Chapter-III

Materials

And

Methods
3.1. Substrate: - Mahua flower (*Madhuca indica*)

Mahua flowers were collected (when flowers are ready to fall to the ground) from harvesters in Bhadrachalam, Khammam District, Andhra Pradesh, India. Traditionally, harvesters preserve these flowers along with Neem leaves (*Azadiractha indica*) and dried mahua flower showed in fig: 3.1.1. In the present investigation, mahua flowers were stored at -80 °C in deep refrigerator for its availability throughout the fermentation studies, to retain its biochemical composition and to avoid microbial contamination. At present, moisture content, total fermentable sugars, total reducing sugars, protein, and fat concentrations were estimated and tested for its suitability as substrate for bioethanol production using estimation procedures as follows:

3.2. Determination of moisture content of mahua flower:

Moisture content of the mahua flower was determined by using the direct method of Association of Official Agricultural Chemists (AOAC, 2000), Method No: 930.15.

Porcelain dish needs drying to be emptied in hot air oven for 2 hours at 135 °C and to be transferred to be desiccators to cool. Then it is necessary to weigh empty porcelain dish with 2 gm of sample and place the dish with 2gm of mahua flower in a hot air oven at 135 °C for about two hours. This process was repeated at one hour intervals until there was no further reduction in weight of mahua flower after drying. Determination of moisture content in sample as follows:

\[
\text{Total moisture content} = \frac{\text{Loss of weight}}{\text{Total weight}} \times 100
\]
3.3. **Estimation of total fermentable sugars in mahua flower by Anthrone method:**

The total content of sugars was estimated by the Anthrone method using glucose as a standard according to the procedure of Yemm and Willis (1954). The total content of sugars was extracted from the mahua flower according to the procedure of Rao. *et al.*, (1961). According to this method, an amount of 100 grams of mahua flower was taken in 100 ml of distilled water and allowed to grind in a rotor until it becomes mixed. This amalgamation is then filtered with glass cloth. Then the mahua flower extract (MFE) was collected in 500 ml of Erlenmeyer flask. This process was repeated with 100 ml of distilled water and mahua flower extract was allowed to determine total sugar concentration.

**Principle:**

\[ \text{H}_2\text{SO}_4 \] dehydrates carbohydrates form furfurals. These furfurals condense with Anthrone to the colored complex, which can be measured at 620 nm by spectrophotometer. Anthrone reacts with mono or di or and polysaccharide.

**Reagents:**

1. Anthrone reagent (0.2%):- 200mg of Anthrone dissolved in 100ml of concentrated \[ \text{H}_2\text{SO}_4 \].

2. Standard glucose Solution: - 100mg of glucose is dissolved in ml of water (1mg/1ml).

3. Stock standard: - 1ml of stock standard is diluted to 200ml with water.

**Procedure:** From the standard solution, different volumes ranging from 0.1 to 0.8 mg/ml of working standard solution of glucose is taken to different test tubes and distilled water is added to bring the volume to 1ml in each test tube. To each test tube 4ml of Anthrone reagent is added and stirred well. These tubes are then covered with marshes, kept in a boiling water
bath for 10 minutes, and then allowed to cool for few minutes. Optical Density of colored solution was measured at 620 nm in a spectrophotometer (Schimadzu-UV160).

The volume of 4.0 ml of the Anthrone reagent was mixed with diluted sample; then heated in boiling water bath for 10 minutes, and then cooled for five minutes in water at room temperature (28 °C). The intensity of the green colour developed was measured by using Spectrophotometer, Shimadzu-UV.

\[
\text{Concentration of total sugars} = \frac{\text{O.D of Unknown}}{\text{O.D of Known}} \times \text{Concentration of Known}
\]

