Cape Peninsula University of Technology

APPLICATION OF BIOPROCESS-SUPERCRITICAL FLUID EXTRACTION TECHNIQUES IN THE PRODUCTION AND RECOVERY OF SOME SELECTED BIOPRODUCTS

by

ABIOLA EZEKIEL TAIWO

Thesis submitted in fulfilment of the requirements for the degree

Doctor of Engineering: Chemical Engineering

In the Faculty of Engineering and the Built Environment,

at the Cape Peninsula University of Technology

Supervisor: Prof T.V Ojumu

Bellville

March 2020

CPUT copyright information

Abstract

The use of bioproducts in different commercial sectors such as medicine, agriculture, cosmetics, food, and chemical industries motivates the need for easy production and recovery techniques of bioproducts at laboratory and pilot scale. This study aims at the production and recovery of some selected bioproducts using supercritical fluid extraction techniques. Three products are chosen as case studies: these are ethanol, acetoin, and vanillin, since the ease of separation is influenced by the concentration of the product in the broth, these compounds were selected based on their concentration in the fermentation broth, according to literature sources. A standard method was developed in a spectrophotometer for quantifying the targeted product in the broth, while the product recovery studies was carried out using a supercritical fluid extraction pilot plant. *Saccharomyces* and *Bacillus* species were chosen for the bioproduction of the selected bioproducts. Experimental design and statistical analysis of results were carried out using response surface methodology (RSM) and artificial neural network (ANN). Studies on each of the selected bioproducts are as justified in the paragraphs below.

Bioethanol production has recently become an increasing trend in research, with a focus on increasing its economic viability. Hence, the need to develop a low-cost fermentation medium with minimum redundant nutritional supplements, thereby minimizing the costs associated with nutritional supplements whereby inoculum preparation becomes necessary for ethanol production. Corn steep liquor (CSL) in glucose fermentation by *Saccharomyces* Type 1 (ST1) strain and Anchor Instant Yeast (AIY), which are low-cost media, are used as replacements for yeast extract (YE). The fermentation process parameters were optimized using artificial neural networks (ANN) and the response surface methodology (RSM). The study shows that for CSL, a maximum average ethanol concentration of 41.92 and 45.16 g/L representing 82% and 88% of the theoretical yield were obtained after 36 h of fermentation in a shake flask for ST1 and AIY respectively. For YE, ethanol concentration equivalent to 86% and 88% of theoretical yield were obtained with ST1 and AIY respectively after 48 h. Although, ANN predicted the responses of ethanol yield better than RSM, optimum conditions for ethanol production were better predicted by RSM.

The consumers' preference for 'naturally' produced aromas drives the development of bioproduction of acetoin from glucose with a view to optimize its production. The results revealed that by using a cheap nitrogen source, corn steep liquor, the yield of acetoin was similar to those of yeast and beef extracts. Furthermore, it was shown that by using Box-Behnken design, the optimum parameters such as glucose concentration, corn steep liquor, and inoculum size to maximize the concentration of acetoin produced were 78.40 g/L, 15.00%

w/v and 2.70% v/v respectively. The validated concentration of acetoin produced in a triplicate analysis, 10.7 g/L, was 0.06% less than the predicted value.

Increasing awareness of consumers of healthy, eco-friendly flavors and fragrances motivates the bioproduction of vanillin. The interactive effects of three variables on vanillin yield were evaluated by response surface methodology (RSM) with Box-Behnken design (BBD) model. The results showed the optimum conditions for the biotransformation of ferulic acid into vanillin can be achieved with maximum overall desirability (D) of 1.0 and a significant (p<0.05) quadratic model with regression coefficient (R^2) of 0.995. Corn steep liquor, initial ferulic acid concentration and pH significantly influence the concentration of vanillin in the broth. The results in triplicate experiments confirmed vanillin yield of 386 mg/L after validation, which was in agreement with the prediction of the model. The maximum vanillin yield of 384.40 mg/L was predicted when corn steep liquor, ferulic acid concentration and pH were 7.72 g/L, 2.33 g/L, and 9.34 respectively.

Fermentation system in a bioreactor has been proven to be an efficient system for the study of controlled fermentation variables when compared to a shake flask study. The influence of agitation, aeration, time and pH were analysed by Taguchi orthogonal array design for the upscale of acetoin in a bioreactor. The optimized parameters in 1.3L of fermentation vessel were as follows: 300 rpm agitation, 1.5 slpm aeration; 2 days' fermentation time and 6.5 pH value. Agitation with above 70% was the most contributing factor and other variables were less than 30% in the percentage analysis of variance of each fermentation variables in the batch study of acetoin. A fourfold gain in acetoin titre (42.30 g/L) was obtained with the same substrate concentration in a lab-scale bioreactor on scaling up when compared with the shake flask batch study. The validated acetoin concentration of 41.72 g/L was obtained after a triplicate experiment to confirm the possibility of reproducing acetoin using the optimized conditions.

Many separation techniques have been proven to recover value-added products from fermentation broth with a preference for several methods above other and new techniques that are emerging. Supercritical fluids separation using CO_2 is one such technique. The feasibility of acetoin concentration and recovery was studied in supercritical CO_2 pilot plant with pressure ranges of 100 to 300 bar, CO_2 feed rate of 5 to 15 kg/h, at a process temperature of 37 and 80 °C in simulated and fermentation broth, respectively. The validated conditions for the fractionation of acetoin by supercritical fluid extraction (SFE) were determined as follows: extraction pressure, 300 bar; CO_2 feed rate, 15 kg/h; extraction temperature 37 °C; and fractionation time of 30 minutes. At these operating conditions, the percentage recovery of acetoin with respect to the feed solution at the raffinate for the simulated and actual

fermentation broth was 77.8% (0.20 g/L) and 77% (0.15 g/L) respectively. A two-fold extract increase was obtained after 30 minutes of fractionation.

The study provides the technical feasibility and the base case data which are critical to the development and design of processes for production and recovery of acetoin. The lesson gleaned from this study may be extended to develop processes for the production and recovery of other bioproducts (ethanol and vanillin).

Keywords: acetoin; bioconversion; Box-Behnken design; corn steep liquor; ethanol; fractionation; fermentation; ferulic acid; recovery; statistical optimization; supercritical CO_2 extraction; Taguchi design; vanillin

Declaration

I, **Abiola Ezekiel Taiwo**, declare that the contents of this thesis represent my own unaided work and that the thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

Abiola Ezekiel Taiwo   _March 5, 2020_

Signed   **Date**

Dedication

Now unto the King eternal, immortal, invisible, the only wise God, be honor and glory forever and ever. Amen (1 Timothy 1:17)

Acknowledgments

I wish to thank the following people and organizations for their immense contribution towards the completion of this thesis:

List of publications, presentations, and training

Publications

- Taiwo, A.E., Ojumu, T.V. & Madzimbamuto, T.N. 2018. Optimization of corn steep liquor dosage and other fermentation parameters for ethanol production by saccharomyces cerevisiae type 1 and anchor instant yeast. *Energies,* 11(7)**:**1740.

- Taiwo, A.E., Ojumu, T.V. & Madzimbamuto, T.N. 2019. Statistical optimization of acetoin production using corn steep liquor as a low-cost nitrogen source by *Bacillus subtilis* CICC 10025. *In:* Jacob-Lopes, E. & Queiroz Zepka, L. (eds.) *Renewable Resources and Biorefineries. IntechOpen.*

- Taiwo, A.E., Madzimbamuto, T.N. & Ojumu, T.V. Development of an integrated process for production and recovery of some selected bioproducts from lignocellulosic materials. In: Daramola, MO. & Ayeni, OA. (eds.) *Valorization of Biomass to Value-Added Commodities. Springer Nature. In press.*

- Taiwo, A.E., Madzimbamuto, T.N. & Ojumu, T.V. Biotransformation of ferulic acid into vanillin using *Bacillus amyloliquefaciens subsp* in a low-cost media: Modeling and optimization. *Agriculture and Food Chemistry Journal.* Drafted.

- Taiwo, A.E., Madzimbamuto, T.N. & Ojumu, T.V. Optimization of Fermentation Process Variables by Taguchi Orthogonal Array Design for Improved Acetoin Production in Bioreactor. *Heliyon.* Drafted.

- Taiwo, A.E., Madzimbamuto, T.N. & Ojumu, T.V. Recovery of acetoin from *Bacillus subtilis* fermentation broth using Supercritical CO_2. *Separation and Purification Technology.* Drafted.

Presentations

- Taiwo, A.E., Madzimbamuto, T.N. & Ojumu, T.V. 2019. Artificial Neural Network: A reliable tool for modelling and optimization of the production of some value-added products 4th World Academy of Sciences (TWAS) and Young Affiliates Network (TYAN) International Thematic Workshop and 1st African Symposium on Big Data, Analytics and Machine Intelligence for Financial, Health and Environmental Inclusion in Developing Countries, 10-15 June 2019, Akure, Nigeria (Poster presentation).

- Taiwo, A.E., Madzimbamuto, T.N. & Ojumu, T.V. 2019. Biotransformation of ferulic acid into vanillin using *Bacillus amyloliquefaciens subsp* in a low-cost media: Modelling and optimization. 4th Green and Sustainable Chemistry Conference, 5-8 May 2019 Dresden, Germany (Poster presentation).

- Taiwo, A.E., Madzimbamuto, T.N. & Ojumu, T.V. 2018. Corn Steep liquor a cheap Nitrogen Source for ethanol and acetoin production. 6th U6 International Conference,

Research, Innovation and Technology for African Development, Cape Town, South Africa, 4-6 September 2018. Pg. 60-61 (Oral presentation).

- Abiola Ezekiel Taiwo, Tafirenyika N Madzimbamuto and Tunde Victor Ojumu. Sustainability in Bioprocessing: A Case Study of Bioprocess-Supercritical Fluid Extraction of Vanillin. The First TWAS Young Affiliate Network (TYAN) International Conference in Rio de Janeiro, Brazil, 22-24 August 2017. TWAS-Elsevier Foundation Sustainability Case Studies Competition, Green and Sustainable Chemistry Award (Oral and Poster Presentation).

Trainings

- A full-day workshop and three days of training with practical on GC-MS and LC-MS at Central Analytical Facilities, Stellenbosch University, South Africa. 18 June 2018 (Training).
- Two-day Training course on Process Modelling and optimization with SuperPro Designer and Schedule Pro at North Carolina State University, United States, 10-11 May 2018 (Training).

Table of contents

List of figures

xviii

List of tables

Glossary

Abbreviations	Definition
AAD	Absolute average deviation
AC	Acetoin
ANOM	Analysis of mean
ANN	Artificial neural network
AIY	Anchor Instant Yeast
ANOVA	Analysis of variance
BBD	Box-Behnken design
BM	Biomass
C_E	Concentration of solute at the extract
C_R	Concentration of solute at the raffinate
CSL	Corn steep liquor
CV	Coefficient of variation
df	Degree of freedom
D	Desirability
DCW	Dry Cell Weight
DF	Desirability function
DNS	Dinitro salicylic acid
DOE	Design of experiment
GC-MS	Gas chromatography mass spectrometry
FA	Ferulic acid
K	Distribution coefficient
M	Molar
MSD	Mass spectrometric detector
N	Normal
OA	Orthogonal array
Pc	Critical pressure
RMSE	Root mean square error
rpm	Revolution per minute
RS	Reducing sugar
RSM	Response surface methodology
R^2	Coefficient of determination
S1	Separator 1
S2	Separator 2
S3	Separator 3

ST1	*Saccharomyces* Type 1 yeast
SD	standard deviation
SFE	Supercritical fluid extraction
scCO$_2$	Supercritical carbon dioxide
S/N	Signal to noise ratio
slpm	Standard litre per minute
Tc	Critical temperature
UV	Ultra violet
VIF	Variance inflation factor
Vp	Voges-Proskauer
Symbols	**Definitions**
eV	Electronvolt
g/L	Gram per litre
h	hour
p	Probability
mL	Milli litre
min	Minutes
m/z	Mass per charge
µL	Micro litre
v/v	Volume per volume
wt%	Weight percentage
%w/v	Percentage weight per volume

Chapter 1 INTRODUCTION

1.1 Introduction

The history of bioprocessing can be traced to early times in human development. The increased attention received is due to its enormous potential for the production of high-value products with inherent attributes as sustainable processes (Heinzle et al., 2007). Bioprocesses make use of renewable feedstocks and facilitate varieties of chemical transformations to products which have found wide application in the chemical industry (Clark and Macquarrie, 2008). This biologically transformed product is of great benefit because it enhances the energy sector, offers improved environmental friendliness, affects economic growth in rural areas and advances the global bio-based market economy (Singh et al., 2003). It has also contributed to the more efficient use of natural resources, which is a requirement for creating a more sustainable economy.

Three products (ethanol, acetoin, and vanillin) were chosen as case studies based on their concentration in the fermentation broth, since the ease of separation, and indeed the economics of the process, are influenced by the concentration of the product in the broth. The motivation for these selected bioproducts is further emphasized.

