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3.1 Microorganisms and microbiological methods

3.1.1 Organisms

Saccharomyces cerevisiae MTCC- 36, 170 and 177 [MTCC (The Microbial Type Culture Collection and Gene Bank)] were used in Magnetic field (MF) induced adaptive evaluation of Ethanol Tolerance study. Saccharomyces cerevisiae MTCC- 170 and 177 are well characterized laboratory strain for ethanol production and MTCC 36 derived from Sugar refinery. MCubc1& Cubc1 Bacillus Spp isolate (microorganisms taken from department of FELM under running DST project IASE/DST-8878/30.03.11/04) were studied the effects of magnetic field on the metabolites of bacterium as well as bacterial growth were studies under different stress like NaCl, pH and temperature. The effect of the magnetic field (MF) on the effectiveness of biogas generation was studied on self generated methanogenic bacteria

3.1.2 Media

Media used in this study was prepared by dissolving adequate medium components in distilled water and sterilized at 121°C for 20 minutes. A solution of glucose was prepared and sterilized separately from the other components of the medium. After sterilization, proportional volumes of medium components were measured using a measuring cylinder, mixed together and the final volume was adjusted to the appropriate level with sterile, de-ionized water. The types of media used in this study are as follows.

YPD contained 3% (w/v) yeast extract (HiMedia), 10 % (w/v) bacteriological peptone (HiMedia), 2% (w/v) D-glucose (Sigma), dissolved in de-ionized water and sterilized as
describe above. YPD solid medium, used for viable plate preparation, was prepared similarly to YPD but with the addition of 15 % (w/v) of bacteriological agar prior to autoclaving.

2xYPD was prepared similarly to YPD but using double the amount of each ingredient.

YPD with 10% ethanol for broth and agar media - contained 3 % (w/v) yeast extract (HiMedia), 10% (w/v) bacteriological peptone (HiMedia) and 20 % (w/v) D-glucose (HiMedia), 15% agar was added for agar petri plate and slant, dissolved in 90 ml distilled, de-ionized water and autoclaved. Afterwards 10ml of 100% ethanol was mixed in broth. In most cases the medium was adjusted to pH of 3.5 to 4 with HCL unless otherwise specified. This medium was used mainly for ethanol stress adaptation treatment in presence of magnetic field and batch incubations designed to analyse ethanol stress tolerance by growing yeast culture on petri plate and preservation.

YPD with 15% ethanol for broth and agar media - contained 3 % (w/v) yeast extract (HiMedia), 10% (w/v) bacteriological peptone (HiMedia) and 20 % (w/v) D-glucose (HiMedia), 15% agar was added for agar petri plate and slant, dissolved in 85 ml distilled, de-ionized water and autoclaved. Afterwards 15 ml of 100% ethanol was mixed in broth. In most cases the medium was adjusted to pH of 3.5 to 4 with HCL unless otherwise specified. This medium was used mainly for ethanol stress adaptation treatment in presence of magnetic field and batch incubations designed to analyse ethanol stress tolerance by growing yeast culture on petri plate and preservation.

YPD with 20% ethanol for broth and agar media - contained 3 % (w/v) yeast extract (HiMedia), 10% (w/v) bacteriological peptone (HiMedia) and 20 % (w/v) D-glucose (HiMedia), 15% agar was added for agar petri plate and slant, dissolved in 80 ml distilled,
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de-ionized water and autoclaved. Afterwards 20 ml of 100% ethanol was mixed in broth. In most cases the medium was adjusted to pH of 3.5 to 4 with HCL unless otherwise specified. This medium was used mainly for ethanol stress adaptation treatment in presence of magnetic field and batch incubations designed to analyse ethanol stress tolerance by growing yeast culture on petri plate and preservation.

**YPD with 25% ethanol for broth and agar media** - contained 3 % (w/v) yeast extract (HiMedia), 10% (w/v) bacteriological peptone (HiMedia) and 20 % (w/v) D-glucose (HiMedia), 15% agar was added for agar petri plate and slant, dissolved in 75 ml distilled, de-ionized water and autoclaved. Afterwards 20 ml of 100% ethanol was mixed in broth. In most cases the medium was adjusted to pH of 3.5 to 4 with HCL unless otherwise specified. This medium was used mainly for ethanol stress adaptation treatment in presence of magnetic field and batch incubations designed to analyse ethanol stress tolerance by growing yeast culture on petri plate and preservation.

