CHAPTER 3

EXPERIMENTAL TECHNIQUES

3.1 INTRODUCTION

In this chapter a detailed description of the chemicals used, experimental setup, sample analysis techniques, and methodology for enhanced production of inulnase enzyme from *Kluyveromyces marxianus* in a Bio reaction calorimeter are given.

3.2 CHEMICALS AND REAGENTS

All the chemicals used in the experiments were of analytical grade (AR) purchased from SD fine chemicals Pvt Ltd, Sigma India Pvt Ltd, RANKEM India Pvt Ltd, Bangalore. All the media components used for microbiological studies were purchased from Hi media Pvt Ltd, Bangalore. All media components were autoclaved at 121 °C for 15 mins and used aseptically thereafter.

3.3 GROWTH MEDIUM

*K. marxianus* strain (MTCC 4139) was maintained on yeast malt agar (YM Agar) slants containing yeast extract (3 g/L), malt extract (3 g/L), peptone (5 g/L), glucose (10 g/L), and agar (20 g/L) at 4 °C. Sub-cultured at 15–21 days intervals in seed culture media containing yeast extract (10 g/L), peptone (20 g/L), sucrose (20 g/L) at 30 °C, 150 rpm pH 3.5 for 16 hr. 30 mL
seed culture served as 2% inoculum for 1500 mL production medium (0.3 gram of dry cell weight per liter (DCW g/L)).

3.4 STRAIN MAINTENANCE

*Kluyveromyces marxianus* strain (MTCC 4139) was maintained on yeast mold agar (YM Agar) slants, sub-cultured at 15–21 days intervals in seed culture media. Figure 3.1 shows the *K. marxianus* slant culture and quadrant streaking on YM agar plates. Cream colored were colonies observed. *K. marxianus* cells were of globose shape, varying in size between (2-6) × (3-11) µm.

![Figure 3.1 Slant culture and subculture plate of *K. marxianus*.](image)

3.5 STRAIN MORPHOLOGY

Figure 3.2 shows the light microscopic image of lactophenol cotton blue stained *K. marxianus*. Small spherical shaped (globose) cells were observed.
3.6 SHAKER FLASK STUDIES

3.6.1 Batch Medium Selection

The batch medium used in this study was selected based on literature studies. From the available literature, four different suitable media were selected (Table 3.1) Media A (Singh & Lotey 2010), Media B (Cazetta et al. 2010), Media C (Kalil et al. 2001), Media D (Schuler et al. 2012b).

Table 3.1 Composition of the media tested for inulinase production by *K. marxianus*.

<table>
<thead>
<tr>
<th>Medium [A]</th>
<th>g/L</th>
<th>Medium [B]</th>
<th>g/L</th>
<th>Medium [C]</th>
<th>g/L</th>
<th>Medium [D]</th>
<th>g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>20</td>
<td>Sucrose</td>
<td>10</td>
<td>Sucrose</td>
<td>14</td>
<td>Sucrose</td>
<td>10</td>
</tr>
<tr>
<td>CaCl&lt;sub&gt;2&lt;/sub&gt;</td>
<td>0.95</td>
<td>MgSO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>0.7</td>
<td>Yeast Extract</td>
<td>10</td>
<td>(NH&lt;sub&gt;4&lt;/sub&gt;)&lt;sub&gt;2&lt;/sub&gt;SO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>5</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>21.7</td>
<td>Yeast Extract</td>
<td>5</td>
<td>Peptone</td>
<td>20</td>
<td>KH&lt;sub&gt;2&lt;/sub&gt;PO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>3</td>
</tr>
<tr>
<td>SDS</td>
<td>0.288</td>
<td>KCl</td>
<td>1.2</td>
<td>K&lt;sub&gt;2&lt;/sub&gt;HPO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>1</td>
<td>MgSO&lt;sub&gt;4&lt;/sub&gt;.7H&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NH&lt;sub&gt;4&lt;/sub&gt;Cl</td>
<td>1.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>KH&lt;sub&gt;2&lt;/sub&gt;PO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Peptone</td>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>5.5</td>
<td>pH</td>
<td>3.6</td>
<td>pH</td>
<td>3.6</td>
<td>pH</td>
<td>5</td>
</tr>
</tbody>
</table>
Experiments were carried out for 72 hours on a rotary shaker at 30 °C, 150 rpm with a uniform inoculum size of 2 %. Tukey’s high significant difference (HSD) statistical test was performed for the determination of significant medium among the four media. Response surface methodology central composite design was employed for the carbon and nitrogen source optimization in the medium.

3.6.2 Tukey’s Test

Tukey’s test determines the individual average values which are significantly different from the set of average values. Tukey’s test is a multiple comparison test applicable if there are two or more average values being compared (utilizes a t test). Initially Analysis of variance (ANOVA) is performed followed by Tukey’s test. Tukey’s test calculates by pairwise comparison of all means. Honest significant difference (HSD) shows the pairwise differences between two means that exceed the HSD value as calculated by equation 3.1.

$$HSD = q\sqrt{\frac{MS}{n}}$$  \hspace{1cm} (3.1)

where MS is the mean square value computed in the ANOVA, n is the number of samples in each group, and q is determined from the studentized range distribution table.

3.7 RESPONSE SURFACE METHODOLOGY (RSM)

RSM attempts to analyze the influence of the independent variables on a specific dependent variable (response). The independent variables, denoted by $X_1, X_2, ..., X_k$, are presumed to be continuous and can be controlled with negligible error. The response ($Y$) is postulated to be a random variable.
The individual variables \((X_1, X_2, \ldots, X_k)\) and the response can be related as follows

\[
y = f(X_1, X_2, \ldots, X_k) + \varepsilon \quad (3.2)
\]

where \(y\) is the response of the system, \(f\) is the response function, \(X_1, X_2, \ldots, X_k\) are the independent variables, \(k\) is the number of independent variables, and \(\varepsilon\) is the statistical error.