Research into the biotechnological production of biofuel such as ethanol has been on the increase as an alternative to address the environmental issues and shortages resulting from the use of fossil fuel. The depletion of finite fossil fuel reserves and their impact on the environment are the main drivers behind research into biofuels production. Further, synthetic flavors can be found in nearly all processed food and food products on the market, which has necessitated the need to keep the taste of such products attractive to the consumers. As a result, flavorings have become indispensable additives for the food industries (Teixeira et al., 2002). Vanillin and acetoin are such commonly used flavors that have been commercially produced using synthetic chemicals. However, the use of such chemically derived products is restricted in the food and cosmetic industries because of safety concerns. Hence, the need to seek an alternative method that will address these issues.

Microbial fermentation of flavor has gained increasing interest due to its safety, environmental friendliness and abundant natural resources available for its sustainable production. The market size of biovanillin was rated at over \$11.5 million in 2015 and the industry was estimated to make profits increasing annually by a 13% compound growth rate, and with consumption predicted to exceed 500 tons by 2023 (Global Market Insight, 2016), as shown in Figure 1.1. In addition to that, the current prices of acetoin are 149 \$ kg^{-1} of natural and 132

$ kg^{-1} of synthetic acetoin, from Sigma-Aldrich W200808 and W200832-1KG-K respectively, which are around two to three times more than bulk market price.
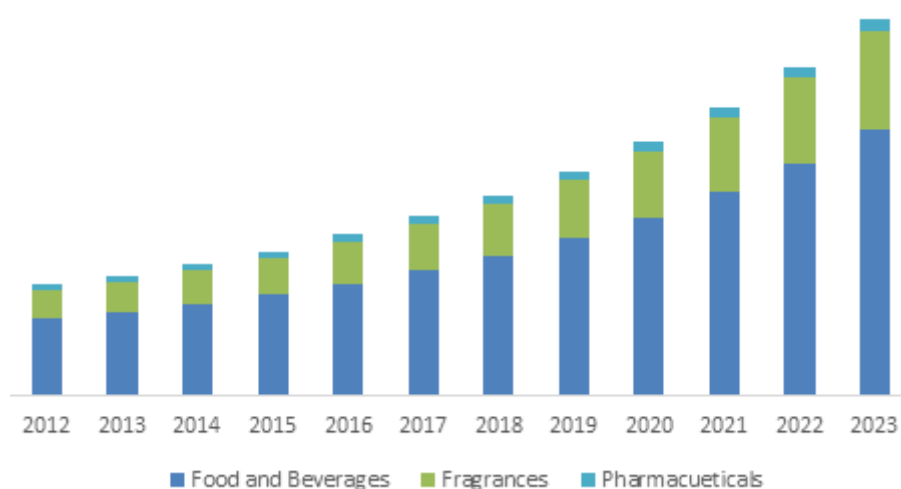
Figure 1.1: US biovanillin market size, by application, 2012-2023 (Tons) (Global Market Insight, 2016)

Further to this, one of the main constraints for industrial fermentation of these selected products (ethanol, acetoin, and vanillin) is the development of effective and economically viable downstream processes for the separation of these products from their fermentation broths. This is because most biological processes take place in aqueous media, and the downstream processing of fermentation products involves the separation of the broth. This necessitates the need for product recovery and purification, such as centrifugation, chromatography, crystallization, dialysis, drying, electrophoresis, filtration, and precipitation which are essential basic unit operation steps in commercial fermentation (Yang, 2011). Fermentation broths in a typical bioprocess operation can be quite complex and often involve sophisticated downstream processing, to meet the high purity required for the targeted bioproduct, which may account for up to 90% of the total production cost (Nielsen, 2008).

To overcome the problem of product inhibition in the broth, several integrated fermentation or product recovery technologies have been investigated recently for product removal to improve fermentation performance (Yang, 2011). Some of the reported separation techniques include salting-out extraction, distillation, ionic-liquid (IL) extraction, pervaporation, liquid-liquid extraction (LLE), perstraction, reverse osmosis, adsorption, and gas-stripping (Maiti et al., 2015). These methods usually require a long time, a large amount of solvent, toxic residue in the final product, and high temperature (Joana Gil-Chávez et al., 2013). Therefore, developing alternative extraction techniques with high efficiency and moderate uniqueness is highly desirable.

Supercritical carbon dioxide (scCO$_2$) extraction has received a lot of attention due to its application at low temperatures, short extraction time and non-toxicity as a solvent (Liu et al., 2009). The main advantages of using supercritical fluids (SCFs) are the tuneable solvation power achieved by manipulating pressure and temperature and their highly selective dissolution ability without damaging or contaminating the target compound. Effendi et al. (2013) reported that carbon dioxide (CO$_2$) is the most extensively used supercritical solvent, owing to its practical advantages, such as being cheap, readily available in high purity, safe handling and storage qualities (non-flammable and non-explosive), environmental friendliness, relatively low critical pressure (7.38 MPa), critical temperature (304.25 K), and ease of separation from the product. The benefits and flexibility of supercritical systems in bioprocessing have been emphasized by Catchpole et al. (2012), although additional separation methods may be critical to fully integrate the scCO$_2$ technique with bioprocess operations (Catchpole et al., 2012). This approach may allow the overall process to be tailored towards better overall fractionation or targeting of specific value-added products.

Although extensive studies have been reported on the application of scCO$_2$ in the processing of dry materials and isolation of targeted bioproduct from the broth, there is little or no report of an integrated process that combines scCO$_2$ with a bioprocess operation toward recovery of a valued product (Catchpole et al., 2009; Knez, 2009; Sarrade et al., 2003; Turner et al., 2004). A critical review of the literature suggests that most of the reported integrated processes were attempts to recover the target product(s) from a dilute aqueous system that mimics typical fermentation broths.

Therefore, studies that focus on the technical feasibility of scCO$_2$ extraction were limited to such processes. There is a dearth of information on a bioprocess-supercritical technology system that involves the production of a value-added product(s). Hence, such study may provide an understanding needed for the design of an integrated system for target bioproducts of high value. Therefore, this study aims at the production and recovery of some selected bioproducts (ethanol, acetoin and vanillin) using supercritical fluid extraction techniques. The study would in addition to providing for the first time, the technical feasibility and the base case data which are critical to the development and design of processes for production and recovery of these selected bioproducts. The lessons that can be gleaned from this study may be extended to develop processes for the production and recovery of other bioproducts.

1.2   Research questions

This research work is intended to answer the following questions:

(i) What are the main fermentation parameters to be considered to maximize the production of the selected bioproducts using a low-cost nitrogen source?

(ii) What is the effect of supercritical fluid extraction process variables (flow rate, fractionating time, pressure and temperature) on the concentration and recovery of the selected bioproduct from the broth?

(iii) Can the bioprocess-supercritical fluid separation system be technically feasible and comparable to the current method in literature, or not?

1.3   Aim and objectives of the study

The study aims to apply bioprocess-supercritical fluid extraction techniques in the production and recovery of some selected bio-products derived from the fermentation of glucose, to investigate the technical feasibility of the recovery of these bioproducts.

Specifically, the objectives of this thesis are:

(i) To develop a low-cost fermentation medium that is definite and devoid of; redundant nutritional supplements for ethanol production.

(ii) To establish the optimum processing parameters for the production of acetoin in a low-cost nitrogen source.

(iii) To establish the optimum processing parameters for the production of vanillin in a low-cost medium formulation.

(iv) To establish the optimum fermentation processing parameters for the scale-up study of acetoin in a bioreactor with a view to improving the yield parameter when compared to a shake flask study.

(v) To investigate the feasibility of concentrating and recovering acetoin from a typical fermentation broth containing acetoin using supercritical carbon dioxide.

1.4   Delineation of the study

This research investigated the application of bioprocess method for the production of three bioproducts (ethanol, acetoin, and vanillin) while supercritical fluid extraction techniques were used for the recovery of acetoin from fermentation broth. Recovery and concentration of ethanol and vanillin was not investigated in this study. While shake flask and bioreactor studies were conducted for acetoin, mass transfer effects did not form part of the parameters investigated. A simulated broth of acetoin was used as a base case for comparative recovery studies in the actual fermentation recovery. The study does not use broth from ethanol and vanillin as feed for recovery studies in supercritical fluid extraction pilot plant studies. This research did not include an economic evaluation of the process investigated.

1.5   Thesis outline

This thesis is divided into 9 chapters. Chapter 1 is the introduction to this research, chapter 2 starts with a review of the relevant literature as related to the objective of the research. Chapter 3 discusses the materials and methods used in the study. Chapter 4 describes the optimization studies of the first selected bioproduct (ethanol) published as Taiwo et al. (2018a). Chapter 5 describes the optimization studies of acetoin production, which was second selected bioproduct, published as Taiwo et al. (2018b). Chapter 6 focuses on optimization studies on bioconversion of ferulic acid into vanillin, the third bioproduct, Chapter 7 describes the scale-up study of the second bioproduct (acetoin) in the bioreactor from the shake flask batch studies. Chapter 8 reports on the recovery study of acetoin using a supercritical fluid extraction pilot plant. Chapter 9 consists of the conclusions and recommendations of this study. The flow diagram of the overall thesis structure and mind map is shown in Figure 1.2.
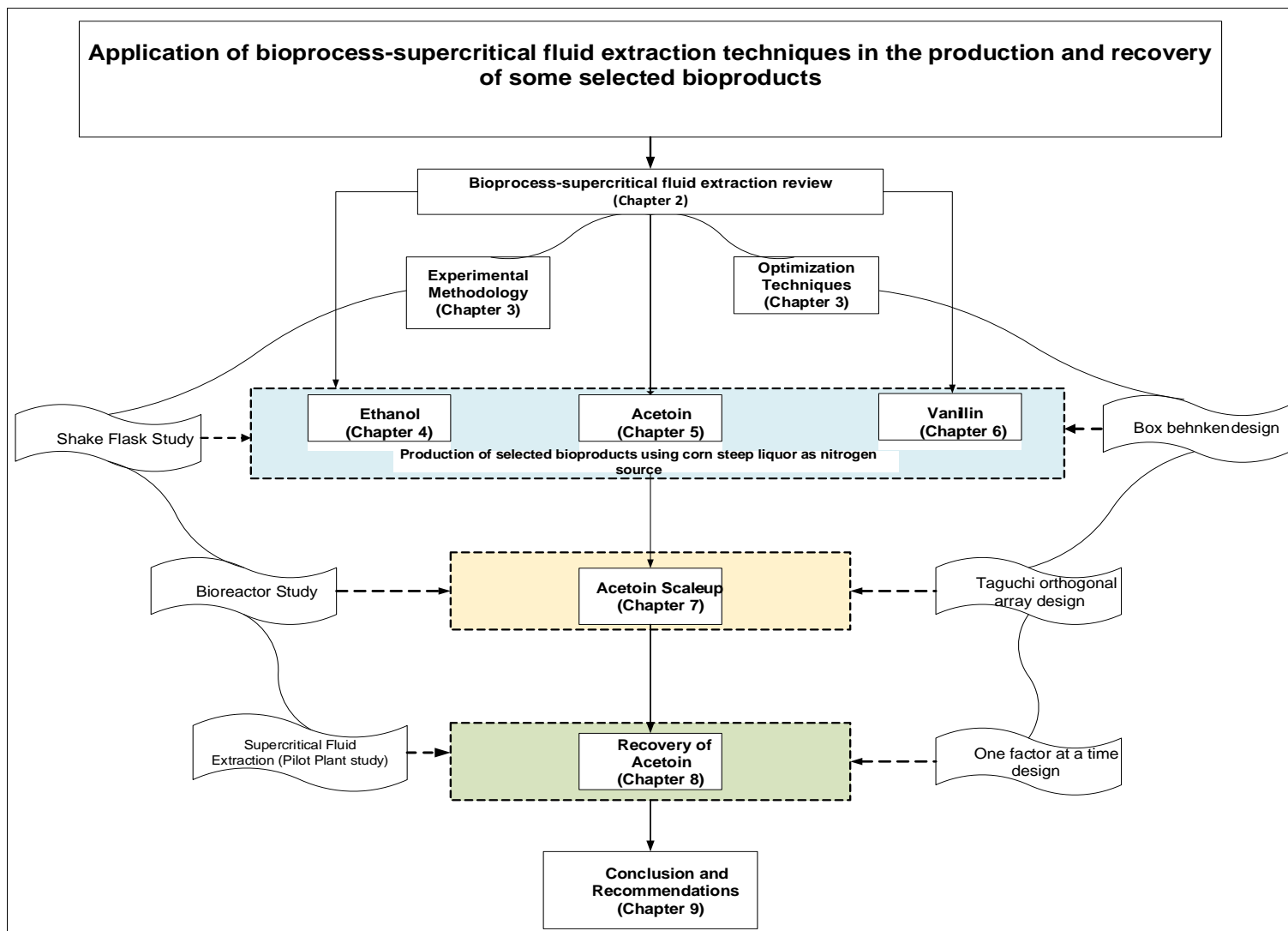
Figure 1.2: Flow diagram of the overall thesis structure and mind map

Chapter 2   LITERATURE REVIEW

2.1   Biotechnology overview

Biotechnology can be seen as the use of biological means, processes, or organisms to produce products that are targeted towards the improvement of human livelihoods. It can be broadly defined as the engineering of organisms for the purpose of human usage. It can also be defined as the skill set required for the utilization of living systems or the induction of natural processes to produce products, systems or environments that will enhance human development. The use of scientific applications in a biological system to produce or alter a particular process for specific use can be referred to as biotechnology (Bhatia et al., 2015). Biotechnology has been identified as a potent source of radical innovation that can address the environmental issues and concerns of industry (Bers et al., 2009). Furthermore, It is a technology of interest as it may present a considerable diversity of industries based upon the application of different kinds of scientific and engineering principles to the processing of raw materials to provide goods and services (Bull, 2001).