3.4. Estimation of total reducing sugars in mahua flower di-nitro salicylic acid (DNS) reagent method:

The total content of reducing sugars of mahua flower extract (MFE) was estimated using Miller method (1959). Initial reducing sugar concentration was estimated after autoclaving the mahua flower. In this method 11 ml of 10% NaOH was added to 5gm of crystalline phenol. This was diluted to 50 ml in 35.5 ml of the alkaline phenol and 3.45 gm of Sodium bisulphate was dissolved. A solution of 150 ml of 4.5 % NaOH, 127.5gm of Rochelli’s salt and 440 ml of 1% di-nitro salicylic acid (DNS) solution were mixed with alkaline phenol solution. These solutions should be mixed well and kept in stopper bottle. Then, 1ml of diluted sample and 3ml of di-nitro salicylic acid (DNS) reagent were taken into a test tube and then heated for 5 minutes in the boiling water bath, then cooled. The solution was diluted with 10 ml of water and measure the optical density (O.D) at 540 nm. The unknown concentration was calculated against a standard graph of glucose (10-100µg/ml) prepared under similar conditions.
3.5.  Estimation of protein in mahua flower extract (MFE):

The total protein content in mahua flower was estimated using Lowry’s method (1951).

Principle:-

The phenolic group of tyrosine and tryptophan residues (amino acids) in a protein will produce a blue purple color complex, with a maximum absorption in the range of 660 nm wavelength. Folin-Ciocalteau reagent consists of sodium tungstate molybdate and phosphate. Thus, the intensity of color depends on the amount of aromatic amino acids present in different proteins. Most proteins estimation method use bovine serum albumin (BSA) universally as a standard protein, because of its low cost, high purity and ready availability.

Reagents:-

(1). Bovine serum albumin (BSA) stock solution 100mg/100ml.

(2). Analytical reagents:

(a). 50 ml of 2 % sodium carbonate mixed with 50 ml of 0.1 N NaOH solution (4 gm in 100 ml of distilled water).

(b). 10 ml of 1.56% copper sulphate solution mixed with 10 ml of 2.37% sodium potassium tartrate solution. Prepare analytical reagents by mixing 2 ml of (b) solution and 100 ml of (a) solution.

(3). Folin – Ciocalteau reagent solution (1N) Dilute commercial reagent (2N) with an equal volume of water on the day of use (2 ml of commercial reagent + 2 ml of distilled water).
Procedure:-

1. Different dilutions of bovine serum albumin (BSA) solutions were prepared by mixing stock BSA solution (1mg/ml) and water in the test tube as given in the table. The final volume in each of the test tubes is 5 ml. The BSA range is 0.05 to 1 mg/ml.

2. From these different dilutions, pipette out 0.2 ml protein solution to different test tubes and add 2 ml of alkaline copper sulphate reagent. These solutions should be mixed well.

3. This solution is incubated at room temperature for 10 minutes.

4. Then add 0.2 ml of reagent folin ciocalteau solution to each tube and incubate for 30 min. Zero the colorimeter with blank and measure the optical density (O.D) at 660 nm.

5. Check the absorbance of unknown sample and determine the concentration of the mahua flower sample using the standard concentration as follows:

\[
Concentration\ of\ Protein = \frac{O.D\ of\ Sample}{O.D\ of\ BSA} \times Concentration\ of\ BSA
\]

3.6. **Estimation of crude fat in mahua flower:**

The total content of crude fat was estimated using American association of cereal chemists (AACC, Method: 30-25, 1983). 2gm of sample was dried in vacuum oven at 95-100\(^\circ\)C under pressure (not to exceed 100 mm of Hg). The sample was placed in the tube of the Soxhlet extraction apparatus. Extraction with petroleum ether (150 drops/min) was done till 6 hour without interruption by gently heating. The flask was cooled and the ether was evaporated on a steam of water bath, and then cooled at room temperature. The flask was removed carefully and sampled was weighed.

\[
Total\ weight\ fat = \frac{Weight\ of\ the\ fat}{Weight\ of\ the\ sample} \times 1(0)
\]
3.7. Microorganisms:

Yeast and Bacterial strains such as *Saccharomyces cerevisiae*-3190 NCIM, *S.cerevisiae*-171 MTCC, *K.thermotolerance*-30 MTCC, *S.cerevisiae*-3288 NCIM, *K.marxianus*-1389 MTCC, *Z.mobilis*-92 MTCC, *E.coli* and *S.cerevisiae*-463 MTCC, which are obtained from National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratories, Pune and (MTCC) Microbial Type Culture Collection, Chandigarh, India) cultures were tested for bioethanol production. These microbial strains are shown in figure no: 3.2.1.