**YPD with 30% ethanol for broth and agar media** - contained 3 % (w/v) yeast extract (HiMedia), 10% (w/v) bacteriological peptone (HiMedia) and 20 % (w/v) D-glucose (HiMedia), 15% agar was added for agar petri plate and slant, dissolved in 70 ml distilled, de-ionized water and autoclaved. Afterwards 25 ml of 100% ethanol mixed was in broth. In most cases the medium was adjusted to pH of 3.5 to 4 with HCL unless otherwise specified. This medium was used mainly for ethanol stress adaptation treatment in presence of magnetic field and batch incubations designed to analyse ethanol stress tolerance by growing yeast culture on petri plate and preservation.
3.1.3 Solutions and buffers

All solutions and buffers were prepared from analytical grade reagents unless otherwise stated. Chemicals were dissolved in autoclaved de-ionized water in a glass container and pH was adjusted to a desire level. Solutions and buffers were sterilized by using autoclave $121^0\text{C}$ for 20 minutes or by filtering using 0.22 µm membrane filters.

3.1.4 Glassware and non-glass vessels preparation

All glassware used in the experiments was washed and acid rinsed. It was then sterilized at $121^0\text{C}$ for 5 minutes and dried at $70^\circ \text{C}$ until water drops and/or vapor was no longer visible inside the glass vessels. Most of the non-glass vessels such as polyester tubes etc were purchased sterile and ready for aseptic use.

3.1.5 Cultures

3.1.5.1 Maintenance of cultures

Yeast strains designated for storage were grown overnight in YPD medium. Cells were harvested by transferring 10 ml aliquots to 10 ml sterile polyester tubes and spinning down at 1780 g for 2 min. After discarding of supernatant, cell pellets were re-suspended in cryoprotective fluid from Protect vials, transferred into the same vials and gently shaken to redistribute cells inside and around internal beads. After 30-60 seconds, excess cell-containing cryoprotective fluid was withdrawn using a sterile pipette. The prepared stock cultures were immediately frozen and stored at $-80^\circ \text{C}$. All these operations were performed using barrier sterile tips to avoid cross contamination of the cultures. For short term storage, yeast stocks were streaked out on YPD agar plates incubated at $30^\circ \text{C}$ for 48 hours and stored at $4^\circ \text{C}$.
3.1.5.2 Inoculums preparation

Cells were transferred from stocks kept at -80°C into 20 ml of YPD in 50 ml polyester tubes and incubated overnight at 30°C/140 rpm. Culture tubes were placed and shaken at approximately a 45° angle to allow adequate mixing of the culture during the incubation period. When the parent cultures reached late exponential phase, the culture OD<sub>600</sub> was used to determine the inoculum volume needed to give an initial OD<sub>600</sub> of 0.1 in the experimental culture. Microscopic examination of the parent culture was conducted prior to experimental culture inoculation to check for contamination. The appropriate volume of parent culture was transferred to 10 ml sterile tubes, centrifuged for 2 minutes at 1780 g and the supernatant discarded, leaving behind the cell pellet. Approximately 1 ml of fresh medium was aseptically removed from total volume of experimental culture medium and used to re-suspend the inoculum cell pellet in the polyester tube. The resuspended inoculum was transferred into the experimental culture medium giving an initial OD<sub>600</sub> of 0.1. The inoculation of all experiments described in this thesis was performed using barrier sterile tips to avoid contamination of the cultures.

For the purposes of experiments described in Chapter 6, the inocula were prepared similarly as described above. However, the parent cultures were grown in sterile 250 ml Erlenmyer flasks covered with aluminum foil and containing 100 ml of YPD medium. Flasks with air locks were used to prepare inocula for anaerobic experiments.