The primary function of RSM is to postulate a suitable approximation for the true functional relationship between the response and the set of independent variables. Initially, a low-order (first-order) polynomial in some region of independent variables is employed. A first-order response surface model can be expressed as follows:

\[
y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \varepsilon \quad (3.3)
\]

For maximization problems, experiments are conducted along the path of steepest ascent until no further increase in the response is observed. The set of values of independent variables where no further increase in response is observed is known as the optimal region. In most cases, a second-order response surface model is used which can be given as (Rodríguez-Nogales et al. 2007):

\[
y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{i,i} X_i^2 + \sum_{i=1}^{k-1} \sum_{j=i+1}^{k} \beta_{i,j} X_i X_j + \varepsilon \quad (3.4)
\]

where \(X_i, X_j\) are coded independent variables,

\(\beta_0, \beta_i, \beta_{ii}, \beta_{ij}(i = 1, 2, \ldots, k), \beta_{ij}(i = 1, 2, \ldots, k)(j = 1, 2, \ldots, k)\) are the regression coefficients for the intercept, linear, quadratic and interaction terms,
respectively, and $\epsilon$ is the statistical error. In the present study, RSM has been used to determine the relation between enzyme activity and operating parameters such as pH, temperature, inoculum size and agitation.

The uncoded variables are converted to coded variables using the following Equation (3.5) (Banik et al. 2007):

$$
X = \frac{x-[x_{\text{max}}-x_{\text{min}}]/2}{[x_{\text{max}}-x_{\text{min}}]/2}
$$

where, $x$ is natural variable and $X$ is coded variable. The significant bioprocess variables chosen were as follows: pH ($X_1$), temperature ($X_2$), inoculum size ($X_3$), and agitation ($X_4$), each variable was assessed at three coded levels (-1, 0 and +1). The mathematical representation of the response $y$ and the variables is given as [Murugesan et al. 2007]:

$$
y = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_4X_4 + \beta_{11}X_1^2 + \beta_{22}X_2^2 + \beta_{33}X_3^2 + \beta_{44}X_4^2 + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{14}X_1X_4 + \beta_{23}X_2X_3 + \beta_{24}X_2X_4 + \beta_{34}X_3X_4
$$

(3.6)

In the present work, Central Composite Design (CCD) module of RSM was adopted for the augmentation of inulinase production. For the response (inulinase activity), four independent variables (pH, temperature, inoculum size, and agitation) were varied simultaneously in relation to the chosen center point, there being six replicates at the center point and a single run for each of the other combinations.

### 3.7.1 Central Composite Design

The most popular method of DoE is central composite design (CCD). A CCD has three groups of design points:

(A) Two-level factorial or fractional factorial design points
(B) Axial points (sometimes called star points)

(C) Centre points

3.7.1.1 **Factorial points**

The two-level factorial part of the design consists of all possible combinations of the +1 and -1 levels of the factors. For the two factor case there are four design points (-1, -1) (+1, -1) (-1, +1) (+1, +1).

3.7.1.2 **Axial points**

The axial points have all of the factors set to 0, the midpoint, except one factor, which has the value +/- Alpha. For a two-factor problem, the axial points are:

(-Alpha, 0) (+Alpha, 0) (0, -Alpha) (0, +Alpha)

3.7.1.3 **Centre points**

Centre points, as implied by the name, are points with all levels set to coded level 0 - the midpoint of each factor range: (0, 0). Centre points are usually repeated 4-6 times to get a good estimate of experimental error (pure error).

3.7.1.4 **Categorical factors**

This will cause the number of runs generated to be multiplied by the number of combinations of the categorical factor levels.
3.7.2 Statistical analysis

The data obtained from RSM on inulinase production were subjected to statistical analysis for validating the model equation.

3.7.2.1 Analysis of variance (ANOVA)

ANOVA is a statistical technique that subdivides the total variation (into sets of data) into component parts associated with specific sources of variation for the purpose of testing the hypotheses on the parameters of the model (Ravikumar et al. 2006). The statistical significance ($SS_p$) is the ratio of mean square variation due to regression; mean square residual error was performed where $m$ is the total number of the experiments, and $\eta_i$ is the S/N ratio at the $i^{th}$ test. The sum of squares from the tested factors, $SS_p$, can be calculated as:

$$SS_p = \sum_{j=1}^{t} \left( \frac{S_{nj}}{t} \right)^2 - \frac{1}{m} \left( \sum_{i=1}^{m} \eta_i \right)^2$$  \hspace{1cm} (3.7)

where $p$ represents one of the tested factors, $j$ the level number of this specific factor $p$, $t$ the repetition of each level of the factor $p$, and $S_{nj}$ the sum of the S/N ratio involving this factor at level $j$.

Degree of freedom ($D$) denotes the number of independent variables. The degree of freedom for each factor ($D_p$) is the number of its levels minus one. The total degrees of freedom ($D_T$) are total number of trials minus one. The degree of freedom for the error ($D_e$) is the number of the total degrees of freedom minus the total degree of freedom for each factor.
Variance ($V$) is defined as the sum of squares of each trial sum result involving the factor, divided by the degrees of freedom of the factor as shown in Equation (3.8)

$$V = \frac{SS_p}{D_p} \times 100$$  \hspace{1cm} (3.8)

The corrected sum of squares ($SS'_p$), is defined as the sum of squares of factors minus the error variance times the degree of freedom of each factor as shown in Equation (3.8)

$$SS'_p = SS_p - D_pV_e$$ \hspace{1cm} (3.9)

Percentage of the contribution to the total variation ($P_p$), denotes the percentage of the total variance of each individual factor as shown in Equation (3.9)

$$P_p = \frac{SS_p}{SS_T} \times 100$$ \hspace{1cm} (3.10)

The statistical software package, design expert was used for the regression analysis of the experimental data, and also to plot the response surface graphs. The statistical significance of the model equation and the model terms was evaluated by Fisher’s test. The quality of fit of the second-order polynomial model equation was expressed by the coefficient of determination ($R^2$) and the adjusted $R^2$. The fitted polynomial equation was then expressed in the form of three-dimensional surface plots, in order to illustrate the relationship between the response and the experimental levels of each of the variables utilized in this study.
3.8 FED-BATCH MEDIUM SELECTION

Three fed-batch media (P, Q, and R) were formulated from medium D in order to study the effect of carbon and nitrogen source on enzyme activity (Table 3.2). The three media were inoculated with 2 % seed culture (0.3 DCW g/L) and fermentation was carried out in a 500 mL conical flask containing 125 mL of the media, for 63 hours at pH 5, temperature 30 °C and with 150 rpm.

3.9 OPTIMIZATION OF FEEDING RATE

In order to optimize the feeding rate, two methods were followed for the concentrated feeding of the carbon, nitrogen, and mixed feeding (Table 3.2). In the first method (P1, Q1, R1) 0.5 mL/hr feed rate and a dilution rate of D= 0.004 hr\(^{-1}\) was maintained. In the second method (P2, Q2, R2) 0.25 mL/hr feeding with a dilution rate of D= 0.002 hr\(^{-1}\) was maintained.