Biotechnology emerged more than three decades ago, and it has been used as an instrument for developing and optimizing the efficiency of different bioproducts which are better substitutes for chemically synthesized equivalents. The green processes and the possibility for production of high value-added products found in biotechnology have the potential for setting a path to industrial sustainability. Likewise, the utilization of renewable resources in bioprocesses without leading to depletion has increased the efficiency and optimization of industrial products, minimized the degradation of the environment and improved quality of life in general. The integration of biotechnology concept with industries like agriculture, pharmaceuticals, food, flavor and energy will not only reduce dependence on synthetic chemicals, but will also help to fulfill the social, economic and other requirements of present and future generations (Herrero and Ibáñez, 2015). Biotechnology can also enable the design of processes and value-added products for which conventional methods of production prove difficult or impossible in the chemical industry. It should be noted that biotechnology techniques and processes are ever-evolving to address the increasing range of valuable products that can be produced through bioprocessing and genetic manipulations (Webb and Atkinson, 1992).

2.2   Bioproducts

A bioproduct is a biologically derived product that is easily described by its chemical composition or structure and its function or application (Heinzle et al., 2007). Recovery of these valuable products from fermentation broth has an added advantage as it helps to

improve the economic value and sustainability of an integrated bioprocess. Integrated bioprocessing focuses on industrial bioconversion of renewable resources to value-added products to attain the sustainable production of food, energy, and industrial products. Some classes of bioproducts are shown in Figure 2.1. Vijayan et al. (1994) have broadly classified the recovery and purification of biological products into three segments: High Value, Low Volume (HVLV); Intermediate Value, Intermediate-Volume (IVIV); and Low Value, High Volume (LVHV). The development of bioproducts has touched almost every aspect of human life, from medicine to agriculture to the production of industrial products in different sectors which are being used to replace the synthetic products of petrochemical origin. A schematic diagram is shown in Figure 2.2 reflecting some of the sectors where bioproducts have found wide applications.
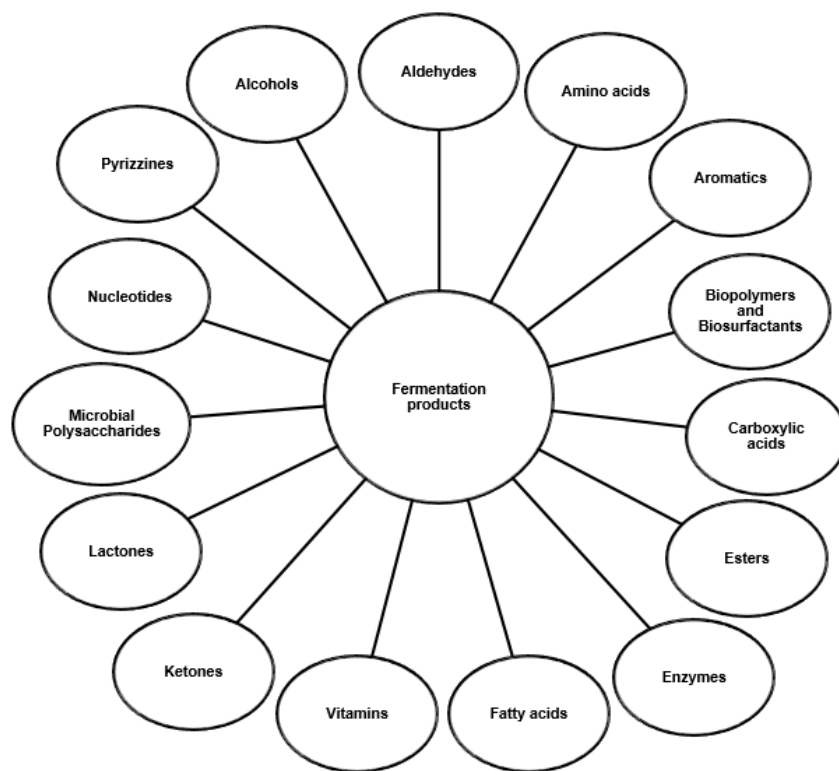
Figure 2.1: Classes of fermentation product
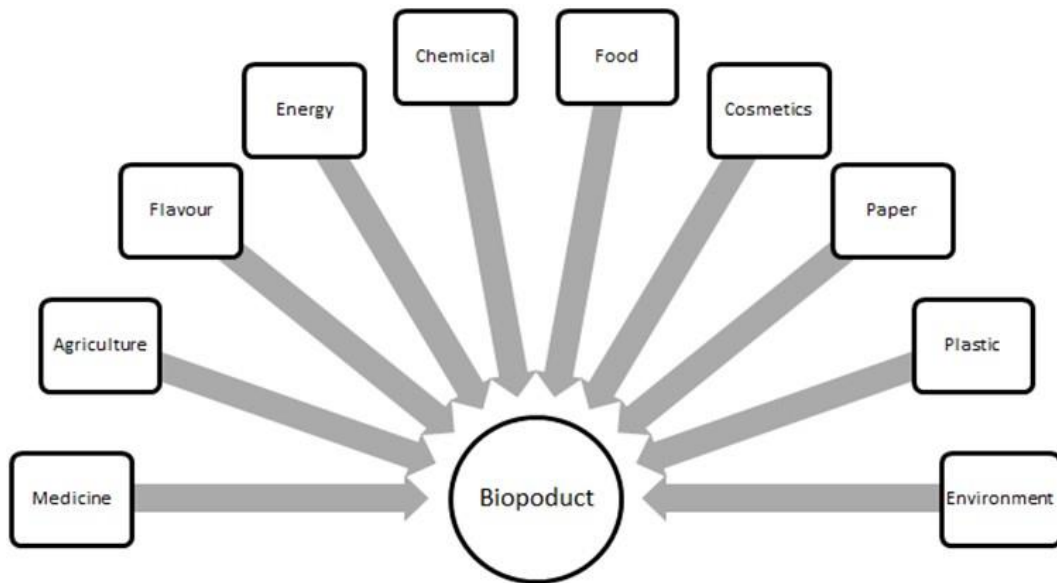
Figure 2.2: Some sectors where fermentation products have found application

2.3 Criteria for targeted bioproduct recovery

The factors that are considered when selecting a technology for the recovery of products from fermentation broths are partition coefficient, selectivity, and biocompatibility, choice of solvent and waste generation (Kim et al, 1999). The desired levels of product purity, density, shape, recoverability, and interfacial tension are also considered. Ghosh (2006) reported on the overview of biological separation and reiterated that several substances which are usually impurities and mostly side-products compete with the targeted bioproduct, and these make separation difficult because they often have the same physiochemical properties. Some of the main criteria for ease separation are discussed below:

2.3.1 Partition coefficient

The presence of a third compound in a two-phase system causes the third compound to distribute itself between the two phases. The property that best describes the distribution of this third compound is known as the partition coefficient or distribution coefficient (Equation 2.1). The regular procedure used to determine the partition coefficient is to measure the equilibrium concentrations of the compound in two immiscible liquid phases which are in contact (Streng, 2012). The partition coefficient is important because it determines the choice of a solvent and the separation quality in the recovery of the targeted product (Menet and Thiebaut, 1999).

$$\text{Distribution coefficient} = \frac{\text{mass fraction in organic phase}}{\text{mass fraction in aqueous phase}} \qquad (2.1)$$

9

2.3.2 Selectivity

The capacity of a solvent to separate a targeted product from water is best described as separation constant or factor. It is defined as the ratio of the distribution coefficient of the product to that of the water (Equation 2.2). In the process of extracting a target product from the fermentation broth, the separation factor greater than unity is an indication that the solvent preferentially extracts the product other than water (Kim et al., 1999). It has been shown that the extraction of the aroma from the broth could be efficient and selective by adjusting the selectivity of the extraction of PEA (2-phenyl ethyl alcohol) and ethanol in the extraction of aroma with supercritical CO_2 (Fabre et al., 1999). Groot et al. (1990) were of the opinion that an increased selectivity factor in separation could help in reducing the cost of recovery in extraction processes. The authors further argued that the high capacity of the solvent and selectivity of alcohol/water separation extraction would favor the extraction processes.

$$\text{Selectivity} = \frac{\text{distribution coefficient in organic phase}}{\text{distribution coefficient in aqueous phase}} \qquad (2.2)$$

2.3.3 Biocompatibility

Biocompatibility is the term used to describe the present state of biomaterial within a physiological environment, without negatively and significantly affecting any of the other body of environment and material (Williams, 1981). The biocompatibility of 62 organic solvents has been tested with yeast cultures to determine experimentally the controlling parameters affecting the product recovery. The author reported that 15 of these solvents were proven to be biocompatible, while 26 were completely toxic, the remainder showing different levels of inhibition (Kollerup and Daugulis, 1985). The highlighted reasons for the discrepancy were a different type of microbial strain, medium formulation, an adaptation of microorganism to solvents, differences in solvents cell contact and the type of fermentor design. In recent work by Zhang et al. (2017), biocompatibility with the fermentative organism has been identified as the criteria required for fermentative product recovery. Likewise, it has been demonstrated that biocompatibility is highly strain-dependent and many solvents are toxic to yeast and bacteria (Knutson et al., 1999). Therefore, biocompatibility is required in the choice of separation techniques in the recovery of bioproduct from the fermentation broth.

2.3.4 Nature of extractant or choice of solvent

The limitation of organic solvents in the recovery of the microbial product when compared to the broad usage in chemical synthesis could be attributed to the degradation of most recovered bioproducts (Ghosh, 2006). Microbial products are usually exposed to denaturing

and degradation that makes the compatibility of solvents selected a necessary factor to be considered in the recovery of the targeted product. Therefore, the nature of extractant is imperative because an extractant with a low partition coefficient will not be effective in the recovery of a fermentation product and a toxic extractant to the microbiological organism will inhibit cell growth and fermentation (Schaechter, 2009). However, the choice of extractant is key to the development of a suitable solvent system for downstream processing of bioproduct as it has implications for the quality and yield of the targeted product (Choudhury et al., 2013). Several solvents have been screened and modes of operation identified for recovery of various products from fermentation broths (Kim et al., 1999).

2.3.5   Environmental acceptability

The compelling regulation, for example, the European Union (EU) environmental policy and legislation have a goal and objectives set from 2010 to 2050 to prioritize the reduction of hazardous solvents in industries. They stand against the common use of carcinogenic or toxic solvents that are detrimental to the health of end-users and create a nuisance to the environment. Therefore, a solvent that will be used for recovery and separation of the product should be derived from either renewable resources or those that show better environmental, health and safety properties (Cvjetko Bubalo et al., 2015).

2.4   Separation techniques for target bioproduct

The diversity of cultured cell types and fermentation processes has led to the design of an array of process schemes for product purification. The basis for choosing separation techniques for bioproduct recovery is a function of the product composition (properties of the impurities and the producing microbes, cells or tissues), desired product purity, yield, and activity requirement. Most bioproduct recovery involves a series of unit operations for purification and concentration of the desired product. Suspended solids, such as cells, can be easily removed from fermentation broth through simple filtration or centrifugation processes, while the removal of dissolved impurities, especially organic acids and inorganic salts, requires much more complicated processes (Van Hee et al., 2006; Harrison, 2014). Figure 2.3 depicts the different stages of separation techniques that may be required in product recovery. The primary recovery stage is mainly concerned with the separation of cells or their debris to reduce the impurities, followed by the intermediate recovery stage that focuses on product concentration, while the choice of the technique is a function of the targeted product characteristics. Sometimes, it may be required to put into consideration a protein refolding step if the targeted product is formed within the cells' inclusion bodies (IBs) (Harrison, 2014). The final stage seems to be the most complex level of separation because it has been reported