3.8. Preparation of yeast culture medium:

The yeast culture medium was prepared in 2 litre Erlenmeyer flasks containing glucose, yeast extract, malt extract and peptone (GYMP) in 1 litre of distilled water and pH was adjusted to 6 using 1 N HCL and 1 N NaOH. The medium was autoclaved at 121°C for about 30 minutes. After autoclave was completed, 10 ml of medium was aseptically transferred to petri plates and 30 ml tubes. Then, the original cultures of yeast were aseptically inoculated with loop on agar slopes. Using this medium composition, cultures were incubated at 30°C for 48 hours. For every 30 days, yeast culture was freshly prepared for maintaining cell viability and the total experiments were carried out with freshly prepared nutrient agar medium and nutrient broth medium. After the completion of growth period of 48 hours, these cultures were used for further experiments. The culture medium was prepared as follows:
Table no: - 3.8.1. Yeast culture medium and nutrient broth medium composition for inoculum preparation:

<table>
<thead>
<tr>
<th>Component</th>
<th>Nutrient agar medium (g.l^{-1}) composition</th>
<th>Nutrient broth medium (g.l^{-1}) composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>10gm/1 litre</td>
<td>20gm/2 litre</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>3gm/1 litre</td>
<td>6gm/2 litre</td>
</tr>
<tr>
<td>Malt Extract</td>
<td>3gm/1 litre</td>
<td>6gm/2 litre</td>
</tr>
<tr>
<td>Peptone</td>
<td>5gm/ llitre</td>
<td>10gm/2 litre</td>
</tr>
<tr>
<td>Agar</td>
<td>20 gm/1 litre</td>
<td>---------------</td>
</tr>
<tr>
<td>pH</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

3.9. Preparation of bacterial culture medium:

Bacterial culture medium was prepared using beef extract of 3 g.l^{-1}; peptone, 5 g.l^{-1}; sodium chloride of 8 g.l^{-1} and 15 grams of Agar were added to 1000 ml of distilled water in 2 litre Erlenmeyer flask. The pH of the medium was adjusted to 7.5 using 1 N HCL and 1 N NaOH with the aid of pH Meter (Systronics). Then, the medium was allowed to sterilize at 121 \(^0\)C for 30 minutes. Then, 10 ml of sterilized medium was aseptically transferred to the culture tubes and rotated at 45\(^0\) for 25 minutes to develop agar slopes. After the solidification completed, one loopfull of original culture of bacterial strains were aseptically streaked on agar slopes and tightly capped with non-adsorbent cotton. Then, these agar slants were incubated at 25 \(^0\)C for 48 hours of growth period.

3.10. Preparation of fermentation medium:

In the present investigation, 2 litres of fermentative medium was prepared for each set of experiment using mahua flower extract (MFE) for bioethanol production in 5 litre batch bioreactor along with the optimized fermentative conditions. Different concentrations of sugars were taken on the basis of weight (w/w) or (grams of total sugars/grams of mahua flower extract). The fermentative medium was sterilized at 121 \(^0\)C for 30 minutes in bioreactor before batch fermentation process.
3.11. Determination of total cell count (TCC):

Determination of total cell count (TCC) per milli litre was determined using plate count technique. It is one of the most routinely used procedures because of the enumeration of viable cells by American public health association (APHA Method, 1967).

**Principle:** - when material containing bacteria are cultured, every viable bacterium develops into a visible colony on a nutrient agar medium. Therefore, the number of colonies is same as the number of microorganisms contained in the sample.