**Table 3.2 Optimization of feed and feeding rate of sucrose for the production of inulinase by K. marxianaus.**

<table>
<thead>
<tr>
<th>Media P</th>
<th>g/L</th>
<th>Media Q</th>
<th>g/L</th>
<th>Media R</th>
<th>g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>KH(_2)PO(_4)</td>
<td>3</td>
<td>KH(_2)PO(_4)</td>
<td>3</td>
<td>KH(_2)PO(_4)</td>
<td>3</td>
</tr>
<tr>
<td>MgSO(_4).7H(_2)O</td>
<td>0.5</td>
<td>MgSO(_4).7H(_2)O</td>
<td>0.5</td>
<td>MgSO(_4).7H(_2)O</td>
<td>0.5</td>
</tr>
<tr>
<td>(NH(_4))(_2)SO(_4)</td>
<td>5</td>
<td>Sucrose</td>
<td>10</td>
<td>(NH(_4))(_2)SO(_4)</td>
<td>2.5</td>
</tr>
</tbody>
</table>

Feed solutions for P, Q, and R contained sucrose 1 g/mL, ammonium sulphate 1 g/mL, sucrose and ammonium sulphate each 0.5 g/mL respectively. From 8\(^{th}\) to 23\(^{rd}\) hr continuous feeds were given at the above
dilution rate to the respective media. Media P1 and P2 were subjected to carbon source feeding while media Q1 and Q2 were subjected to nitrogen source feeding (Cazetta & Contiero 2013). A combination of carbon and nitrogen sources was fed to media R. Enzyme Activity (IU/mL) and DCW (g/L) at the end of 24, 36 and 63 hours were estimated. These feed solution concentrations were optimized by several pilot studies Hi-Media Pvt Ltd, Bangalore.

3.10 INULINASE ENZYME ASSAY AND QUANTIFICATION

3.10.1 Inulinase Assay by Dinitro Salicylic Acid (DNS) Method

This method tests for the presence of free carbonyl group (C=O) in reducing sugars. This involves the oxidation of the aldehyde functional group present in, for example, glucose and the ketone functional group in fructose. Simultaneously, 3, 5-dinitrosalicylic acid (DNS) is reduced to 3-amino, 5-nitrosalicylic acid under alkaline conditions.

3.10.2 List of Reagents

1) Inulin Acetate Buffer (0.01 mol/L), pH 4.6

To 0.65 mL acetic acid, 0.35 mL sodium acetate (1M) was added and made up to 100 mL with distilled water. To this, 2% inulin was added.

2) DNS Reagent

To 100 mL 1% NaOH, 1 g DNS was added and completely dissolved. Following this, 200 µL crystalline Phenol and 25 mg Sodium Sulphite was added. The reagent was stored it at 4 °C.
3) **Rochelle’s Salt**

To 100 mL of distilled water, 40 g of Sodium Potassium tartarate was added and mixed well.

4) **Fructose Stock Solution**

1% Fructose - 1g Fructose in 100mL of distilled water

Working Standard: 1mg/L

3.10.3 **Procedure**

The fermented broth was centrifuged (2500 rpm, 10 minutes, 4°C) in a refrigerated centrifuge and supernatant was used as the crude enzyme. To 0.1 mL enzyme (supernatant culture, diluted, if necessary), 0.9 mL of inulin acetate buffer was added and incubated at 50°C for 15 minutes (Kalil et al. 2001). After incubation, the tubes were kept in a water bath (90°C) for 10 minutes to inactivate the enzyme and then cooled to room temperature. The reaction mixture was assayed for the reducing sugar by the DNS method (Miller 1959) by reading the absorbance at 540 nm on a spectrophotometer. A calibration curve between concentration and optical density was plotted with known strength fructose solution. Blanks were run simultaneously with the enzyme and substrate solutions.

3.10.4 **Inulinase activity and Specific Enzyme Activity Definition**

*One unit of inulinase activity (IU/mL) was defined as the amount of enzyme that produced 1µmol of fructose per minute under the aforementioned assay conditions* (Kalil et al. 2001). Specific enzyme activity gives an idea about the purity of the enzyme, it is the amount of enzyme present in the total protein present in the sample, expressed in IU/mg.
3.11 SUBSTRATE DEPLETION

Sucrose is first hydrolyzed in an acid solution at 90°C for 5 minutes to yield glucose and fructose (Cazetta et al. 2010). The reducing sugar concentration in the sample is determined by DNS method. The sucrose concentration can then be calculated from a calibration curve based on the difference in the absorbance between the acid treated sample and the untreated sample.

3.11.1 List of Reagents

1) Dinitrosalicylic Acid Reagent

2) Potassium Sodium Tartrate, 40%

3) Concentrated HCl solution

4) KOH, 5N solution

3.11.2 Procedure

1. To 1mL of enzyme solution, added 20µl of concentrated HCl and hydrolysis was allowed to take place in a hot water bath at 90°C for 5 mins.

2. Following this, 50µl of 5N KOH was added to neutralize the acid, as the DNS method works only in an alkaline condition and develops the red brown color which represents the presence of reducing sugars.

3. The DNS reagent added and thereafter DNS method followed.
3.12 ESTIMATION OF PROTEINS BY BRADFORD METHOD

3.12.1 Principle

Total protein was determined quantitatively by Bradford method with Bovine serum albumin standard (Bradford 1976), which uses the concept - protein’s capacity to bind to the dye. The assay is based on the ability of the proteins to bind to Coomassie Brilliant Blue G-250 and form a complex whose extinction coefficient is much greater than that of the free dye.

3.12.2 List of reagents

1) Bradford Reagent

Dissolve 100 mg of Coomassie Brilliant Blue G250 in 50 mL of 95% ethanol. Add 100 mL of 85% phosphoric acid and make up to 600 mL with Distilled water. Filter the solution and add 100 mL of glycerol, then make up to 1000 mL. The solution can be used 24 hr later.

2) Protein Solution

Stock Standard: BSA Standard – 1 mg/L

Working solution: 0.1 mg/L of BSA

3.12.3 Procedure

1) Prepare various concentration of standard protein solutions from the working stock solution (say 0.2, 0.4, 0.6, 0.8 and 1.0 mL) into series of test tubes and make up the volume to 1mL.

2) A tube with 1 mL of water serves as blank.

3) Add 5.0 mL of Coomassie Brilliant Blue to each tube and mix by vortex or inversion.
4) Incubate for 30 minutes and read each of the standards and each of the samples at 595 nm.