3.1.5.3 Aerobic growth

For each set of experimental conditions, triplicate cultures were conducted. Cultures for aerobic experiments were inoculated at an initial OD<sub>600</sub> of 0.1 using inocula taken from the
same parent culture at the same time. Inocula were prepared as described in (Section 3.1.5.2). These incubations were conducted using 250 ml Erlenmeyer flasks with a working medium volume of 100 ml. unless otherwise stated. Flasks were covered with aluminum foil that allowed free gas exchange between external and internal flask space. Aerobic experiments were carried out in shaking water baths at 30°C/140rpm unless otherwise stated. The temperature of all water baths was adjusted and monitored using one thermometer for all the experiments. This practice ensured a consistent temperature was used for all incubations. The incubations described in Chapter 6 were conducted in 1000 ml Erlenmyer flasks covered with aluminum foil, and with a working medium volume of 500 ml. The cultures were incubated in shaking incubators at 30°C/140 rpm.

3.1.5.4 Semi-anaerobic growth

Semi-anaerobic incubations used in this study refer to growth conditions in which there was limited access to oxygen during incubation. For each set of experimental conditions, triplicate cultures were conducted. For all semi-anaerobic experiments, cultures were inoculated at an OD$_{600}$ of 0.1 using inocula taken from the same parent culture at the same time. Inocula were prepared as described in Section 2.1.5.2. Incubations were conducted in 250 ml Erlenmeyer flasks with air-locks, which did not permit free gas exchange during incubation. Oxygen that was initially present in the incubation flasks was metabolised in the early incubation stages and CO$_2$ production by the cells ensured that the cultivation became anaerobic. The fermentations were conducted with a working volume of 100 ml of medium in shaking water baths at 30°C /140 rpm. The temperature of all water baths was adjusted and monitored using one thermometer for all of the experiments.
3.1.5.5 Anaerobic growth

For each set of experimental conditions, triplicate cultures were conducted. Medium used for anaerobic incubations was transferred (500 ml) into sterile 1000 ml Erlenmyer flasks supplemented with magnetic stirrers and covered with aluminum foil. These flasks were subsequently transferred into an anaerobic hood, placed on a magnetic stirrer and left stirring at 140 rpm for 24 hours to de-oxygenate the medium. Parent cultures used for the anaerobic experiments were incubated overnight under semi-anaerobic conditions as described in Section 2.1.5.4. Subsequently, pre-determined volumes of parent cultures (Inoculums), needed to provide an initial OD$_{600}$ of 0.1 in the anaerobic experimental cultures, was measured into sterile tubes, and centrifuged for two minutes at 1780 g. The supernatant was discarded and the pellets in the tubes de-oxygenized in the anaerobic pre-chamber, and subsequently transferred into the anaerobic hood. The cell pellets were then re-suspended in the anaerobic experimental medium. Each triplicate culture was inoculated using inocula taken from the same parent culture at the same time. The cultures were incubated anaerobically at 30°C/140 rpm. Samples of the cultures were taken anaerobically at regular intervals.

3.1.5.6 Micro plate growth

Parent cultures for micro plate experiments were prepared as described in Section 3.1.5.2. The volume of the parent culture used for the inoculum was calculated so that the inoculum preparation had an initial OD$_{600}$ of 0.4. The inoculum was prepared in 10 ml of experimental medium in polyester tubes and 50 u1 of inoculum was transferred to wells in a 96-well micro-plate. of which every well was filled with 150 u1 of experimental medium. The micro
plate was sealed with Breathe-Easys® (U.S. Patent No. 5,858,770) gas permeable sealing membrane for inerplates, and incubated at 30°C in a thermo multiscan Ascent plate reader. An OD$_{600}$ of the culture was measured every 30 minutes. Data from micro plate experiments were processed using Microsoft® Office Excel 2007.

3.2 Instruments for Magnetic field facility and there process.

3.2.1 Line diagram of magnetic field apparatus

Fig 3.1 Schematic illustration of the apparatus
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The selected bacterial strains need their exposure to magnetic field of various strength for which a device (Machine) was develop by the author having following components. (Fig 3.1) (3) Cylindrical hollow core (Ductile iron pip) around which copper winding of 300 turns was coiled. (2) The cylindrical core & copper winding were housed in the iron frame and the frame was covered by iron plates to increase the magnetic strength. (1) The central hollow was used for keeping the sample in 50 ml test tube. Where it can be exposed to magnetic field of 100µT to 4500µT. (8) The magnetic field may be regulated by DC power supply. In the instrument we do need homogeneous but variable magnetic field strength according to bacterial strain. For that a machine was constructed in which cylindrical hollow core was used (Ductile iron pipe). Around the cylindrical hollow core copper winding of around 3000 turns was placed. To support cylindrical core a plated iron frame was constructed on the edges of cylindrical hollow core and to increase magnetic strength in free cylindrical hollow core we cover this frame using iron plates Fig 3.2. Iron frame decreases the loss of magnetic flux in outside of cylindrical core. The sample loading site posses space for 50 ml test tube and 100 µT to 10000µT homogeneous magnetic field. Magnetic field can be adjusted by varying the current and voltage using variable D.C power supply.
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Fig 3.2 Apparatus developed for magnetic field treatment for microorganism’s adaptive evaluation generation