as difficult, expensive and requires different separation techniques to attain the desired level of purity. The recovery of products from fermentation broths is regarded as the last difficulty to be overcome in closing the gap between laboratory-scale and commercial production of most potential products in the broth (Harrison, 2014; Harrison et al., 2015; Wu et al., 2017).
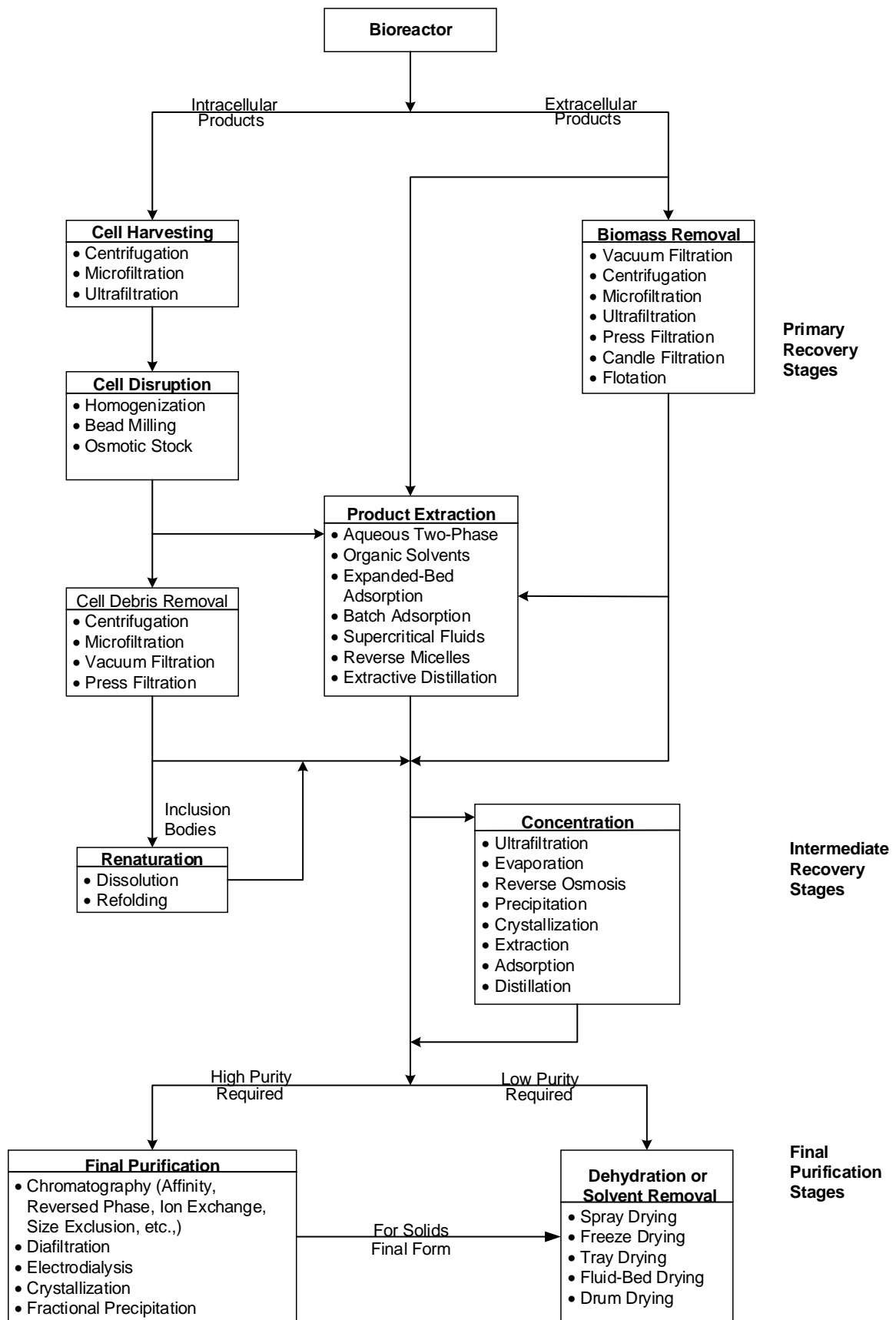
Figure 2.3: Generalized block diagram of downstream processing (Harrison, 2014)

2.5 Supercritical fluid extraction

2.5.1 Overview of supercritical fluid

The supercritical state is said to have been reached for fluid when its pressure and temperature are beyond their respective critical states, Tc and Pc which are critical temperature and critical pressure respectively (Khosravi-Darani and Vasheghani-Farahani, 2005). A critical point (CP) is defined as the endpoint of the vapor-pressure curve of the fluid, as shown in Figure 2.4. At a critical state, the liquid and vapor phase cannot be distinguished. The boundary line between vapor and liquid phases disappears at this point. A point in which all the three phases of solid, liquid and gases coexist in phase equilibrium is called triple point (TP). The thermodynamic and transport phenomena of supercritical fluids can be manipulated by changing the operating pressure and temperature (Welti-Chanes and Velez-Ruiz, 2016).
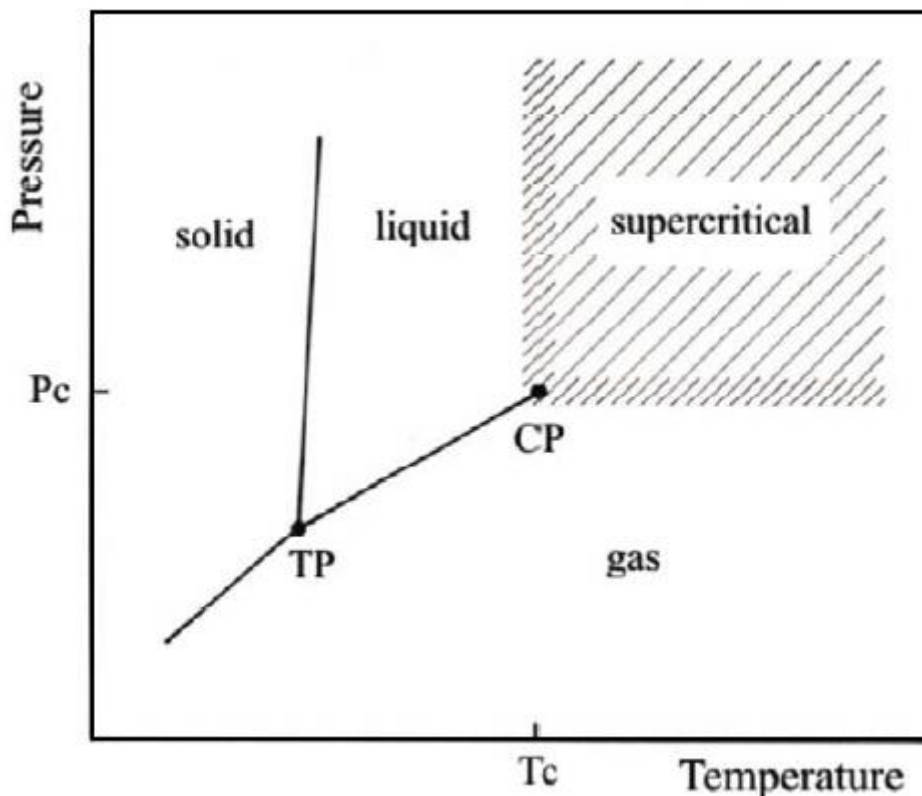
Figure 2.4: P-T diagram of a pure substance (Brunner, 2013)

2.5.2 Properties of supercritical fluid

The supercritical fluids (SCF) properties (Table 2.1) can be simply represented in terms of liquid-like densities, gas-like viscosities and diffusion coefficients located in the range between gas and liquid states. Owing to these unique features, specifically high solvent power

associated with gas-like transport properties, supercritical fluids have found several applications in the engineering field (Hrnčič et al., 2018).

Table 2.1: Physical properties of supercritical fluids (Brunner, 2013)

Fluid	P/T (MPa)/(K)	Density ρ (kg/m^3)	Diffusion coefficient D_{ij} (cm^2/s)	Viscosity η (g/cm*S)
Gas	0.1/298	0.6-2.0	0.1-0.4	$(1\text{-}3) \times 10^{-4}$
Supercritical fluid	Pc/Tc	200-500	0.7×10^{-3}	$(1\text{-}3) \times 10^{-4}$
Liquid	0.1/298	600-1600	$2\times 10^{-6} - 10^{-5}$	$(0.2\text{-}3) \times 10^{-2}$

Among several gases used as supercritical fluids (Table 2.2), special attention is drawn towards CO_2 as a good solvent in separation technique. In comparison to other substances, CO_2 has a critical temperature (Tc) close to room temperature (31.1 °C) and a relatively low critical pressure (Pc = 7.38 MPa). Besides, it is abundant, relatively cheap, inert, in high purity, non-flammable, non-explosive, non-toxic as a solvent and is generally regarded as safe (GRAS) which makes it compatible with environmental and health organizations' rules on the use of solvents for industrial purposes (Pansuriya and Singhal, 2009).

Table 2.2: Critical data of some pure components (Brunner, 2013)

Component	Tc (°C)	Pc (MPa)
Ethylene	9.4	5.04
Carbon dioxide	31.1	7.38
Ethane	32.3	4.87
Nitrous oxide	36.6	7.26
Propane	96.8	4.25
N-Hexane	234.5	3.01
Acetone	235.1	4.70
Methanol	239.6	8.09
Ethanol	240.9	6.14
Ethyl acetate	250.2	3.83
Water	374.1	22.06

The difference in pressure and temperature can be used to change the solubility of a substance in supercritical carbon dioxide (scCO$_2$). Owing to its compressibility, an increase in pressure will increase the density, which causes an interaction between the solute and solvent for easy extraction (Capuzzo et al., 2013). Figure 2.5 illustrates the CO_2 density as a function of temperature and pressure. Close to the critical point, a slight change in the operational

conditions (pressure and temperature) may cause a drastic variation in its density, affecting consequently the solubility of the solute in the supercritical phase (Effendi et al., 2013).
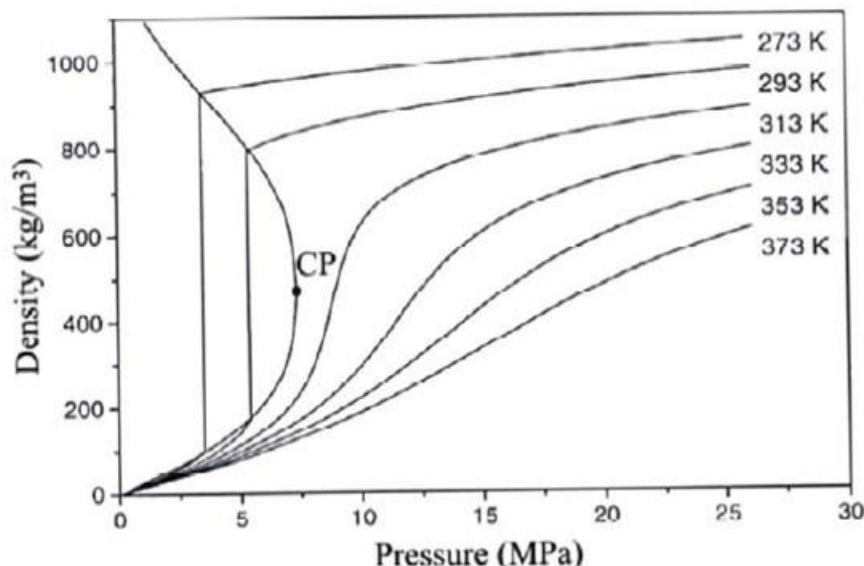
Figure 2.5: Density of pure CO_2 at different conditions (Danielski, 2007)

The polarity of the fluid can enhance the solvent power used during the extraction process. Non-polar solvents decrease the solubility of chemical compounds with increasing molecular weight, polarity and functional groups in the compound. CO_2 is a non-polar solvent, which makes it difficult to dissolve polar substances. A co-solvent usually refers to as entrainer is added to CO_2 to increase the efficiency of the process through the enhancement of the solvent´s polarity (Manjare and Dhingra, 2019; Zhang et al., 2014). Liquid substances like ethanol, ethane, and propane can be employed. These factors (density, pressure, and temperature) are crucial in the extraction control process and relationships between them and solubility are needed to predict the overall feasibility of extraction or initial extraction condition.

2.5.3 Supercritical carbon dioxide extraction

2.5.3.1 Supercritical fluid extraction from a solid feed

The application of supercritical fluid to extract from solid materials has gained wide applications in the field of biotechnology. The extraction is performed when a continuous contact is established around the solid feed and solvent at elevated pressures. The feed is placed into the extractor, creating a fixed bed of particles, and the solvent flows through it, allowing the targeted compounds to be solubilized. The targeted compounds are then continuously extracted by the supercritical phase until the solid matrix is exhausted.