5) Plot the absorbance of the standards verses their concentration.

6) Plot graph of optical density versus concentration. Using slope value, calculate the concentration of protein in samples.

3.13 DENATURING PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as per the Laemmli method (Laemmli 1970, Kushi et al. 2000) with standard protein molecular marker (Merck GeNei marker comprises of Phosphorylase b 97.4 kDa, bovine serum albumin 66 kDa, Ovalbumin 43 kDa, Carbonic anhydrase 29 kDa, Lysozyme 14.3 kDa). SDS-PAGE was performed for the Crude inulinase sample, which showed a band at 57 kDa, corroborating with Kushi et al. (2000). The other band noticed at 98 kDa could be due to other proteins. The molecular weight of inulinase varied with the strain and the assay method further purification was required for determining exact molecular weight (Kushi et al. 2000).

3.14 BIOMASS DETERMINATION

Biomass dry cell weight (DCW) was estimated by taking 2 mL of culture in a pre-weighed 2 mL tubes, centrifuged at 2500 rpm for 10 minutes, pellets were washed by re-suspending in sterile saline and washed twice by centrifuging it. Pellets were oven dried at 70 °C for 12 hr and weighed for dry cell weight estimation.

3.15 CHN ELEMENTAL ANALYSIS FOR K. MARXIANUS

C, H, N analysis of the biomass was carried out using Vario MICRO V2.0.3 elemental analysis system. CHN Calculations were made as
follows, initially nitrogen (N), carbon (C), hydrogen (H), oxygen (O) mole fractions (f) were calculated 3.3. In Table 3.3 the CHN data mole % per C mole are given.

Biomass CHN mole fraction (per carbon mole) calculated were $X_C$ (2.7704), $X_H$ (5.2412), $X_N$ (0.3309). The ash content was found to be 8.1%. Water content calculation given in Table 3.4.

From these values oxygen mole fraction calculated (Equation 3.11)

$$F_o = 1 - (f_C + f_H + f_N + f_{water} + f_{ash})$$

where $F_o$ calculated as 0.4874 (48.74 %) and $X_o$ is 1.52.

**Table 3.3 Biomass CHN value per Carbon Mole**

<table>
<thead>
<tr>
<th></th>
<th>N %</th>
<th>C %</th>
<th>H %</th>
<th>S %</th>
<th>C/N ratio</th>
<th>C/H ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4.6328</td>
<td>33.2453</td>
<td>5.2412</td>
<td>0.9067</td>
<td>7.1760</td>
<td>6.3430</td>
</tr>
</tbody>
</table>

**Table 3.4 Water content calculation in biomass CHN analysis**

<table>
<thead>
<tr>
<th>Tube wt</th>
<th>Wet wt (W)</th>
<th>Tube wt + dcw g/2 mL (T)</th>
<th>DCW g/2 mL</th>
<th>Water content(W-T)</th>
<th>Water mole fraction f_{water}</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.2074</td>
<td>1.2529</td>
<td>1.2214</td>
<td>0.014</td>
<td>0.0315</td>
<td>0.000315</td>
</tr>
</tbody>
</table>

**Table 3.5 C Mole conversion**

<table>
<thead>
<tr>
<th>Xc</th>
<th>Xh</th>
<th>Xn</th>
<th>Xo</th>
<th>Mol wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.7704</td>
<td>5.2412</td>
<td>0.3309</td>
<td>1.523328</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.89185677</td>
<td>0.11944124</td>
<td>0.5498585</td>
<td>3.56</td>
</tr>
<tr>
<td>14</td>
<td>1.89185677</td>
<td>1.6721773</td>
<td>8.79773607</td>
<td>26.36</td>
</tr>
</tbody>
</table>
Thus, the CHNO per C mole for *Kluyveromyces marxianus* (Table 3.5) was obtained as $C_{1.9}H_{1.9}N_{0.12}O_{0.55}$

3.16 BIOREACTION REACTION CALORIMETER (BioRc1e) EXPERIMENTS

3.16.1 Set-up of BioRc1e

The enthalpy change for microbial growth is measured using a BioRc1e (Mettler-Toledo AG, Switzerland) (Figure 3.3). It is a double-jacketed 2.2 litre glass reactor with an operating pressure of 29 psi. The reactor is equipped with real-time calibrator (RT-cal) which helps in real time monitoring of the process. The instrument has inbuilt online dissolved oxygen (DO), pH and turbidity probes. Pure oxygen (99.9% Iolar grade) is purged into the medium by a metal L-shaped sparger (procured from Bioengineering Switzerland); the flow is controlled by a rota-meter (0.66 VVM) and filtered through a 0.2 µm membrane filter (Sigma Aldrich, Bangalore). The glass top plate of the calorimeter was replaced with a metallic double jacketed plate, as per the details are given in 3.10.2. The reactor was operated in an isothermal condition at 37 °C throughout the experiment. The temperature of the reactor ($T_r$) was held constant by controlling the jacket temperature ($T_j$) by circulating silicone oil through the reactor jacket. The constant blending of hot and cold silicone oil was achieved through electronically controlled metering valves. Aeration and agitation dissipate or take up heat resulting in a decrease or increase in $T_j$, which leads to a temperature gradient across the reactor wall is directly proportional to the thermal flux of the process ($q_r$) (Equation 3.12)

$$q_r = UA (T_r - T_j) \quad (3.12)$$

Here $UA$ is the overall heat transfer coefficient, $T_r$ and $T_j$ are the reactor and jacket temperatures (°C). The metabolic heat flow ($q_{RTC}$) was calculated online by inbuilt sensor bands (vertical and horizontal). The heat
flux sensor attached to the outer wall of the reactor measured the specific heat flow through the horizontal sensor band. The vertical sensor band measured the fill level in the reactor (Figure 3.4). With these parameters, the heat flow through the reactor wall could be calculated using the Equation (3.13).

\[ q_{RTC} = A \cdot q_{iso} \]  

(3.13)

Here \( q_{RTC} \) was the heat flow through the wetted part of the reactor vessel (W), \( A \) the heat exchange area, determined by the sensors for the vertical band (m\(^2\)) and \( q_{iso} \) the specific heat flow through the horizontal sensor band (W/m\(^2\)).