Fig 3.3 Apparatus developed for magnetic field treatment to ongoing fermentation
Fig 3.4 Experimental design for fermentation under various conditions
3.2.2 Design anaerobic biogas digester and pretreatment plant

The present study was carried out under laboratory conditions using a modified anaerobic biogas digester with magnetic field facility (MFF). Cow dung was collected from GVM, IASE Deemed University gosala, sardarshahr.

3.2.2. A Pretreatment- Cow dung was mixed with approx. equal amount of water and mechanical size reduction was done through wet grinding machine. The thermal pretreatment with temperature 80°C were selected to pretreat the substrate (slurry). The slurry was treated with temperatures of 80°C for 3 hour with intermittent gentle shaking to ensure the homogeneity of temperatures. Substrate that was not exposed to these temperatures, but left under room temperature was considered as control.

3.2.2.B Anaerobic biogas digester- Two (200 L) airtight plastic drums equipped with pH probe, stirrer, sampling ports and temperature controller (bobbin element with temperature control unit) used as digester. MFF (Magnetic field facility) Recycled laminated transformer core which was made out of cast iron and were assembled in ‘E’ shape, submersible 16 gage copper wire coiled on ‘E’ shape with 3.5 ohm resistance and connected with DC regulated power supply 0-32/5A. The core was fixed on plastic base and fitted on the bottom of one 200L plastic drum. The drum that was not facilitated with magnetic field was considered as control. Both digesters were painted black.

3.2.2.C Start-up and operation - digesters equipped with pH probe, stirrer, sampling ports and temperature controller (bobbin element with temperature control unit) was used in this study. The working volume of the bioreactor was maintained at 150 Liter and ran under uncontrolled pH, which was without acid or base addition. Experiments were carried out at
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mesophillic temperature of 42°C to 45°C and mixing was aided by a mechanical stirrer set at a speed of 150rpm and a semisolid liquid pump’s sucking point was fixed just above the magnetic core and slurry dropping point on the top to get rid of dry slurry layer on the top ensuring proper homogeneity of magnetic field treatment. The system was started up as batch to achieve an active acidifying culture by loading the substrate seeded with palm oil mill effluent, then sealed and purged with Nitrogen gas for 15 minutes. Semi-continuous feeding started from day 10, where a known volume of slurry was withdrawn daily from the reactor and replaced with fresh feedstock via the slurry sampling ports. In addition, approximately 50 ml of the sample was taken daily from the bioreactor through the sampling port, which then underwent series of analysis. Biogas production was measured by water displacement method.

Fig 3.5 Apparatus developed for biogas generation. A Magnetic field facility digester and B control digester
3.3 Analytical methods

3.3.1 Optical Density Measurement

Measurements of culture absorbance were conducted at 600 nm using a Thermo UV/Vis Spectrophotometer. Absorbance measurements were conducted in Greiner Bio-One 3ml Semi-Micro-Cuvettes, (10 x 10 x 45 mm), manufactured from crystal clear polystyrene. The spectrophotometer was calibrated against sterile medium of the same composition as was used for yeast incubations. At high culture densities, samples were diluted with filtered water (0.2 um filter MiliQ™) to give OD_{600} readings in range of 0.1-0.5. Medium used for spectrophotometer calibration was diluted with filtered MiliQ™ water according to the dilution factor used for sample preparation. Data was plotted and analyzed using Microsoft® Office Excel 2003.

