CHAPTER 8

SUMMARY AND MAJOR FINDINGS

This chapter summarizes the major conclusions drawn from the biocalorimetric batch, linear and exponential fed-batch studies for the production of inulinase from *K. marxianus*. The study involves

- A statistical design approach for media optimization
- Effect of aeration and agitation on inulinase production
- Effect of feeding strategies on inulinase production
- Modeling of exo-inulinase biosynthesis.

8.1 A STATISTICAL DESIGN APPROACH TO MEDIA OPTIMIZATION FOR INULINASE PRODUCTION FROM *Kluyveromyces marxianus*

- A successful and significant enhancement in the production of inulinase by *K. marxianus* was accomplished using a simple medium containing inorganic nitrogen (Ammonium sulphate) and sucrose as carbon source.

- *K. marxianus* strain morphology, growth kinetics were studied for understanding growth behavior.
• Four different medium compositions were screened for maximum inulinase activity. Significant differences among the four different media of comparison were established by Tukey’s Test Statistical Analysis.

• Medium D favored biochemical metabolism resulting in greater inulinase production and so was chosen for further optimization.

• The critical process parameters such as carbon source, nitrogen source, pH, inoculum size of the strain were critically examined using the five-level CCD. Regression equations were developed for enzyme production using experimental data and solved.

• Further two level CCD was employed for optimization of important media components such as sucrose and ammonium sulphate. The regression equation was developed for inulinase production using trial run results and the optimum trial was chosen.

• It was observed that model predicted was in good agreement with experimental observations ($R^2$ is 0.969) and inulinase production was significantly influenced by the listed bioprocess parameters.

• The optimum levels of each variable were: pH (5), inoculum size (2 %), potassium di hydrogen phosphate (3 g/L), sucrose (10 g/L) and ammonium sulphate (7.5 g/L).

• SDS PAGE confirmed the presence of inulinase at 57 kDa.
8.2 EFFECT OF AERATION AND AGITATION ON BATCH INULINASE PRODUCTION BY K. marxianus AND ITS METABOLIC HEAT FLUXES STUDIED IN A BioRC1e.

- Heat based monitoring of inulinase production with various parameters such as aeration rate and agitation rate showed inulinase was strongly influenced by shear stress and led to poor dissolved oxygen levels.

- A similar trend could be noticed between the heat, OUR and CER implying that heat could be an alternative parameter for monitoring the bioprocess.

- Optimum aeration and agitation rates for inulinase production were found to be 1 LPM and 150 RPM respectively.

- Maximum enzyme activity of 20.98 IU/mL was achieved under this condition.

- The undergoing metabolic process and effective substrate utilization were effectively visualized by real-time heat evolved. Heat yield on substrate uptake, oxygen consumption, and biomass production gave real-time insight to ongoing bioprocess at different aeration and agitation rates.

- The biothermokinetic approach has been successfully adopted for determination of enthalpy efficiency of respiration linked energy transformation and bioenergetics of growing and resting yeast.
Biothermodynamics data were not only a useful parameter for identifying the best condition for industrial level production of yeast inulinase but also a check on the consistency of the ongoing bioprocess.

Further, a heat based model of optimization and the bioprocess monitoring control can be adopted from this study in scale up of the bioprocess.

Heat based fed batch design has been adopted for the enhanced inulinase production.

8.3 EFFECT OF FEEDING STRATEGIES ON INULINASE PRODUCTION ANALYZED IN A BIOCALORIMETER

Batch and fed-batch synthesis of inulinase by *K. marxianus* were monitored in a bio reaction calorimeter for the first time.

Feed and feeding rates have been optimized for *K. marxianus* inulina production. Sucrose feeding was found enhancing the inulinase yield.

The strategic feeding of the substrate (3 ghr⁻¹ at a dilution rate of 0.002 hr⁻¹) in fed-batch helped not only to improve the inulinase activity by extending its stationary growth phase but also maintain a growth rate of 0.2 ghr⁻¹ and specific growth rate above 0.002 hr⁻¹ after 28th hr.

We hypothesize that maintenance of specific growth rate is important for obtaining maximum enzyme production.
• The maximum enzyme activity for the batch process was 20.98 IU/mL while for linear fed-batch it was 355 IU/mL i.e., 17 times more than the batch.

• Further exponential feeding appears to be the best option to maximize the enzyme production with reduced bio reaction duration. In exponential feeding strategy, maximum enzyme activity 728 IU/mL was observed at 20\textsuperscript{th} hr i.e, a reduction of 12 hours reaction time with two fold enzyme activity could be achieved.

• Metabolic and physiological correlation of \textit{K.marxianus} growth and its inulinase production with OUR, CER, and Q were studied.

• Depletion of sucrose, or the catabolite-repressor effect of the glucose so produced, may turn-off the inulinase induction, further carbon source starvation turns on the inulinase induction in \textit{K.marxianus}.