**Procedure:** - A measure of volume or weight is mixed with a large volume of sterile water or saline called the diluents or dilution blank. Dilutions are usually made in multiples of ten. A single dilution is calculated as follows:

\[
\text{Dilution} = \frac{\text{Volume of the sample}}{\text{Total volume of the sample and diluent}}
\]

Serial dilutions are prepared by transferring a known volume of the dilution to second dilution blank and so on. Once diluted, the specified volume of the dilution sample 1 ml from various dilutions is added to sterile petri plates (in triplicate for each dilution) to which molten and cooled (45-50 °C) suitable agar medium is added. The colonies are counted on quebec colony counter.

The number of organisms developed on the plates after an incubation period of 24 h or 48 h or 72 h per ml is obtained by multiplying the number of colonies obtained per plate by the dilution factor, which is reciprocal of the dilution. To facilitate calculations, the dilution is written in exponential notation. For example, 1.1000; 1.2000; 1.3000; 1.4000; 1.5000; 1.6000; 1.7000 ml dilution would be represented as 10\(^{-1}\), 10\(^{-2}\), 10\(^{-3}\), 10\(^{-4}\), 10\(^{-5}\), 10\(^{-6}\) and 10\(^{-7}\). Total number of cells per ml can be calculated as follows:
Therefore, Microorganism per millilitre/ gm of the sample can be computed as follows:

\[
\text{Number of cells per ml} = \frac{\text{Number of colonies}}{\text{Amount plated} \times 1/ \text{Dilution}}
\]


Methylene blue is the most commonly used stain for the determination yeast cell viability. Viable cells are able to reduce the stain and making the yeast colorless, whereas non-viable cells are unable to reduce the stain rendering them a deep blue-purple shade. Viable yeast cell count was determined using a hemocytometer and a light microscope in less than ten minutes. When buffered and supplemented adequately, methylene blue has no effect on yeast cell viability. Methylene blue staining is considered to be an accurate method only when yeast cell viability is greater than 90% (Bonara and Mares. (1982)).

3.13. Fermentation with 5 litre bioreactor:

Bioreactor (B-Lite, Sartorious Private Limited, Mumbai, India) is batch scale bioreactor that can ferment up to 5 Litres of fermentative medium. In the present study, 2 litres of Mahua fermentative medium was used for bioethanol production under controlled conditions like pH, temperature, and agitation. During the batch fermentation process, bioreactor was sterilized at 121 °C for 15 minutes. For inoculum volume, one loop full of yeast culture was aseptically transferred to the GYMP broth medium at the controlled conditions of pH 5 using 0.1 M HCL and 0.1 M NaOH. Bioreactor is shown in figure no: -3.10.1.
3.14. Determination of bioethanol by gas chromatography:

Quantitative analysis was performed using a gas chromatography (Shimadzu: GC-17A, Software: GC solutions) with an auto injector. The segments of chromatography are wax column (30mm length, 0.55mm ID, 1 µm film thickness) and flame ionization detector (FID). The peak heights are monitored and integrated using GC-solution software. Bioethanol analysis was carried out using gas chromatography (British Pharmacopeia, 2007).

Reagents: n-Butanol (20 ± 1 °C).

Standard solution: - 1 ml of absolute ethyl alcohol is dissolved in 100 ml of n-Butanol in 500 ml Erlenmeyer volumetric flask.

Sample solution: - the volume 1 ml of fermented bioethanol sample to be analyzed was collected after distillation of fermented samples. Then, the 1 ml of sample volume was diluted in 100 ml of n-Butanol in 500 ml Erlenmeyer volumetric flask. The conditions of gas chromatography were used for determination of bioethanol concentration in fermentative medium in the present investigation as follows:

Injector temperature : 230 °C
Detector temperature : 240 °C
Column flow : 2 ml/mn
Injection volume : 2 µl
Carrier gas : Helium