Supercritical fluid extraction (SFE) from a solid feed process occurs in two stages: the extraction and the separation between substance extracted and the solvent. Figure 2.6 shows a flowsheet of SFE from solid materials. Once an equilibrium is reached, the solvent is fed at the chosen pressure and temperature settings, flowing through the loaded extractor. When the solvent reaches a saturated state, separation begins by increasing the temperature or decreasing the pressure. Then, the CO_2 could be flared into the atmosphere or recycled and fed again into the extractor.
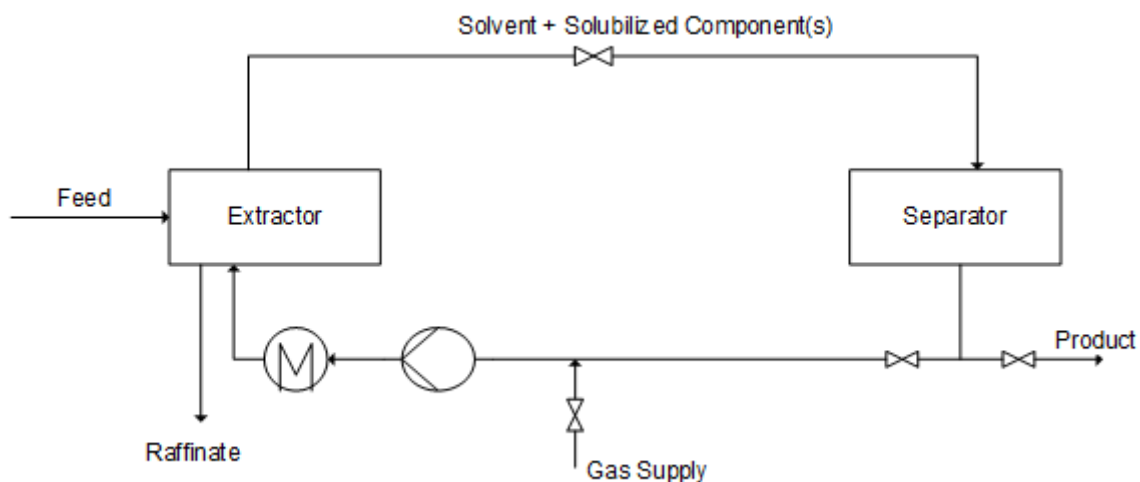
Figure 2.6: Flowsheet of SFE from solid materials (Danielski, 2007)

2.5.3.2 Supercritical fluid extraction from a liquid feed

The liquid feed and SFE solvent can be introduced counter-currently into one separation column. Such a separation column will allow multiple equilibrium stages through the enrichment of the mass-transfer area between gas and liquid phases using structured packing, which accelerates the efficiency of the extraction process (Keskinen et al., 2002). Supercritical fluid extraction of liquid fluid is a continuous separation process referred to as supercritical fractionation. The fractionation process operates according to solubility differences between the compositions of a liquid feed in a solvent at working conditions where the solvent and feed are not miscible and exceeding the critical pressure and temperature of the solvent (Pieck et al., 2015). After physical separation and a pressure drop, each separating phase will yield a different product stream: the fraction dissolved by the solvent will yield the extract while the remaining part in the feed components will form the raffinate.

A basic flowsheet of a counter-current multistage extraction process is shown in Figure 2.7. The set-up of most counter-current equipment is composed of a packed column, a separator, feed and reflux pumps, a solvent pump or compressor (based on the physical state of the

solvent), and auxiliary devices for recovering top and bottom products, extract and raffinate products respectively. The multistage extraction processes usually act like rectification and it could function with or without the use of reflux. The performance of reflux causes enrichment of the volatile components in the extract fractions. When employing extract reflux, a stripping section will be confined below the liquid-phase feed inlet, where the extracts (high volatile components) are separated from the raffinate and then conveyed to the enriching section. The enriching section is positioned between the liquid feed and extract reflux entry points, where the raffinate (low volatile components) is separated from the extract components and transported to the stripping section) lower section of the column (Danielski et al., 2008).
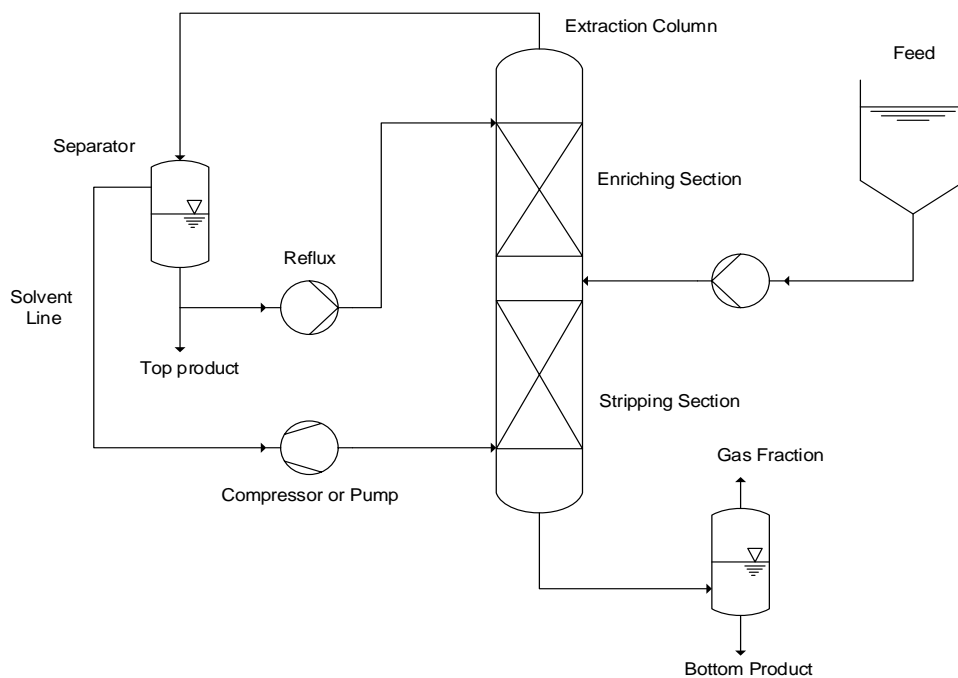
Figure 2.7: Simplified process scheme of a counter-current multistage extraction apparatus (Danielski et al., 2008)

2.5.4   Applications of supercritical carbon dioxide

Supercritical carbon dioxide (scCO$_2$) has found a wide range of applications in industrial and research fields because of its unique features. Table 2.3 highlights some of the uses of scCO$_2$ and the various fields of its application.

Table 2.3: Application of Supercritical CO_2 in various field

S/N	Uses	Field of application	Authors
1	Cleaning and photoresist stripping	Microelectronics processing	(Weibel and Ober, 2003; Kohli, 2019)
2	Particle design and drug formulation	Pharmaceutical industry	(Perrut, 2003; Long et al., 2019)
3	Dyeing	Textile processing	(Banchero, 2013; Eren et al., 2017)
4	Scaffold fabrication, Polymerizations	Tissue engineering Medical	(Bhamidipati et al., 2013; Zhao et al., 2017)
5	Natural products recovery	Biotechnology	(Darani and Mozafari, 2010; Zhang et al., 2018)
6	Solvent and anti-solvent, Material processing	Nanotechnology	(Zhang et al., 2014; Ruphuy et al., 2018)
7	Analysis, extraction and processing	Food industry	(Clark and Winter, 2015; Tyśkiewicz et al., 2018)
8	Essential oil extraction	Flavour and fragrance	(Capuzzo et al., 2013; Shukla et al., 2019)
9	Extraction of pesticides; air analysis and soil samples	Environmental analysis	(Benner Jr, 2015; Patnaik, 2017)
10	Maize Stover biorefinery	Energy	(Attard et al., 2015; Boock et al., 2019)

2.6 Bioprocess-supercritical fluid extraction case studies

2.6.1 Ethanol

The increase in oil prices and the depletion of fossil fuels have promoted the development of biofuels such as biobutanol and bioethanol (Águeda et al., 2013). Globally, consumption of bioethanol as a transport fuel obtained from starch, molasses and lignocellulose fermentation technology is continuously increasing (Gupta and Verma, 2015). Pieck et al. (2015) reported that research focus has been on proving the feasibility of difficult separations as a means of overcoming the thermodynamic restraints on the distillation processes of the water-ethanol-azeotrope. This process drew the attention of many authors because fermentation processes produce aqueous ethanol and its separation has been carried out through a series of distillation processes with high-cost implications. Therefore, a search for a novel separation technique needs to be conducted that will bring about efficient recovery at minimum cost without degrading the product quality. Supercritical carbon dioxide extraction (scCO$_2$), proposed in recent years, seems to be a promising green-solvent (de la Vega et al., 2016;

Feng and Meier, 2015). The removal or extraction of ethanol by $scCO_2$ has gained wider applications in processes such as the dealcoholisation of beverages (Fornari et al., 2009), ethanol production or recovery (Güvenç et al., 1998). The equilibrium data for scCO2 extraction of ethanol has been shown (Budich and Brunner, 2003; Ikawa et al., 1993; Lim et al., 1994) with the working temperature and pressure of 333 K and 10.1 MPa respectively. The study conducted by Güvenç et al. (1998) focused on ethanol extraction from a fermentation broth with $scCO_2$ using a continuous system mode of operation. The authors investigated the effects of CO_2 feed rate, extraction time and pressure on the extraction yield. Khosravi-Darani and Vasheghani-Farahani (2005) reported that $scCO_2$ in situ extractive fermentation has been limited by its inhibitory effect on the metabolism of a variety of yeasts and bacteria, which is attributed to acidic pH resulting from the increased solubility of CO_2 under high-pressure conditions.

Furthermore, the extraction and mass transfer rates in a CO_2-ethanol-water ternary system have been investigated by several authors (Bernad et al., 1993; Budich and Brunner, 2003; Lim et al., 1995; Pieck et al., 2015). The authors fractionate the ternary system at a pressure of 10.1 MPa and temperature of 333 K and reported that recovery fraction is increased with pressure at a constant temperature of 313.2 K. Lang and Wai (2001) reported that the modeling of supercritical fluid extraction processes can help to better understand the mechanism and optimize the extraction conditions. Sovová (2012) reviews different types of models applied to supercritical extraction in the last decades and the areas that demand attention. The techno-economic analysis of ethanol has gained attention in areas such as feedstock supply system (Aden and Foust, 2009), inoculum propagation strategies (de Castro et al., 2014), pretreatment of biomass (Baral and Shah, 2016), hydrolysis (Shah and Darr, 2016), with few exception to the evaluation of technical and economic feasibility of the whole production. Pereira et al. (2013.) describe the technical and economic evaluation of phorbol esters extraction from Jatropha curcas seed cake using supercritical carbon dioxide. The authors evaluate the production cost and conclude that supercritical technology is a promising technology when integrated with bioprocessing.

2.6.2 Acetoin

Acetoin (3-hydroxy-2-butanone) is a natural flavor usually present in wine, butter, honey, garnet berry and strawberry as a food additive (Xiao et al., 2007). It is also a precursor for the synthesis of valuable fine chemicals and widely used as an intermediate chemical that can be converted to a wide range of chemicals (Liu et al., 2011). Acetoin can be produced by traditional chemical synthesis. However, being a food additive, the naturally produced product has good commercial prospects. Compared to chemical synthetic methods and enzyme

conversion methods, microbial fermentation is the most competitive method of acetoin production due to its richer content as a source of raw materials, mild process conditions, environmental friendliness and high safety of food processes (Xu et al., 2011). The biotechnological productions of acetoin investigated by different authors are illustrated in Table 2.4.

Table 2.4: Biotechnological production of acetoin adapted and updated (Sarma et al., 2014)

Microorganism	Major substrate	Production scale	Maximum concentration	Reference
Lactococcus lactis subsp. lactis 3022	Glucose	100 mL	1.054 mol/L	(Kaneko et al., 1990)
Hanseniaspora guilliermondii	Glucose	-	367 mg/L	(Teixeira et al., 2002)
Bacillus subtilis CICC 10025	Molasses	5 L	35.4 g/L	(Xiao et al., 2007)
Bacillus licheniformis MEL09	Glucose	-	41.26 g/L	(Liu et al., 2011)
Serratia marcescens H32	Sucrose	3.7 L	60.5 g/L	(Sun et al., 2012)
Bacillus subtilis TH-49	Glucose	100 L	56.9 g/L	(Xu et al., 2011)
Bacillus amyloliquefaciens FMME044	Glucose	7 L	51.2 g/L	(Zhang et al., 2013)
Paenibacillus polymyxa CS107	Glucose	5 L	55.3 g/L	(Zhang et al., 2012)
Bacillus subtilis SF4-3	Glucose	5 L	48.9 g/L	(Tian et al., 2016)
Escherichia coli	Mesocarp fiber	-	29.9 g/L	(Yusoff et al., 2017)

Tian et al. (2016) reported that acetoin and 2,3-butanediol are always generated concomitantly in the fermentation broth and that several attempts have been made to improve the yield and process recovery of acetoin through statistical optimization, two-stage speed control strategy and reversible transformation. However, all these measures for acetoin recovery still seem challenging because acetoin shares similar properties with its analog by-product (2,3 butanediol) in production. Exploring the application of supercritical fluid extraction may prove efficient for acetoin recovery above the conventional extraction method since it has distinct properties such as low density and viscosity, and high diffusivity. Manipulating change of pressure and temperature in supercritical fluid extraction (SFE) could also influence the

solubility of the targeted compound and may increase the recovery efficiency (Tyśkiewicz et al., 2018). To date, supercritical fluid CO_2 extraction has not been used for the recovery of acetoin. This could be due to a lack of solubility measurement and the evaluation of phase behavior of acetoin in a supercritical CO_2 system which was not made available in the literature until the work of Effendi et al. (2013). The author reported that acetoin is readily soluble in supercritical CO_2 due to its low dipole nature and its behavior as a lipophilic solvent in supercritical conditions. This was the first fundamental report on acetoin in the $scCO_2$ system.

The previous work of Lu et al. (2011) shows only the process optimization for the recovery of various aroma-active compounds in a synthetic broth of Zhenjiang aromatic vinegar of which acetoin was reported to be part of the recovered aromas in a supercritical CO_2 fluid extraction. Tian et al. (2016) affirmed that the commercial production of acetoin by microbial fermentation is yet to be promoted in the market. The authors were of the opinion that its industrialization would bring about vast economic change and social benefits to consumers who desire natural products. Thus, on the above basis, it can be deduced that the recovery of acetoin using $scCO_2$ technique is worth investigating, as it would find application in the chemical industry, and in food and fermentation technology. In addition, the technical and economic feasibility of the process should be appraised with the possibility of reducing the process cost.