• A Michaelis-Menten type empirical model was developed for fed-batch that can be adopted for controlling specific growth rate based on metabolic heat responses, towards achieving maximum enzyme production and release.

• An empirical model predicting the exo inulinase yield and biomass concentration could be successfully extended to the fed-batch inulinase production of BioRc1e.

• The usefulness of biocalorimeter in fingerprinting the metabolite production (more valuable than OUR and CER
measurements) is demonstrated in this study. Heat flux measurement can be profitably used at industrial levels too for in-situ monitoring.

- The model can be used for heat based metabolite and growth prediction and further has the potential to be applied as bioprocess control system.

8.4 MODELING OF EXO-INULINASE BIOSYNTHESIS BY Kluyveromyces marxianus IN FED-BATCH MODE: CORRELATING PRODUCTION KINETICS AND METABOLIC HEAT FLUXES

- A complete profile of the product synthesized by K. marxianus has been obtained using GC-MS studies, the study confirmed the existence of the overflow metabolism of K.marxianus during inulinase production.

- The relationship between specific production rate of inulinase by K. marxianus and its specific growth rate was simulated well by the Luedeking-Piret equation.

- A robust mathematical model (a modified form of the Luedeking-Piret equation) which has parameters of biological significance and utilizes biocalorimetric (BioRc1e) data as inputs has been developed to describe the inulinase production kinetics by K. marxianus.

- MATLAB software (The MathWorks, Inc, Massachusetts, USA) was used for simulation of the inulinase production. A
Matlab code was developed for the proposed model, which took the metabolic heat as an input.

- Validation experiment proved that the model predictions are well within the permissible limits.

- Merits of the model lie in the fact that the enzyme production kinetics were monitored solely through the real-time metabolic heat evolved.

- Biocalorimetry has a big potential in monitoring complex fermentations where existing techniques of online monitoring are at a disadvantage. The proposed model can help control the fed-batch culture to optimize the biosynthesis of inulinase.

- Heat flux profile correlates better with enzyme release profile in comparison to the OUR and CER profiles.

- A similar metabolic heat based approach can be adopted in the production of various metabolites for real-time quantification purposes.

- The work is significant in its attempt at quantifying a metabolite (inulinase enzyme) by linking it with metabolic heat flows.

- *K. marxianus* can be used as a model host organism for metabolite prediction with heat based model prediction as a real-time bioprocess monitoring and control tool. It has been found to produce more inulinase than other yeasts, fungal and bacterial strains and also has a high potential for producing commercially acceptable yields.
8.5 SUGGESTIONS FOR FUTURE WORK

- Heat flux measurement can be profitably used at industrial levels for in-situ monitoring.

- *K. marxianus* can be used as a model host organism for metabolite prediction with heat based model prediction as a real-time bioprocess monitoring and control tool.

- Controllers can be programmed using the proposed model which can help in regulating a bioprocess. The utility of the model lies in error diagnosis of a bioprocess. One would be able to ascertain if there are any faults or anomalies in the bio-reactions with the help of the proposed model, which can enable to take corrective action using a process controller.

- The model developed was focused on the exo-inulinase that has been secreted out into the culture fluid, and metabolic heat as measured by BioRc1e has been used as the nodal parameter around which the production estimation has been carried out. However, there is a good amount of intracellular and cell bound inulinase to facilitate the substrate consumption.

- Metabolic heat is a comprehensive factor considering all heat released from the metabolic pathways of the *K. marxianus* can serve as a viable, albeit a more complex, alternative for predicting the intracellular enzyme production patterns, if the central metabolic pathways and the energetics associated, in terms of ATP, NADH or FADH$_2$ that are formed, are known.
• Heat balance in line with central metabolic pathway energy balance can be the best method for bioprocess monitoring and control of any system.

• Quantifying the metabolite with metabolic heat flow can improve the overall efficiency of bioprocess thereby contributing to QbD (quality by design) and use of biocalorimetry as an efficient PAT tool (Process analytical tool).
APPENDIX I

MATLAB CODE USED FOR SIMULATING MODEL

function f
    global Y xo Vo Qo t Q a b i
    xo= m;
    Vo= n;
    Qo= x;
    a=y;
    b=z;
    for i=1:j
        Q=[c d ..............];
        q=[e f ..............];
        Y=[g h ..............];
        f(i)=[a*q(i)/[xo*Vo*Y(i)+(Q(i)-Qo)]]+b;
    end
    disp(f)
    t = linspace(0,p,j);
    f(1,:)
    plot(t,f(1,:),'-or')
    grid on
    title('Inulinase Production kinetics')
    xlabel('Time(hr)');
    ylabel('Specific Inulinase production rate(hr⁻¹)');
    legend('Model')