An operating volume of 1.5 L of the mineral salt medium was loaded in the reactor. The metabolic heat measured for the bioprocess (\( q \)) is given by the Equation (3.1). The heat liberation was allowed to stabilize and the constant baseline heat (\( q_b \)) was corrected with the Equation 3.3. Baseline heat was the sum of external heat loss (aeration & environment heat loss) and heat input (heat accumulation and stirring power) into the system. The metabolic heat during the bioprocess (\( q \)) was determined by deducting the measured baseline heat from the measured \( q_r \)

\[ q = q_r - q_b \]  

(3.14)

The BioRc1e was monitored and controlled by a data logger system equipped with iControl 4.0 software (Mettler Toledo, AG Switzerland). The working principle of BioRc1e and method for calculating heat flow are well described in the literature (García-Payoa et al. 2002, Turker 2004). Samples were collected in an aseptic condition from the reactor at an even time interval. During the initial phase of the process high foaming was observed, and in order to control the foaming 0.1 mL of antifoaming agent per litre of
medium (Aqueous silicone emulsion – Sigma Aldrich, sterilized independently) was added manually to the medium.

Figure 3.3 Schematic functional diagram of BioRc1e

3.16.2 Upgraded BioRc1e Top Plate for Improved Sensitivity

In order to improve the sensitivity of the heat signal, the original glass top plate of the calorimeter was upgraded with a double jacketed SS 316 plate with additional ports (eight in total) and provision for temperature measurements at the location of coolant entry (\(T_{\text{in}}\)) and coolant out (\(T_{\text{out}}\)). The photograph of the modified BioRc1e with the top plate is given in Figure 3.3. The original glass stirrer was replaced with a SS four-blade pitched rushton turbine stirrer with four baffles along the wall of the reactor. The provision of additional ports helped to monitor and measure parameters such as, pH, turbidity, Tr, DO, and exhaust gas. The temperature of the top plate was held constant by swirling coolant in the hollow region of the top plate to retrain...
heat exchange between broth and top plate. This setup reduced the disruption in the measurement of heat signal from the surroundings. The temperature of the top plate was upheld at +2 °C of the operating temperature (Tr).

![Figure 3.4](image1.png)

**Figure 3.4** Photo of Bioreaction Calorimeter (BioRc1e) (a) stock glass top plate (b) upgraded steel top plate with temperature control

3.16.3 Measurement of Baseline Heat

Estimation of baseline heat flow (accounting all heat gain and loss effects) at defined operating conditions of a bioprocess system has been widely adopted in almost all heat-flux based biocalorimetric measurements.

A basic description of calorimetry is presented here to facilitate the understanding of the measurement principle and modifications made for heat flow measurement. Heat flows are defined here as positive if heat is released
inside the bioreactor. For an aerobic process, the heat balance around the reactor can be written as:

\[ q_{ac} = q_r - q_j + q_s - q_g - q_e - q_f - q_a - q_{CO_2} \]  \hspace{1cm} (3.15)

In the above Equation (3.4), \( q_{ac} \) is the heat accumulation in the bulk, \( q_r \) the heat flow of the running process, \( q_j \) the heat flow through the reactor wall to the jacket oil, \( q_s \) the stirring power, \( q_g \) the heat flow induced by aeration, \( q_e \) the heat flow to the environment through the non-jacketed part of the reactor, \( q_f \) the heat flow of the feed, \( q_a \) the heat flow of the acid or base addition and \( q_{CO_2} \) the heat flow of the CO\(_2\) vaporization.

For bioprocesses with a good bioreactor temperature controller, the heat accumulation term can usually be neglected. The jacket heat flow is the most important to monitor since it contains all other heat flows, especially the heat flow generated by the (bio) processes. In the isothermal mode, the temperature of the reactor will be maintained constant by the temperature controller. In the case of BioRc\(_{1\text{e}}\) it can be written:

\[ q_j = UA(T_r - T_j) = UA \Delta T \]  \hspace{1cm} (3.16)

When all other heat flows are constant, the heat transfer coefficient can be determined with a known or measured calibration heat flow (\( q_e \)). In case of the BioRc\(_{1\text{e}}\), calibration was carried out by means of an electrical heater which releases a measured quantity of heat in the reaction (20 W).

The heat flow due to stirring (\( q_s \)) is important compared with the process heat flow and is usually not directly measured. Here, a torque meter was used to measure it and the heat flow can be calculated as:

\[ q_s = 2\pi \frac{R}{60} Mt \]  \hspace{1cm} (3.17)
where, $R$ is rotation per minute and $Mt$ is torque in Nm

The heat flow induced by aeration ($q_g$) is a complex function depending on parameters such as mass flow rate, temperature and relative humidity of inlet and outlet gas described as

$$q_g = f\left(T_{gi}, T_{go}, RH_{i}, RH_{o}, m_{gi}\right)$$  \hspace{1cm} (3.18)

The heat losses to the environment ($q_e$) can be calculated with a single relation, considering one lumped heat transfer coefficient ($K_e$) for the upper part of the bioreactor. It has to be used only for high ambient temperature variations.

$$q_e = K_e(T_r - T_e)$$  \hspace{1cm} (3.19)

Any matter added to the bioreactor will also induce a heat flow ($q_f$). The heat flow for substrate feed is given as

$$q_f = m_f C_{pf} (T_r - T_e)$$  \hspace{1cm} (3.20)

where, $m_f$ and $C_{pf}$ are mass flow rate and specific heat capacity of the feed.

During fermentation, carbon dioxide is produced and the associated heat term is given as follows:

$$q_{CO_2} = n_{CO_2} \Delta H_r$$  \hspace{1cm} (3.21)

The heat flow of an on-going (bio) process, $q_r$, is the result of the energy dissipated by all biochemical reactions. It cannot be directly measured but can be determined from the following relationship.

$$q_r = q_j - q_s - q_e + q_g + q_f + q_a + q_{CO_2}$$  \hspace{1cm} (3.22)
When there is no biochemical activity in the bio reactor, the sum of all the heat flows on the right side of Equation (3.22), should always be zero, provided they are properly measured and calibrated. In practice, the equation can be reduced by lumping all the constant heat flows in a “baseline” term, $q_{bl}$, which has to be determined before and after any experiment. If culture conditions are not varied, $q_s,q_e,q_c$ and $q_f$ are potentially constant. Finally, for monitoring purposes, $q_s$ and $q_{CO_2}$ can be lumped with $q_r$, since they relate to the metabolic activity of cells. They have to be separated only for the thermodynamic evaluation of $q_r$. Equation (3.22) can be reduced to Equation (3.23), which is used with the basic (BioRc1e) setup:

$$q_r = q_j - q_{bl}$$  \hspace{1cm} (3.23)

For BioRc1e experiments, the process heat flow is obtained from Equation (3.12) and the following procedure is applied. The bioreactor is maintained under culture conditions and stabilized to determine the initial base-line ($\Delta T_{bl}$). A UA calibration is made immediately before inoculation.

