Belgium.