3.3.2 Cell population

A hemocytometer (Precicolor, HBG, Germany) was used to calculate cell number at particular stages of yeast cultivation. Prior to use, the hemocytometer and cover slip were cleaned with ethanol, wipe-dried, and the cover slip placed on top of the hemocytometer. A cell suspension (diluted in to be in the OD_{600} range of 0.1 - 0.5) was mixed by vortexing and loaded between hemocytometer and cover slip, filling entirely both hemocytometer chambers, The loaded hemocytometer was left for approximately 1 minute to allow the cells to settle. It was then placed under a microscope (40x magnification) and the middle 1 mm² square was counted. Samples were calculated in both chambers of hemocytometer. The cell population was calculated taking into account the sample dilution and number of counted fields.
3.3.3 Biomass measurement

Biomass of cultures was estimated based on the dry cell weight (g/1). A suspension of 10 ml of the thoroughly mixed cells was filtered through 0.22um membrane filters. The cells were collected on the surface of filter, dried and weighed on an Adam AMB 50 drying balance. The total mass of the dried cells was estimated by subtracting the initial weight of the dried filter.

3.3.4 Reducing Sugar

Carbohydrates are dehydrated with concentrated H₂SO₄ to form “Furfural”, which condenses with anthrone to form a green color complex which can be measured by using colorimetrically at 620nm (or) by using a red filter. Anthrone react with dextrins, monosaccharides, disaccharides, polysaccharides, starch, gums and glycosides. But they yields of color where is to form carbohydrate to carbohydrate.

Anthrone reagent: Dissolve 200mg of anthrone reagent in 100ml of concentrated H₂SO₄.

Standard Glucose solution: a) Stock standard: Weigh 100mg of Glucose and transfer it carefully into a 100ml with Distilled water. (100mg of Glucose in 100ml of Distilled water). Working standard: Dilute 10ml of stock standard solution in 100ml with distilled water in a volumetric flask.

Procedure: To take 0.2 to 1ml of working standard solution of five different test tube and add water to bring the volume to 1ml in each test tube add 4ml of anthrone reagent and mix the contents as well and cover the test tube with bath for 10 min then cool the test tube to the room temperature and measure the optical density in a photoelectric colorimeter at 620nm (or) by using a red filter. Simultaneously prepare a blank with 1ml of distilled water and 4ml
of anthrone reagent. Construct a calibration curve on a graph paper, by plotting the glucose concentration (10 to 100mg) on x-axis and absorbance at 620 nm on the y-axis. Compute the concentration of the sugar in the sample from the calibration curve. While calculating the sugar concentration in the unknown sample, the dilution factor has to be taken into account.

3.3.5 Analytical methods for biogas generation

The samples taken were analyzed for volatile solids (VS), total solids (TS) and chemical oxygen demand (COD) using the Standard Method [4]. Ammonia nitrogen (NH3-N) content was examined using the hanna HI96715 Ammonia Medium Range Portable Photometer. The composition of methane in the biogas produced was analyzed using a gas chromatography equipped with a thermal conductivity detector (TCD). The column used was a HP Molesieve 30m × 0.53mm × 0.05mm capillary column. The injector, oven and detector temperatures were set at 150°C, 160°C and 200°C, respectively. Argon served as the carrier gas while nitrogen was used as the makeup gas.

3.3.6 Analysis method Study of magnetic field treatment on rhizosphere

15 minutes at 2 hour intervals for 36 hours. The bacterial strains were given magnetic treatment called MCUbc1. The growth rates of magnetic field treated (MCubc1) and control (Cubc1) were studied through measuring the absorbance at wavelength 600 nm of the viable cells and then plotted as a function of time. Best-performed MCubc1 under selective magnetic field intensity were subjective to compare with the control using different stress factor like Growth studies under salt (NaCl) stress, Growth studies at different pH. Stress tolerance of MCubc1 for NaCl, pH and temperature was studied in relation to its growth afterwards IAA production by bacterial isolates was determined following the methods of
Gordon and Weber (1951), ACC deaminase enzyme (EC 4.1.99.4) activity was assayed according to method of Glick et al. (1995), Siderophore production was determined on Chrome-azurol S (CAS) medium following the method of Schwyn and Neilands (1987). And control Cubc1) by inoculating equal volume (107cells/ml) of overnight grown cultures in nutrient broth and incubated in shaker incubator at 30°C for 36 hour. Three different magnetic fields ranges 500µT, 1700µT and 3000 µT were used. The magnetic field treatment was given at intervals of 2 h till 36h and the Growth was determined at various time intervals by measuring absorbance at 660 nm.