Procedure: It is being injected 2 µl of standard solution into the injection port and recorded the standard chromatograms. Then, it is being injected 2 µl of the fermented bioethanol
samples into the injection port and recorded sample peak height of ethyl alcohol and n-butanol. In this method, n-Butanol was used as internal standard. Standard curve was developed using different bioethanol concentrations. Bioethanol volume (v/v) is converted to (w/v %) the percentages by multiplying with the standard potency of ethanol (99.5% dehydrated ethyl alcohol). The percentage of bioethanol was calculated as follows:

$$\text{Bioethanol (\%) = } \frac{\text{Peak Area of Bioethanol}}{\text{Peak Area of n-Butanol}} \times \frac{\text{Wt of Std}}{\text{Std Volume}} \times \frac{\text{Sample Volume}}{\text{Wt of sample}} \times \text{Std Potency}$$

3.15. Computation of percentage of bioethanol in fermented medium:

Theoretically, 1.0 gm of glucose can be converted to 0.511 gm of ethanol and 0.488 gm of carbon dioxide (CO₂) (Lucilia Domingues., et al, 2010) and Pratyoosh Shukla, et al, 2012). The theoretical and experimental yields of bioethanol, Percentage of bioethanol in fermentative medium (Alvarenga, Raquel Mendonca, et al, (2011), and the improved percentage of bioethanol during the batch fermentations are computed as follows:

Theoretical bioethanol recovery = Total concentration of sugars X 0.511 gm

Experimental bioethanol recovery = Actual bioethanol recovery by experiments

Total bioethanol recovery (%) = \frac{\text{Experimental bioethanol recovery}}{\text{Theoretical bioethanol recovery}} \times 100

Improved bioethanol (%) = Bioethanol recovery of medium II – Bioethanol recovery of medium I.
Figure No :- 3.10.1. Bioethanol fermentation in 5l bioreactor.

Figure No :- 3.13.1. Gas chromatography (GC-Shimadzu).
Figure No: 3.1.1. Dried mahua flower (*Madhuca indica*):

![Dried mahua flower](image1.png)

Figure No: 3.2.1. Pure cultures of yeast and bacterial strains.

![Pure cultures](image2.png)
3.16. Optimization studies using Box-Wilson response surface methodology (RSM):-

To develop a novel method for the production of higher bioethanol, optimization of medium constituents and fermentation conditions were performed using response surface methodology by “Statistica8” software package. Optimization of media constituents by laboratory method is a single – dimensional search involving changing one variable while fixing the others at a certain level is laborious and time consuming, especially when the number of variables is in large. Therefore, an alternative and potential method in microbial system is the function of statistical methods. Statistical inference techniques can be used to evaluate the importance of individual factors, as well the appropriateness of functional form and sensitivity of the response to each factor (Mason, et al. 1989). The effect of medium components on bioethanol production studied at three different levels such as low concentration level (-1), Middle level (0) and High level (+1) and three independent variables represented as X₁, X₂, X₃ and dependent variable is production of bioethanol (g.1⁻¹) of a design.

Recently, many statistical experimental design methods have been employed in bioprocess optimization. Among them, response surface methodology (RSM) is one of the suitable methods for identifying the effect of individual variables and optimizing the conditions for a multivariable system efficiently. Multiple regression and correlation analysis are used as tools to assess the effects of two or more independent factors on the dependent variables. This method has been successfully applied to optimize alcoholic fermentation and other fermentation media (Maddox & Reichert, (1977); Zertuche and Zall (1985); Ayyanna, et al, (1998), Ratnam, et al., (2003), Popa, et al, (2007), Park, et al., (2010) and Shihao Zhao et al, (2012). It is has been applied successfully for the optimization of fat, oil including Madhuca indica oil for the production of biodiesel (Raheman, et al, 2006). Karuppaiya, et al.,
(2010) reported that the optimum fermented conditions were determined; substrate concentration 62 % (v/v), pH 6.5, temperature 32°C and fermentation time 37h. A maximum ethanol concentration of 15.64 g.l⁻¹ was obtained at the optimized process conditions by solving the regression equation.