2.6.3   Vanillin

The significance of aromatic compounds such as flavor in food industries has been known for a long time. These compounds impart fruity fragrances to foods and beverages, making them appealing to consumers (Di Gioia et al., 2007). Vanillin (4-hydroxy-3-methoxy benzaldehyde) is one such commercially important flavor. Setbacks associated with the cultivation and processing of vanilla plants have necessitated a search for alternatives (Priefert et al., 2001). Currently, the commercial production for vanillin is met by synthetic chemicals such as eugenol or guaiacol extract (Carlquist et al., 2015). However, the high cost of vanilla flavor of plant origin along with consumer preference for natural extract has created a concern within the flavor industries to route the production of vanillin by bioprocessing from other renewable sources.

Vanillin and vanilla extracts have an estimated annual total volume of 16,000 metric tonnes, worth some $650 million in total (Kennedy, 2015). Natural vanilla extract represents less than 1% by volume, though it is more important in terms of value. Sales prices range from about $1,500 per kg for natural vanilla extract to $10-20 per kg for synthetic vanillin (Evolva, 2017). To make the biological production process economically viable, it is necessary to find a precursor chemically close to vanillin, cheap and easily available (Converti et al., 2010).

Ferulic acid is a phenolic compound present in the cell wall of lignocellulosic materials, where it is covalently linked with a variety of carbohydrates such as a glycoside conjugate, ester or amide, with special treatments necessary to release sufficient ferulic acid (Paz et al., 2018). Ferulic acid is a cheap feedstock to produce natural value-added compounds like vanillin when extracted from lignocellulose wastes like cotton, corncobs and rice bran.

Therefore, environmentally friendly routes and various agriproducts have been investigated for vanillin production (Table 2.5). However, due to the complex nature of the fermentation broth and its host toxicity in the isolation of vanillin, there is a need for improvement in purification and recovery processes. Carroll et al. (2016) reported that chemical volatility can be addressed using an organic bi-layer gas stripping system but the technical and economic viability is not certain. Hence there is a need for an alternative separation technique that will increase the microbial tolerance for this compound. Supercritical fluid extraction (SFE) with CO_2 has been regarded as an all-natural process, and the products allowed for food applications have GRAS (Generally Regarded As Safe) status (Díaz-Reinoso et al., 2006). Liu et al. (2006) describe the phase behavior of solid vanillin in CO_2 and this is part of the information needed to design the $scCO_2$ extraction process. Knez et al. (2007) present three-phase equilibria of binary systems composed of vanillin and compressed gases, fluorinated hydrocarbons and sulfur hexafluoride. The research data describes the feasibility of a high-pressure particle formation process using other viable solvents apart from CO_2. Nguyen et al. (1991) studied the extraction of oleoresin from vanilla beans using sc-CO_2 at 307 K and pressures between 110 and 140 bar. The authors obtained a 95% yield of vanillin from vanilla beans with supercritical carbon dioxide.

A further study was carried out on the sc-CO_2 fractionation process of vanilla oleoresin to evaluate its extraction at different temperatures from 310.5 to 348.3 K and at moderate pressures (140 and 180 bar) which resulted in higher values of vanilla oleoresin solubility in CO_2 at the highest operating conditions (de la Vega et al., 2016). The authors evaluated the effect of extraction time and material pretreatment on the oleoresin yield and its composition. In general, there is a limited number of applications in which the SFE system is incorporated with bioprocessing to isolate and purify vanillin. This could be due to the high solubility of vanillin in water, which makes most separation techniques less efficient or feasible (Carlquist et al., 2015).

Table 2.5: Biotechnological production of vanillin

Microorganism	Major substrate	Production scale	Maximum Concentration	Reference
Escherichia coli strain JM109	Ferulic acid	100 mL	2.52 g/L	(Barghini et al., 2007)
Streptomyces sp. strain V-1	Glucose, ferulic acid	500 mL	19.2 g/L	(Hua et al., 2007)
Engineered *Saccharomyces Cerevisiae*	Glucose	2 L	500 mg/L	(Brochado et al., 2010)
Aspergillus niger CGMCC0774 and *Pycnoporus cinnabarinus* CGMCC1115	Ferulic acid (from waste residue of rice bran oil)	25 L	2.8 g/L	(Zheng et al., 2007)
Staphylococcus aureus	Ferulic acid and Glucose	100 mL	45.7 mg/L	(Sarangi et al., 2010)
Bacillus species	Eugenol and Dimethyl sulfoxide	500 mL	0.32 mg/L	(Sindhwani et al., 2012)
Strain KK-02	Ferulic acid	50 L	15 g/L	(Yiyong and Hong, 2011)
Bacillus aryabhattai BA03	Ferulic acid	250 mL	147.1 mg/L	(Paz et al., 2018)
Aspergillus niger I-1472	Isoeugenol	250 mL	0.137 g/L	(Tan et al., 2015)

Chapter 3　　　　　MATERIALS AND METHODS

This chapter presents a detailed description of the materials used for the experiments, the experimental procedures followed and the analytical techniques. The theoretical aspects of the data analysis generated in this study are also discussed.

3.1　Ethanol shake flask study

3.1.1　Feedstock and materials used

Two types of yeast strain were used for this study:

(i)　Anchor Instant dry yeast, a product of Rymco (Pty) Ltd., 22 Bunsen Street, Industrial, South Africa.

(ii) *Saccharomyces* Type 1 (Baker's yeast) purchased from Sigma Aldrich.

The yeast strains were subjected to the same treatment and used throughout the studies.

3.1.2　Microorganism and inoculum preparation

The activation of the baker's instant dry yeast was done by adding a spatula of the yeast to 150 mL distilled water in a 250-mL Duran flask which was placed on a shaking incubator (Labcon FSIM SP016) for 18 h (Betiku and Taiwo, 2015). The yeast was then plated and maintained on malt extract agar. The yeast was stored and maintained at a temperature of 4 °C with a sub-culturing every two weeks. For the inoculum, the inoculating loop was used to scoop cells from a colony on the agar plate and transferred aseptically into a 250 mL Duran flask containing 50 mL of sterilized medium. The inoculated flask was placed in the incubator shaker at 200 rpm and 30 °C for 18-20 h before it was used to inoculate the media for the fermentation studies of bioethanol (Betiku and Alade, 2014; Betiku and Taiwo, 2015).

3.1.3　Media composition

A modified method of Davis et al. (2006) was adopted as an inoculum and fermentation medium for this study, in which yeast extract was replaced with corn steep liquor. The inoculum medium was composed of the following constituents (g/L): glucose 50; corn steep liquor 5; KH_2PO_4 2; $MgSO_4.7H_2O$ 1; $(NH_4)_2SO_4$ 1. The fermentation medium was composed of the following (g/L): glucose 100; KH_2PO_4 2; $MgSO_4.7H_2O$ 1; $(NH4)_2SO_4$ 1; corn steep liquor (0.5-5.5% w/v) as specified in fermentation variable design (Table 3.1). The media and flasks were sterilized in an autoclave maintained at 121 °C for 15 min.

3.1.4 Submerged fermentation study

The preliminary experimental study of ethanol production, 50 mL of glucose solution (100 g/L) was measured into a 250 mL Duran flask and nutrients were added as appropriate. The pH of the medium was regulated with 3 M NaOH and 1 N HCl buffer solution. Consequently, a 5% volume per volume of the inoculum size was added aseptically to the flask. The flasks were transferred into the Labcon Shaking incubator (model: FSIM SP016) at 30 °C and 198 rpm (Figure 3.1). Fermentation was performed for 72 h and sampling done every 6 h interval.

Figure 3.1: Labcon Shaking incubator (model: FSIM SP016)

3.1.5 Ethanol production experimental design by Box-Behnken using DOE

A Box–Behnken design was used to study the relationship between five fermentation variables. A factorial design with five variables and three levels, including six replicates at the center point, was used for fitting a quadratic response surface. A total of 46 runs were used to optimize the range and levels of chosen variables which were corn steep liquor (% w/v), pH, time (h), temperature (°C), and inoculum size (% v/v). The range and the levels of the independent variables investigated using the Box–Behnken experimental design in this study are shown in Table 3.1. The variables chosen for the study have been reported as part of the growth variables that supported the specific rate of yeast growth and ethanol production. Subsequently, their values were carefully chosen to fall within previously reported values (Tesfaw and Assefa, 2014).

Table 3.1: Variables and their levels for Box-Behnken design

Variables	Units	Symbols	Coded factor levels		
			-1	0	1
CSL	(% w/v)	X_1	0.5	5	5.5
pH		X_2	4	4.5	5
Time	(h)	X_3	12	24	36
Temperature	(°C)	X_4	25	30	35
Inoculum Size	(% v/v)	X_5	0.5	3	5.5

3.2   Acetoin shake flask study

3.2.1   Precursor and reagents

B. subtilis CICC 10025 purchased from the China Centre of Industrial Culture Collection was used in this study. All other chemicals were of analytical grade and purchased from Sigma-Aldrich (St Louis, MO).

3.2.2   Microorganism and inoculum preparation

B. subtilis CICC 10025 was maintained on agar slants with the following media (g/L): glucose 10, beef extract 10, peptone 10, sodium chloride 5, and agar 16 at pH 7.0. The seed culture was prepared by growing the bacterium in 50 mL of the following media in a 250 mL shake flask for 10 h with agitation of 150 rpm and temperature at 37 °C: glucose 60 g/L, beef extract 10 g/L, peptone 10 g/L yeast extract 10 g/L and sodium chloride 5 g/L at pH 7.0 (Xiao et al., 2007).

3.2.3   Medium composition

The fermentation medium used for this work was the modified optimized medium of Xiao et al. (2007). The nutrients used comprised the following (g/L): glucose 150, K_2HP0_4 0.5, CH_3COONa 0.5, NaCl 5, $MgSO_4.7H_2O$ 0.5, while 1 g/L of three different nitrogen sources (corn steep liquor, yeast extract and beef extract) was used for the preliminary experiment. The medium was adjusted to pH 7.0 and autoclaved at 121 °C for 15 min. The flasks were incubated at 37 °C with an orbital shaker at 150 rpm for 7 days.

3.2.4   Batch fermentation study

In the preliminary experimental study of acetoin production, 50 mL of glucose solution (100 g/L) was measured into a 250-mL Duran flask, and the growth effects of three complex nitrogen sources (yeast extract, beef extract, and corn steep liquor) were tested for acetoin

fermentation nutrients. The pH of the medium was adjusted with 120 g/L NaOH and 36.5 g/L HCl buffer solutions. Subsequently, a 5% volume fraction of inoculum size was added aseptically to the flask. The flasks were transferred into the environment-controlled incubator shaker (platform shaker, model: FSIM SP016) at 30 °C and 150 rpm. Fermentation was performed for 168 h at 12 h sampling intervals.

3.2.5   Acetoin production experimental design by Box-Behnken using DOE

A three-level-factor was employed to generate 17 experimental runs by considering the effect of glucose concentration (g/L), inoculum size (% v/v) and corn steep liquor (% w/v). The range and the levels of the independent variables investigated using the Box–Behnken experimental design is shown in Table 3.2. The fermentation variables chosen for this study had been previously reported to influence acetoin production (Tian et al., 2014; Sharma and Noronha, 2014). The minimum, center point and maximum levels of each variable were coded as −1, 0, and +1 respectively.

Table 3.2: Factors and their levels for Box-Behnken design

Factor	Unit	Symbols	Coded factors		
			-1	0	1
Glucose	(g/L)	X_1	50	100	150
CSL	(% w/v)	X_2	5	10	15
Inoculum Size	(% v/v)	X_3	2	3.5	5

3.3   Vanillin shake flask study

3.3.1   Precursor and reagents

Ferulic acid (≥ 99%, product number W18301), vanillin (≥ 97% natural, FCC, FG. W310727), vanillic acid sodium carbonate (BioXtra ≥ 99%), Folin & Ciocalteu's phenol reagent, corn steep liquor, yeast extract, and beef extract were purchased from Sigma Aldrich. All other chemicals were of analytical grade and purchased from Sigma-Aldrich (St Louis, MO).

3.3.2   Microorganism and Inoculum preparation

Bacillus amyloliquefaciens subsp. CICC 10025 purchased from the China Center of Industrial Culture Collection was used in this study. It was maintained on agar slants with the following media (g/L): glucose 10, beef extract 10, peptone 10, sodium chloride 5, and agar 16 at pH 7.0 (Xiao et al., 2007). The seed culture media (S1) were prepared by growing the bacterium in 50 mL of the following media in a 250 mL shake flask for 24 h with agitation of 150 rpm and

temperature at 35°C: glucose 5 g/L, ferulic acid (FA) 0.5 g/L, peptone 10 g/L yeast extract 3 g/L and sodium chloride 5 g/L at pH 8.0 (Yan et al., 2016). The seed culture media of Yan et al. (2016) were modified as the media (S2) where peptone and yeast extract was replaced with 13 g/L of corn steep liquor (CSL).