UA calibration was performed during an experiment at regular time intervals by means of in situ calibration heater (20 W) and accordingly, the measured heat signal ($q_r$) was corrected. When the culture conditions were modified (aeration rate, temperature, stirring speed etc.), the baseline stabilization and UA calibrations were repeated for each set of culture conditions. Heat effects due to inlet aeration, stirring speed and other heat losses were eliminated by pre-thermostatting and insulating the respective streams. The exhaust gas stream was allowed into another membrane filter (0.2 m) to remove the bacterial species trapped in the gas stream and sterile condition was maintained inside the lab.
After the experiment, cells had to be inactivated and the BioRc1e stabilized to determine the final baseline. A second UA calibration was then initiated for the offline evaluation of the process heat flow. At this stage several situations could occur,

1. UA did not vary and the final base-line heat flow \( (q_{bl}) \) was equal to the initial one. In this case, \( q_r \) was not corrected offline.

2. UA varied but the final \( q_{bl} \) was equal to the initial one. In this case, UA was re-calculated linearly with time or volume (if available) to correct \( q_r \) offline.

3. UA did not vary but the final \( q_{bl} \) was different from the initial one. In this case, \( q_{bl} \) was re-calculated linearly with time or ambient temperature (if available) to correct \( q_r \) offline.

4. UA varied and the final \( q_{bl} \) was different from the initial one. In this case, both UA and \( q_{bl} \) were re-calculated as described under points 2 and 3.

However, the heat flow evaluation was carried out using a user-friendly evaluation software (supplied with the equipment) known as iControl 4.0. Heat loss terms and calibration for obtaining heat flux data were done in an automatic mode of the instrument.

### 3.17 HEAT YIELD ESTIMATION

The power-time pattern indicated the growth and metabolism of the bacterial consortium, but the quantitative mass of substrate \( (Y_Q/S) \), oxygen\( (Y_Q/O) \) utilized and biomass \( (Y_Q/X) \) produced were studied by integrating the heat values. The cumulative heat yield per unit of substrate, biomass, and oxygen utilized was calculated (Shanmugam & Mahadevan 2015). The measured heat
yield values can then be used to predict the on-going biological process and justify the process parameters optimized for better results.

Heat dissipation can be indirectly used for prediction of biomass concentration (Von-Stockar & Marison 1989, Sandler & Orbey 1991)

\[
Y_{Q/X}' = \frac{\Delta H^*_S - Y_{PIS}' \Delta H^*_X}{Y_{X/S}'} - \Delta H^*_X
\]

(3.24)

In Equation (3.13), the modified heats of combustion could be estimated.

\[
Y_{Q/X}' = Q_0 \left[ \frac{\gamma_S - Y_{PIS}'}{Y_{X/S}'} - \gamma_X \right]
\]

(3.25)

where \( Q_0 \) is about 115 kJ/C-mol (degree of reduction).

The amount of heat released per C-mol of biomass formed is thus expected to increase for microbial growth on more reduced substrates. Further, more heat will be dissipated per unit biomass in less efficient growth processes, i.e., with decreasing \( Y_{X/S}' \). The appearance of a by-product will probably lower \( Y_{X/S}' \), thereby tending to increase the heat dissipation.

The heat dissipated per mole of oxygen consumed, designated by \( y_{Q/O}' \), can be predicted (Singh 1996, Cooney et al. 1968, Gustaffson et al. 1994).

\[
y_{Q/O}' = \frac{\Delta H^*_S - Y_{X/S}' \Delta H^*_P}{1/4(Y_S' - Y_{X/S}' Y_X' - Y_{PIS}' Y_P')} \]

(3.26)

\[
y_{Q/O}' = 4Q_0 = 460 \text{ kJ/mol}
\]

(3.27)
The metabolic heat release per mole of oxygen utilized is mostly the same for all the aerobic growth processes, irrespective of the substrate, product and microbial strain used and several researchers have proved this statement (Roels 1983, Erickson et al. 1978).

The carbon and other nutrients (substrate) play a crucial role in the heat released by a microbial culture. The amount of heat generated per unit biomass formed ($y'_{Q/X}$) depends on the enthalpy content of the substrate as shown by Equation (3.25)

$$y'_{Q/X} = Q_0 \left[ \frac{\gamma_S - \gamma'_{PS} - \gamma_X}{\gamma'_{X/S}} \right]$$  \hspace{1cm} (3.28)

The heat yield $y_{Q/X}$ ranges from about 10 to 15 kJ/g cell dry weight when biomass is grown on substrates with an average degree of reduction, such as hexose ($\gamma_S = 4$). As predicted by Equation (3.25) much higher values of 16-26 kJ/g for $y_{Q/X}$ are obtained with more reduced substrates such as ethanol, methanol, and hexadecane.

A study of microbial heat generation using a range of substrates with widely different degrees of reduction has been published by Birou et al. (1987). Their experiments were conducted under strongly aerobic conditions in order to suppress the formulation of products as much as possible. The results of these experiments (Table 3.6) reflect the influence of $\gamma_S$ on the heat yield and may be compared with the influence predicted by Equation (3.28).

The heat yield $Y_{Q/X}$ ranges from about 10 to 15 kJ/g cell dry weight when biomass is grown on substrates with an average degree of reduction, such as hexose ($\gamma_S = 4$). As predicted by Equation (3.15) much
higher values of 16-26 kJ/g for $Y_{Q/X}$ are obtained with more reduced substrates such as ethanol, methanol, and hexadecane.