**Similarity of regression analysis and response surface methodology (RSM)**

The regression analysis assists the assessment of variable quantities that effect of the factors on the behaviour of the system. From the set of experimental data, a mathematical model can be established the relationship between the response variables and the factors that influence the fermentation process. It is one of the effective tools used for investigating affect of relationships having applications of physical, chemical, engineering, and biotechnology, as well as in social sciences.

Response surface methods are additional techniques employed before and after a regression analysis performed on the data. The experiment must be designed using regression analysis that selects input variables and their values during the actual experimentation assigned. After the regression analysis is performed, certain model testing procedures and optimization techniques were applied. Thus the subject RSM includes the application of regression as well as other techniques in an attempt to gain a better understanding of the characteristics of the response system under study.
Central Composite Design (CCD)

The central composite design (CCD) is an alternative class of 3k factorial design and it was introduced by Box and Wilson (1951). A central composite design consists of:

- A complete (or a fraction of a) $2^k$ factorial design, where the factor levels are coded to the Usual -1, 0, +1 values. This is called factorial portion of the design.
- $n_o$ = Center points ($n_o(3)$)
- Two axial points on the axis of each design variable at a distance of $\alpha$ from the design center. This portion of the design. Thus the total number of design points in central composite design (CCD) consisting of k variables are given as follows:

$$N = 2^k + 2k + n_o$$

To make the design rotatable, the variance of the predicted response remains constant at all points which are equidistant from the design center. To make central composite design (CCD) rotatable, it is chosen the value of $\alpha$ that satisfies the condition:

$$(iii) = 3 \{ ijj \}, I (1,2......k; i=j)$$

Where, “$k$” is the number of Variables. Let “G” be the scale factor given by the equation

$$G = \{ N/ (F+2\alpha^2) \}^{1/2}$$

Where, “$F$” is the number of points in the factorial portion of the design given 2k and “$n_o$” is the total number of points in the central composite design (CCD):

$$Fg^2 + 2\alpha^4 g^4 = 3 Fg^4$$

Or equivalently $\alpha = F^{1/4}$, $\alpha = F^{k/4}$
The center points are repeated for every block of experiments to have a check on whether the experiments are conducted under same conditions in every block:

The variables are coded according to the equation $X_i = (X_i - \bar{x}_i)/(\Delta x_i)$

Where $x_i$ is the dimensionless value of an independent variable, $X_i$ is the real value of an independent variable, $x_i$ is the real value of the independent variable at the center point and $\Delta x_i$ is the step change. The independent variables are the process parameters to be optimized.

**Experimental designs for the determination of optimum concentrations of medium components and fermentation conditions for the production of bioethanol by submerged batch fermentation:**

Central composite design (CCD) was used to optimize the medium constituents and the conditions of fermentation of bioethanol separately by submerged fermentation using 5l bioreactor. The experiments were designed by using the Design Expert (Stat Ease Inc., USA). The carbon source, nitrogen source and the physical conditions were reported to be highest influenced conditions were chosen as major constituent factors to optimize the medium to enhance bioethanol production.

In the present investigation, the concentration of substrate (g.l$^{-1}$), Temperature ($^0$C) and pH were kept constant in the fermentative medium throughout the study. A $2^3$ factorial experimental designs or central composite designs (CCD) with four axial points (with $\alpha = \sqrt{3}$) and three replications at the centre points ($n_o = 3$) leading to a total number of seventeen experiments were applied for the optimization of both the medium constituents and the conditions of fermentation.
Coding of the variables was done according the equation as follows:

\[ X_i = (X_i - \bar{x}_i) / (x_i) \] .......................... 3.1

Where

- \( x_i = \) Dimensionless value of an independent variable.
- \( X_i = \) Real value of an independent variable.
- \( \bar{x}_i = \) Real value of an independent variable at the center point, and
- \( x_i = \) Step change

The design matrix and levels of the independent variables chosen for the study are shown in tables 3.1 and 3.2. Regression analysis was performed on the data obtained using MATLAB software. A second order polynomial equation (3.2), which includes all interaction terms were used to calculate the predicted response as follows:

\[ Y_i = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{33} x_3^2 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 \] .......................... 3.2