3.3.3   Medium composition

The biotransformation medium (M1) comprised the following (g/L): peptone 10, yeast extract powder 3, NaCl 5 and 2 ferulic acid (FA) (Yan et al., 2016). The medium was adjusted to pH 9.0 and autoclaved at 121 °C for 15 min. The flasks were incubated at 37 °C in an orbital shaker at 150 rpm for 72 h. The modified biotransformation media (M2), substitute corn steep liquor (13 g/L), was used to replace peptone and yeast extract in the fermentation medium.

3.3.4   Batch fermentation study

Preliminary experiments were conducted to evaluate the operational conditions on vanillin production and ranges were selected based on the optimized medium of different authors (Yan et al., 2016; Chen et al., 2017b; Chen et al., 2016). The effects of media (M1 and M2), pH (5, 8, 8.5, 9.0, 9.5) corn steep liquor (3, 6,9,12, 15) and FA (0.5, 1.0, 1.5, 2.0, 2.5) concentrations were studied. The pH of the medium was adjusted with 5 M NaOH and 10 M HCl buffer solutions. Subsequently, a 5% volume fraction of inoculum size was added aseptically to the flask. The flasks were transferred into the environment-controlled incubator shaker (platform shaker, model: FSIM SP016) at 35 °C and 150 rpm. Fermentation was performed for 72 h.

3.3.5   Vanillin production experimental design by Box-Behnken using DOE

In this study, the Box-Behnken design (BBD) approach from RSM was used to determine the relationship between the bioconversion variables. A three-level-factor was employed to generate 17 experimental runs by considering the effect of corn steep liquor (CSL) (g/L), ferulic acid concentration (FA) (g/L) and pH. The range and the levels of the independent variables investigated using the BBD are shown (Table 3.3). The minimum, center point and maximum levels of each variable were coded as −1, 0, and +1 respectively.

Table 3.3: Factors and their levels of Box-Behnken design

Factor	Unit	Symbols	Coded factors		
			-1	0	1
CSL	(g/L)	X_1	3	6	9
FA Conc.	(g/L)	X_2	0.5	1.5	2.5
pH		X_3	8.5	9.0	9.5

3.4   Acetoin bioreactor scale-up study

3.4.1   The theory of bioreactor and its geometry

Bioreactors are usually represented in a cylindrical vessel with hemispherical top and/or bottom, ranging in size from some liters to cubic meters, and are made of stainless steel and glass. They contain a mechanical stirrer (agitator) or stirrers that rotate around the axis of the tank. The geometrical ratios of different bioreactor vessels have been presented in the literature (Doran, 2012; McCabe et al., 1993; Katoh et al., 2015; Alade and Betiku, 2010). The system geometry of a bioreactor is shown in Figure. 3.2. A typical bioreactor is made up of glass vessel (heat or water blanket), motor (direct drive fermentation), probes (foam, dissolved oxygen, pH at 12mm compression), exhaust condenser, thermowell, harvest/sample tube, sparge ring/cooling coil, impeller (6-blade Rushton type 52mm (fermentation) and baffle.

The agitator system consists of the shaft, baffle, and impeller, which is also made of impeller disc and blade. It allowed an interfacial reaction between bulk fluid and gas-phase during mixing and create room for air dispersion, oxygen, and heat transfer. The agitation vessel helps introduce gases or air in the fermentation vessel. Up to four gases, including air, nitrogen, carbon dioxide and oxygen, can be introduced into the media through the sparge ring or optional microsparger. Gas under pressure is supplied to the sparger through a ring or tube with a single orifice into the vessel.

The mixture in a bioreactor is realized with the help of an impeller mounted on the vessel. The standard measures of the impeller to the various vessels in a bioreactor are available in the literature. The most commonly used impeller in fermentation is the 6 flat blades disc-mounted Rushton turbine (Doran, 2012).

To enhance the distribution and dispersion of gases, baffles are connected inside the vessel. They are placed in agitated vessels of different sizes to enhance aeration efficiency. Baffles are strips of metal about one-tenth of the vessel diameter that is connected radially to the wall of a bioreactor.

The vessel dimension varies for different bioreactors, and 1.3 L capacity was considered for this study (V_T=0.0013m^3), while other measurements are related to the diameter (D_T) of the vessel. Details about the bioreactor geometric ratio have been reported in the literature (Doran, 2012). The height-to-diameter ratio is a primary factor in vessel design for a bioreactor. A ratio of two to three is more appropriate and in other instances, where stratification of the tank content is not considered or a mixer is used, the higher ratio could be used (Chandrashekhar and Rao, 2010).

The volume of the cylindrical vessel for a bioreactor is estimated using equation (3.1)

$$V_T = \pi \left(\frac{D_T^2}{4} \right) H_T \qquad (3.1)$$

With the height to diameter ratio (aspect ratio) of 2.0, vessel capacity. $V_T = \pi \left(\dfrac{D_T^3}{2} \right)$, vessel diameter D_T= 0.15 m. Other vessel geometric details such as the height of the vessel, height of the liquid, width of the baffle, height of impellers are clearly stated in the work Alade and Betiku (2010).
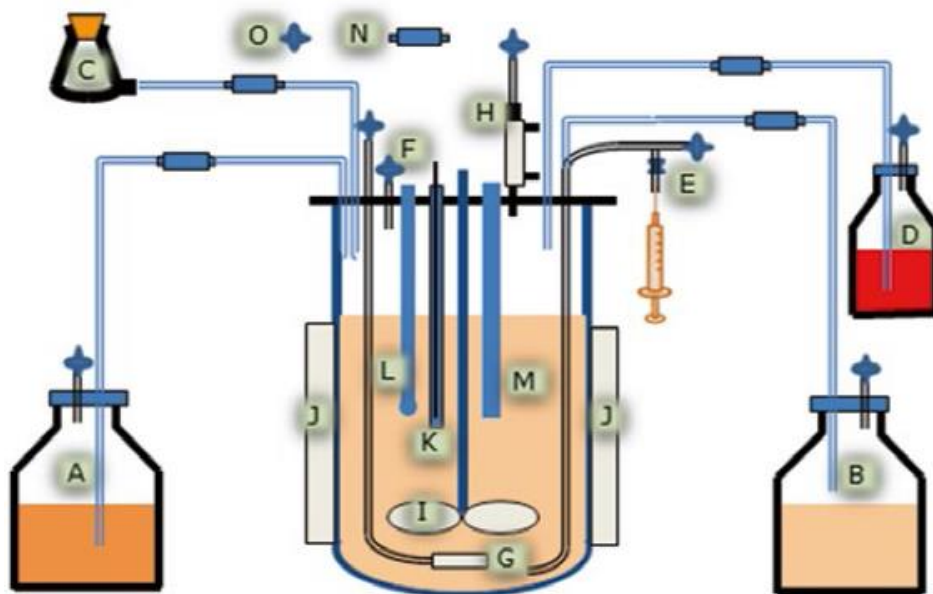
Figure 3.2: A schematic drawing of a laboratory-scale bioreactor setup (Martens et al., 2014)

(**a**) *A*, medium bottle; *B*, waste bottle; *C*, inoculation; *D*, base addition; *E*, sampling; *F*, overlay gas; *G*, sparger; *H*, gas outlet with condenser; *I*, impeller; *J*, heating blanket; *K*, temperature probe; *L*, pH probe; *M*, dissolved oxygen probe; *N*, connector; *O*, air filter.

3.4.2 Microorganism and Inoculum preparation

The procedure of microorganism and inoculum preparation is the same as reported earlier in section 3.2.2.

3.4.3 Medium composition

The same medium composition as previously mentioned in section 3.2.3 was used in this study.

3.4.4 Batch fermentation study of acetoin production in bioreactor

The optimized process conditions for acetoin production in the shake flask study (Taiwo et al., 2018b) were used as the fermentation medium in the bioreactor studies. These media are glucose concentration, corn steep liquor, and inoculum size as 78.40 g/L, 15% w/v, and 2.70% v/v respectively.

3.4.5 Acetoin fermentation experiment in the bioreactor

A 1.3 L laboratory-scale fermentor (BioFlo/CelliGen 115, New Brunswick Scientific Co., and Eppendorf, Germany) was employed for acetoin scale-up study. The bioreactor was equipped with a top driven stirrer and two six-blade Rushton impellers placed onto the agitation drive shaft (i.e., diameter 52 mm, width 16 mm, length 18 mm). To ensure gas bubble dispersion and provide a high rate of oxygen transfer, a removable 316 L stainless steel was positioned inside the vessel. The maximum adjustable levels of stirrer and aeration were 1200 rpm and 5 standard litre per minute (slpm). A standard two-point calibration method (pH 4 and 7 buffer standards) was performed to calibrate the pH electrode. The pH of the medium was kept constant with a measurement error of 0.01 by automatic addition of base (2 M NaOH) or acid (1 N HCL). The bioreactor control station was connected directly with a recirculating chiller (FL300) that helped to cool the condenser and maintain temperature stability. The bioreactor was maintained at 37 °C with operating conditions (agitation, aeration, time and pH) sets as designed by the experiment. The bioreactor was inoculated with a bacterial seed culture of 3% v/v inoculum size. Seed culture was grown in a 250 mL flask with 100 mL cell suspension volume for 24 h under 37 °C. The bioreactor was sterilized before inoculation with seed culture. Biocommand software (New Brunswick Scientific) was used for controlling the bioprocess and data acquisition (Seifan et al., 2017). The set-up of the bioreactor is displayed in Figure 3.3.

Figure 3.3: Experimental setup of bioreactor

3.4.4 Taguchi orthogonal array design

Taguchi L9 orthogonal array (OA) design was used in this experiment. Agitation, aeration, pH and fermentation time were used as variables and each factor was held at three levels, as shown in Table 3.4. In the Taguchi L9 OA, however, the required experiments are only 9 in number. The design of experiment (DOE) using the Taguchi method provides a simple, efficient, and systematic approach to determine the optimum conditions (Shahavi et al., 2016).

Table 3.4: Selected process variables and respective levels in the experimental design

	Process variables	Units	Level 1 (L_1)	Level (L_2)	Level 3 (L_3)
(A)	Agitation	rpm	150	300	400
(B)	Aeration rate	Slpm	0.5	1.0	1.5
(C)	Time	days	2	4	5
(D)	pH	-	Uncontrolled	6.5	7.0

3.5   Supercritical fluid extraction study of acetoin recovery from the broth

3.5.1   Chemicals

Acetoin (natural, purity ≥ 95%, CAS Number: 513-86-0) was purchased from Sigma-Aldrich, South Africa. The acetoin procured was a crystalline dimer with a molecular weight of 88.11 g/mol and utilized in the preparation of the simulated broth. Liquid CO_2 (99.98% pure) was supplied by Air Liquide (Pty) Limited, South Africa, as food-grade in a dip-tube supply cylinder. Ethanol absolute (99.9%, B&M Scientific) was used in cleaning the pilot plant prior to the plant operation for the experiment.

3.5.2 Feed preparation

3.5.2.1 Simulated broth

A known mass (1g) of acetoin was dissolved in deionized water to add up to a volume in litre. A 25 L volume of the broth of 1g/L concentration was prepared for the study. The solution was thoroughly stirred on a magnetic stirrer to ensure the homogeneous effect on the mixture.

3.5.2.2 Fermentation broth

Freeze-dried *Bacillus subtilis* CICC 10025 (China Center of Industrial Culture Collection) was revived in sterile *Bacillus* medium and periodically subcultured to maintain strain viability and purity. The optimized fermentation conditions described in section 3.4.3, were used in the broth preparation (Taiwo et al., 2018b).

3.5.3 Supercritical CO_2 equipment

Pilot plant scale supercritical CO2 equipment (SEPAREX SFE-5) designed for the processing of solids and liquid feed was used in this study (see Appendix C). It consisted of two units, namely an extraction section for the processing of solids, and a counter-current fractionation section for liquid feed. The two sections employed the same separating vessels for the separation of the extract from the solvent. For this study, the counter-current fractionation section was used in the recovery of the acetoin broth.

3.5.3.1 Fractionation unit

The counter-current fractionation unit was used for this study with the equipment parts, code, and specifications detailed in Table 3.5. All through the studies, the fractionation column temperature and pressure were varied according to the designed specifications for the study (Table 3.6). A process flow diagram of the plant fractionation unit is shown in Figure 3.4 (details about the valves and equipment parts functions are provided in Appendices C and E).

This fractionation unit included a packed column (C42) with an internal diameter of 28 mm and 4 m height with a viewing film located at the base of the column, below the solvent injection nozzle. The column had a maximum pressure of 350 bar, and its temperature is regulated using four separate heating jackets.

During operation, carbon dioxide under roughly 4.5 MPa was cooled to 275 K in a double tube heat exchanger before being pumped and then heated to the working temperature in an electrical heater. A high-pressure piston pump with a maximum capacity of 300 g/min (18kg/h) of liquid carbon dioxide and a maximum attainable pressure of 700 bar delivered the

supercritical solvent at the appropriate flow rate. Once the supercritical solvent left the column, an electrical heater was used to reduce the solvent power of the solvent by the application of heat, although it was not used for the fractionation of the broth. The overhead current was depressurized through a backpressure regulator to recover the extract in a pressurized cyclonic separator. The recovered solvent was recycled and condensed into the cooler to reduce carbon dioxide consumption. The solvent was recycled in this study. The broth was fed by a feed pump with 0-50 mL/min flow rate and maximum pressure capacity of 700 bar.