### Table 3.5 Heat yields as a function of reductance degree of substrates

<table>
<thead>
<tr>
<th>Organism</th>
<th>Substrate</th>
<th>$\gamma_X$</th>
<th>$Y'_{X/S}$ (g/g)</th>
<th>$Y_{Q/X}$ (g/g)</th>
<th>$Y_{Q/O}$ (kJ/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. lipolytica</td>
<td>Citrate</td>
<td>3</td>
<td>0.290</td>
<td>14.99</td>
<td>423</td>
</tr>
<tr>
<td>C. lipolytica</td>
<td>Succinate</td>
<td>3.5</td>
<td>0.344</td>
<td>16.76</td>
<td>492</td>
</tr>
<tr>
<td>K. fragilis</td>
<td>Glucose</td>
<td>4</td>
<td>0.520</td>
<td>12.51</td>
<td>447</td>
</tr>
<tr>
<td>K. fragilis</td>
<td>Glucose (1)</td>
<td>4</td>
<td>0.465</td>
<td>12.51</td>
<td>456</td>
</tr>
<tr>
<td>K. fragilis</td>
<td>Glucose (2)</td>
<td>4</td>
<td>0.388</td>
<td>15.16</td>
<td>-</td>
</tr>
<tr>
<td>C. pseudotropicalis</td>
<td>Glucose (3)</td>
<td>4</td>
<td>0.465</td>
<td>13.50</td>
<td>408</td>
</tr>
<tr>
<td>C. utilis</td>
<td>Glucose</td>
<td>4</td>
<td>0.481</td>
<td>11.66</td>
<td>421</td>
</tr>
<tr>
<td>E. coli</td>
<td>Glucose</td>
<td>4</td>
<td>0.416</td>
<td>8.70</td>
<td>-</td>
</tr>
<tr>
<td>Ent. cloacae</td>
<td>Glucose</td>
<td>4</td>
<td>0.345</td>
<td>9.35</td>
<td>-</td>
</tr>
<tr>
<td>K. fragilis</td>
<td>Galactose</td>
<td>4</td>
<td>0.494</td>
<td>13.63</td>
<td>418</td>
</tr>
<tr>
<td>K. fragilis</td>
<td>Lactose</td>
<td></td>
<td>0.485</td>
<td>14.58</td>
<td>495</td>
</tr>
<tr>
<td>C. utilis</td>
<td>Acetate</td>
<td>4</td>
<td>0.406</td>
<td>17.78</td>
<td>385</td>
</tr>
<tr>
<td>C. utilis</td>
<td>Glycerol</td>
<td>4.67</td>
<td>0.562</td>
<td>10.79</td>
<td>474</td>
</tr>
<tr>
<td>C. boidinii</td>
<td>Ethanol</td>
<td>6</td>
<td>0.790</td>
<td>16.7</td>
<td>-</td>
</tr>
<tr>
<td>C. utilis</td>
<td>Ethanol</td>
<td>6</td>
<td>0.557</td>
<td>19.91</td>
<td>421</td>
</tr>
<tr>
<td>M. methylotrophus</td>
<td>Methanol</td>
<td>6</td>
<td>0.406</td>
<td>23.7</td>
<td>437</td>
</tr>
</tbody>
</table>

### 3.17.1 Bioenergetic parameters and Specific growth rate estimation

The energy present in the substrate is dissipated as heat during the formation of 1 C-mol of biomass. A relationship between the biomass in
terms of C-moles and the heat evolution rate \( Y_{Q/X} \), relationship between the 
substrate utilization and the heat evolution rate \( Y_{Q/S} \), amount of heat 
produced due to unit gram oxygen consumed \( Y_{Q/O} \) Yield of biomass defined 
as the maximum increase in cell mass per unit time biomass formed per gram 
of sucrose consumed \( Y_{X/S} \) were determined and tabulated. Several 
physiological attributes of cells vary as a function of the specific growth rate 
since \( \mu \) depends on substrate concentration. From the experimental data, \( dx/dT \) 
is calculated and then a maximum of \( (dx/dT)/X \) is determined numerically as \( \mu_{\text{max}} \).

3.18 ANALYSIS OF METABOLIC PATHWAYS AND 
ENERGETICS

3.18.1 Metabolic Pathways

In the experiments carried out, the substrate used is sucrose, which 
is broken down into Glucose and Fructose by enzymatic action. Sucrose 
cannot be metabolised by the Kluyveromyces marxianus directly as it is a non-
reducing sugar and hence it is broken into reducing sugars.

\[
\text{C}_{12}\text{H}_{22}\text{O}_{11} \rightarrow \text{C}_{6}\text{H}_{12}\text{O}_{6} \text{ (G)} + \text{C}_{6}\text{H}_{12}\text{O}_{6} \text{ (F)} \quad (3.29)
\]

The central and oxidative response metabolism pathway for 
Kluyveromyces marxianus (Gao et al. 2015) is shown in Figure 3.5. Due to 
the action of Kluyveromyces marxianus on glucose, after undergoing 
glycolysis, two molecules of pyruvate are formed under aerobic conditions. 
This is then converted to acetyl CoA which in turn enters the citric acid cycle 
(TCA cycle). For 1 mole of glucose the energy released due to the above 
process is around 30-32 ATP. The energy released due to metabolism of 1 
mol of fructose is 30-32 ATP as well, as fructose is also metabolized in the 
same manner as that of glucose. Hence, the net amount of ATP released if one 
mole of sucrose is aerobically oxidised via glycolysis, pyruvate
dehydrogenase reaction, citric acid cycle and oxidative phosphorylation is 60-64 ATP.

Table 3.6 The standard free energies of the hydrolysis of ATP, ADP and AMP (Cox & Nelson 2000)

<table>
<thead>
<tr>
<th>Reaction</th>
<th>$\Delta G^0$ (kJ/mol)</th>
<th>$\Delta G^0$ (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP $\rightarrow$ ADP + P&lt;sub&gt;i&lt;/sub&gt;</td>
<td>-30.5</td>
<td>-7.3</td>
</tr>
<tr>
<td>ATP $\rightarrow$ AMP + PP&lt;sub&gt;i&lt;/sub&gt;</td>
<td>-45.6</td>
<td>-10.9</td>
</tr>
<tr>
<td>ADP $\rightarrow$ AMP + P&lt;sub&gt;i&lt;/sub&gt;</td>
<td>-32.8</td>
<td>-7.8</td>
</tr>
</tbody>
</table>

Table 3.7 Stoichiometry of coenzyme reduction and ATP formation

The aerobic oxidation of glucose via glycolysis, pyruvate dehydrogenase reaction, the TCA cycle and oxidative phosphorylation (Cox & Nelson 2000).