The optimum conditions of independent variables were determined by optimizing the second order polynomial equation (equation 3.2) using MATLAB Software.
3.17. Design of medium-I using standard optimization process and medium –II with statistical optimization of fermentative conditions:

Initially, fermentative parameters were optimized and designed a medium which is represented as medium-I. Based on these results, optimization of fermentative parameter conditions were studied using central composite design (CCD) by response surface methodology (RSM). A cost effective fermentative medium was designed to enhance bioethanol production and it was represented as medium-II. In this study, central composite design (CCD) consisted of (i) A complete \(2^3\) factorial design, (ii) center point \(n_0=3\) (iii) two axial points on the axis of each design variable at a distance of \(\alpha = 1.67332\) from the design center. Hence, the total number of design points of \(N = 2^k + 2K + n_0\) leading to a total of 17 sets of experiment was formulated with three independent variables \((X_1, X_2, X_3)\) at -1(High value), 0 (Center value), +1 (Low value) levels were conducted. Central coded value was considered as zero for all variables.

The Predicted response \((Y_i)\) of each design and optimum values of each variable on bioethanol productions were analyzed using response surface methodology (RSM). Analysis of variance (ANOVA) is important in determining adequacy and significance of the quadratic model. Under statistically optimized conditions of the medium-II, bioethanol productions were performed using \(S.cerevisiae\)-3190 in batch bioreactor and the distilled samples of fermented broth were analyzed using gas chromatography. The results of initial batch fermentation process, statistical optimization of various physico-chemical and nutritional parameters as well as the comparative studies of medium-1 and medium-II on bioethanol production were discussed in the Chapter-IV.
3.18. Coded values of central composite design of variables (X₁), (X₂) and (X₃) on bioethanol production:

<table>
<thead>
<tr>
<th>S.no</th>
<th>No of experiments with CCD</th>
<th>Coded values (X₁)</th>
<th>Coded values (X₂)</th>
<th>Coded values (X₃)</th>
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<tbody>
<tr>
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</tr>
</tbody>
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3.19. The central composite design matrix applied for the optimization of different medium design factors of the medium-II on bioethanol production:

<table>
<thead>
<tr>
<th>S.no</th>
<th>Central composite design (CCD)</th>
<th>Independent variables</th>
<th>Coded Values</th>
</tr>
</thead>
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<td>Design-I</td>
<td>Substrate concentration, g.l⁻¹</td>
<td>-1 0 +1</td>
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<td></td>
<td></td>
<td>Temperature, °C</td>
<td>-1 0 +1</td>
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<tr>
<td></td>
<td></td>
<td>pH</td>
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</tr>
<tr>
<td>2</td>
<td>Design-II</td>
<td>Inoculum volume, v/v</td>
<td>-1 0 +1</td>
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<tr>
<td></td>
<td></td>
<td>Agitation, RPM</td>
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<td></td>
<td>Inoculum age, Hours</td>
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<td>Design-III</td>
<td>Inorganic nitrogen, g.l⁻¹</td>
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<td>Copper chloride, g.l⁻¹</td>
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<td>Manganese, g.l⁻¹</td>
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<td>Design-IV</td>
<td>Magnesium chloride, g.l⁻¹</td>
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<td>Zinc sulphate, mg.l⁻¹</td>
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<td>Vitamin, mg.l⁻¹</td>
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<td>Design-V</td>
<td>Amino Acids, g.l⁻¹</td>
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<td>Phosphorous, g.l⁻¹</td>
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<td>Metal chelaters, g.l⁻¹</td>
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<td>Design-VI</td>
<td>Potassium phosphate, g.l⁻¹</td>
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<td>Calcium chloride, g.l⁻¹</td>
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<td>Cobalt chloride, mg.l⁻¹</td>
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<td>Ferrous sulphate, g.l⁻¹</td>
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<td>Oxygen, mg.l⁻¹</td>
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<td>Peptone, g.l⁻¹</td>
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<td>Yeast Extract, g.l⁻¹</td>
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