<table>
<thead>
<tr>
<th>Reactions</th>
<th>No of ATP/ reduced coenzymes directly formed</th>
<th>Number of ATP ultimately formed*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose $\rightarrow$ Glucose-6-Phosphate</td>
<td>-1 ATP</td>
<td>-1</td>
</tr>
<tr>
<td>Fructose-6-Phosphate $\rightarrow$ Fructose-1,6-Biphosphate</td>
<td>-1 ATP</td>
<td>-1</td>
</tr>
<tr>
<td>2 Glyceraldehyde-3-Phosphate $\rightarrow$ 2 1,3-Bisphosphoglycerate</td>
<td>2 NADH</td>
<td>3-5</td>
</tr>
<tr>
<td>2 1,3-Bisphosphoglycerate $\rightarrow$ 2 3-Phosphoglycerate</td>
<td>2 ATP</td>
<td>2</td>
</tr>
<tr>
<td>2 Phosphoenol Pyruvate $\rightarrow$ 2 Pyruvate</td>
<td>2 ATP</td>
<td>2</td>
</tr>
<tr>
<td>2 Pyruvate $\rightarrow$ 2 Acetyl-CoA</td>
<td>2 NADH</td>
<td>5</td>
</tr>
<tr>
<td>2 Isocitrate $\rightarrow$ 2 $\alpha$-ketoglutarate</td>
<td>2 NADH</td>
<td>5</td>
</tr>
<tr>
<td>2 $\alpha$-ketoglutarate $\rightarrow$ 2 succinyl-coA</td>
<td>2 NADH</td>
<td>5</td>
</tr>
<tr>
<td>2 Succinyl-coA $\rightarrow$ 2 succinate</td>
<td>2 ATP (or 2GTP)</td>
<td>2</td>
</tr>
<tr>
<td>2 Succinate $\rightarrow$ 2 fumarate</td>
<td>2 FADH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>3</td>
</tr>
<tr>
<td>2 Malate $\rightarrow$ 2 oxaloacetate</td>
<td>2 NADH</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>30-32 ATP</td>
</tr>
</tbody>
</table>

*Calculated as 2.5 ATP per NADH and 1.5 ATP per FADH<sub>2</sub>.
Figure 3.5  Profiles of central and oxidative stress response metabolism pathways of *Kluyveromyces marxianus*.

Glycolysis (EMP), Pentose Phosphate Pathway (PPP), Ethanol formation pathway, Glycerol formation pathway, Citric Acid Cycle (TCA Cycle), Oxidative Stress Response pathway Red highlight- Upregulated gene, Green highlight- Downregulated gene, Yellow highlight- Mixture of Upregulated and Downregulated.
3.18.2 Energy Due to Different Pathways

3.18.2.1 Energy from sucrose metabolism due to glycolytic degradation and TCA cycle

When glucose is completely oxidized to carbon-dioxide and water, the total standard free-energy change is -2840 kJ/mol. We observe that in the glycolytic degradation of glucose to two molecules of pyruvate, the value of change in free energy is -146 kJ/mol, which is only 5.2% of the total energy that can be released from glucose (Cox & Nelson 2000).

$$\frac{146}{2840} \times 100 = 5.2\%$$

This is because pyruvate contains most of the chemical potential energy of the glucose molecule, which is extracted by oxidative reactions in the Citric Acid cycle and oxidative phosphorylation.

From the Experiments, the concentration of sucrose in the substrate media used is 10 g/L. The working volume of the reactor is V equal to 1.5 L.

$$Number\ of\ moles\ of\ sucrose\ per\ litre = n = \frac{10g}{342\ g} mol = 0.029\ mol$$

For 1 mole sucrose, total ATP in the glycolytic degradation and TCA cycle = 64 ATP

The standard free Energy change due to ATP hydrolysis = $$\Delta G_{ATP}^o = -30.5\ kJ/mol$$

Standard Free energy change due to ATP hydrolysis = $$n \times \Delta G_{ATP}^o \times Number\ of\ ATP^* V$$

= $$0.029 \times (-30.5) \times 64 \times 1.5 = -84.912\ kJ/mol$$

3.18.2.2 Pentose phosphate pathway (PPP)

Glucose-6-Phosphate +2 NADP$^+$ +H$_2$O $\rightarrow$ ribose-5-phosphate + CO$_2$ + 2NADPH +2H$^+$

(3.30)
The Pentose Phosphate pathway ends with the production of NADPH, a reductant for biosynthetic reactions and ribose 5-phosphate, a precursor for nucleotide synthesis (Cox & Nelson 2000).

Calculating as 2.5 ATP per NADH, we get total ATP count as 5 ATP for the PPP.

### 3.18.2.3 Ethanol formation pathway

\[
\text{Glucose} + 2\text{ADP} + 2\text{P}_1 \rightarrow 2\text{Ethanol} + 2\text{CO}_2 + 2\text{ATP} + 2\text{H}_2\text{O} \quad (3.31)
\]

If we have to consider ATP count only from Pyruvate to ethanol formation, we will have to discount the ATP synthesized in the glycolytic degradation. However from the breakdown of glucose, ethanol is formed along with the release of two carbon dioxide molecules and 2 molecules of ATP (Cox & Nelson 2000).

### 3.18.2.4 Glycerol formation pathway

The glycerol formation pathway is driven by a gene which has been downregulated. Hence we can consider no energy to have been released due to this particular pathway in our degradation of sucrose using *Kluyveromyces marxianus*.

### 3.19 EXHAUST GAS ANALYSIS; OXYGEN UPTAKE RATE (OUR) AND CARBON DIOXIDE EMISSION RATE (CER)

The exhaust gas evolved during the bioreaction was analyzed using a gas analyzer (Siemens – ultramat 23, Mumbai) that reads the percentage of \(\text{CO}_2\) and \(\text{O}_2\) in the exhaust gas. The analyzer is equipped with internal auto calibration setup. The exhaust outlet from the BioRc1e reactors top plate is connected to a condenser to prevent moisture entering the analyzer. All the
connective pipes were insulated to prevent water condensing in the pipelines. The reactor was equipped with dissolved oxygen probe (Mettler-Toledo model M 700, Switzerland). The dissolved oxygen level in the reactor was monitored to prevent anaerobic conditions. The OUR and CER were calculated by mass balancing method (Doran 1995).

OUR and CER are calculated by mass balancing method (Doran 1995) by the equation 3.32.

$$N_A = \frac{1}{RV_L} \left[ \left( \frac{FgP_{AG}}{T} \right)_i - \left( \frac{FgP_{AG}}{T} \right)_o \right]$$  \hspace{1cm} (3.32)

where, \( N_A \) - oxygen uptake rate (mg.L/s); \( R \) - universal gas constant (L atm/gmol k); \( V_L \) - the volume of liquid in the fermenter (L); \( Fg \) - the volumetric gas flow rate (L/s); \( P_{AG} \) - the oxygen partial pressure in the gas (atm); \( T \) - absolute temperature (k); \( i \) - inlet, \( o \) - outlet.