Table 3.5: Fractionating pilot plant equipment parts, code, and specifications

Equipment parts	Code	Specifications
Column	C42	350 bar, 150 °C, 4 m height, 28 mm internal diameter
Cyclonic separators	S50, S 51 & S52	200 bar, 150 °C, 0.6 L
CO_2 pump	P200	300 g/min (18 kg/h), 700 bar max.
Feed pump	P210	0-50 mL/min, 700 bar max.
Reflux pump	P400	0-50 mL/min, 400 bar max.
Cold Exchangers	CE1000 & CE2000	Cooled by water and glycol mix
Heat Exchangers	HE3000	250 °C
Chiller	C2000	4 kW, 20 °C

3.5.3.2 Experimental design for the recovery of acetoin from the broth in SFE pilot plant

The design (one factor at a time) and the operating conditions for the study were based on a preliminary experimental study trend and literature survey (Solana et al., 2016; Qureshi and Eller, 2018). Further, these experimental conditions were chosen because the density of $scCO_2$ is near to the normal liquid density under these conditions and consequently it has a high solvent power (Güvenç et al., 1999). Both simulated broth (SB) and fermentation broth (FB) were used for this study. The experimental design showing the column operating conditions and the feed used is displayed in Table 3.6.

Table 3.6: Column operating conditions and the type of feed using in recovery experiment

Runs	Feed	Temperature (°C)	Pressure (bar)	CO_2 Feed rate (kg/h)
1	SB	80	200	10
2	SB	37	200	10
3	SB	37	300	10
4	SB	37	300	15
5	FB	37	100	5
6	FB	37	200	10
7	FB	37	300	15

3.5.3.3 Experimental run for the recovery of acetoin from the broth in SFE pilot plant

The plant start-up procedure is the first step in running the supercritical fluid pilot plant (details can be found at the separation research group manual attached as Appendices C, D, and E). Once the steady-state was reached by the pilot plant, the liquid feed (acetoin broth) was introduced by the feed pump, CO_2 then passed through the chiller to maintain its liquid state to be pumped into the column. The flow rates of these pumps were controlled on a graphical user interface computer program connected to the pilot plant and the setpoints for the flow rate were based on the experimental design specifications. The condenser served the purpose of cooling the pump. As the CO_2 approached the column it was heated via a heating block so that it had a vapor phase change before entering the column. The feed (acetoin broth) flowed to the column, after which the CO_2 was bubbled upward and the liquid feed dripped downward. Heating jackets were found around the column for temperature regulation. The CO_2 collected the desired component from the liquid feed (acetoin broth) and disposed it into the three separator vessels, and the remaining liquid flowed into the raffinate section on the column.

A heat exchanger was placed after the column to account for the Joule Thomson effect (a temperature change that occurs in a gas due to a sudden pressure change over a valve). A recycle stream was introduced to collect the CO_2 that then would flow back to the CO_2 pump. Sampling was done at the raffinate at 5 min interval while extract was collected after 30 minutes. Under each experimental condition, according to the design matrix, the remaining samples were drained out of the column and at the three separation vessels. Thereafter, the shutdown procedure was followed (Appendix D). The process flow diagram of the pilot plant is displayed in Figure 3.4 below.

3.5.3.4 Sampling

The raffinate (DMRV 420) and the extracts (DMRV 500, DMRV 510 and DMRV 520) were sampled at the bottom of the column and separation vessels respectively (Figure 3.4). The sampling of the broth at raffinate was done at a constant interval of 5 min while sampling was done every 30 min at the separation vessel. The samples collected were labeled and designated according to temperature, pressure and CO_2 flow rate.
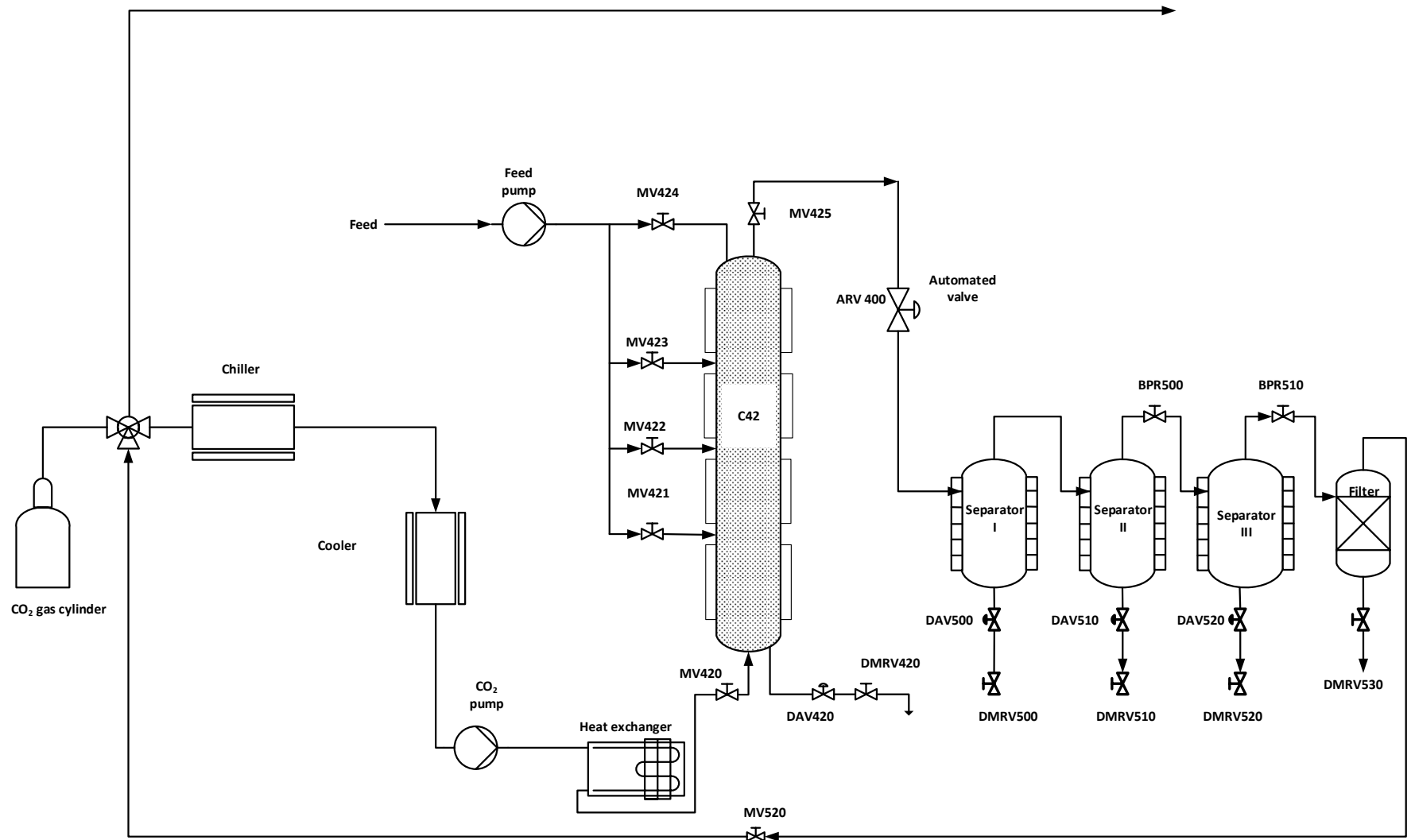
Figure 3.4: Process flowsheet of the pilot plant for the fractionation of fermentation broth using supercritical carbon dioxide

3.6   Analytical procedure: colorimetric method and GC-MS

The standard graphs, tables, and results from colorimetric and gas chromatography can be found in Appendices A and B.

3.6.1   Biomass concentration determination

Dry Cell Weight (DCW) was obtained by centrifuging a known volume of the fermentation broth followed by drying the cell residue to constant weight using an electric oven (Scientific, series 2000) in a pre-weighed centrifuge tube, at 105 °C for approximately 24 h. Thereafter, it was cooled in a desiccator. The biomass concentration was estimated based on the volume of the fresh broth as the difference between the weight of the unused tube and the final weight of the tube with the dried cells after cooling (Harris and Kell, 1985).

3.6.2   Reducing sugar concentration determination

The dinitrosalicyclic acid (DNS) method of Miller (1959) was used to ascertain the reducing sugar concentration and the results were expressed as glucose equivalent. To 1 mL of the supernatant broth, 3 mL of the DNS solution was added in the test tube and was heated for 15 min and cooled and diluted appropriately, after which the absorbance was measured at a wavelength of 540 nm using a UV-Visible Spectrometer (GBC Cintra 2020).

3.6.3   Ethanol concentration determination

The ethanol concentration in the fermentation broth was estimated using the dichromate method with spectrophotometry (Sumbhate et al., 2012). In a 50 mL volumetric flask containing 1 mL of the broth, 5 mL of sodium dichromate solution, 5 mL of acetate buffer of pH of 4.3 and 25 mL of 1 N HCl were added appropriately. A vortex mixer was used to shake the mixture for 1 min and placed at room temperature for 2 h until the green-colored reaction product was observed in the solution. The absorbance was read at 578 nm using a UV-Visible Spectrometer (GBC Cintra 2020).

3.6.4   Acetoin concentration determination

Acetoin concentration was determined by the modified Voges-Proskauer (VP) reaction of Westerfield (Speckman and Collins, 1982). An aliquot of the sample solution was pipetted into a 25 ml calibrated flask after which 2.5 mL of 1-naphthol solution and 1.0 mL of creatine solution were added in succession. After adjusting the mixture to volume and shaking vigorously, the solution was kept at 30 °C. The color intensity of the complex was determined

by measuring the absorbance after 40 min at 530 nm using a UV-Visible Spectrometer of 2020 GBC Cintra model (Campo and CarmenáLajo, 1992).

3.6.5   Vanillin concentration determination

5 mL of 24 wt% HCl solution, 2 mL of 1 wt% thiobarbituric acid solution and a 0.5 mL sample were added to distilled water to make 10 mL in a 10-mL colorimetric tube. It was heated in a 55 °C water bath for 10 min and subsequently stored at room temperature for 20 min (Kaur and Chakraborty, 2013). The ultraviolet absorbance was then determined with a blank solution as a reference at a wavelength of 434 nm using a UV-Visible (GBC Cintra 2020) Spectrophotometer. The concentration of vanillin in the liquid broth was calculated using a standard graph built with Cintra software in the spectrophotometer (Wu et al., 2008; Rana et al., 2013) at a wavelength of 438 nm.

3.6.6   Ferulic acid concentration determination

Ferulic concentration was analyzed using the reaction mechanism of Folin-Ciocalteu reagent. To 1 mL of the supernatant, 2 mL of 15 wt% Na_2CO_3 solution and 0.5 mL of Folin-Ciocalteu reagent were added, mixed thoroughly with a vortex, and cooled and diluted appropriately, after which the absorbance was measured at a wavelength of 718 nm against a blank solution using a UV-Visible Spectrometer (GBC Cintra 2020). (Rojas et al., 2005; Jadhav et al., 2012).

3.6.7   Vanillic acid concentration determination

Vanillic acid was measured using the same protocol in section 3.6.6 above, with the exception that vanillic acid was used as a standard for calibration (Appendix A).

3.6.8   The GC-MS analysis of acetoin broth

The gas chromatograph was coupled to a Thermo Triplus RSH autosampler. Chromatographic separation of the compounds was performed on a polar Zebron ZB (30 m, 0.25 mm ID, 0.25 µm film thickness) capillary column. Helium was used as the carrier gas at a flow rate of 1 mL/min. The injector temperature was maintained at 250 °C. 1µL of the sample was injected in a splitless mode. The oven temperature was programmed as follows: 35 °C for 6 min; then ramped up to 70 °C at a rate of 3 °C/min and held for 5 min, and again up to 120 °C at a rate of 4 °C/min and held for 1 min, and finally ramped up to 240 °C at a rate of 20 °C/min and held for 3 min. The mass spectrometric detector (MSD) was operated in full scan mode. Both the source and transfer line temperatures were maintained at 250 °C. The mass spectrometer was operated under electron impact mode at ionization energy of 70 eV, scanning from 35 to 550 m/z. (Appendix B).

Chapter 4   OPTIMIZATION OF CORN STEEP LIQUOR DOSAGE AND OTHER FERMENTATION PARAMETERS FOR ETHANOL PRODUCTION BY *SACCHAROMYCES CEREVISIAE TYPE 1* AND ANCHOR INSTANT YEAST

4.1   Introduction

The environmental and economic benefits of the dependence on bioethanol, like other biofuels, and its production from a variety of biomass materials have been well reported (Hahn and Cecot, 2009; Hattori and Morita, 2010). The view of most researchers on bioethanol production is to increase yield at a reduced cost (Hahn and Cecot, 2009). Apart from carbon, nitrogen is another significant nutritional element needed for active microbial growth in the fermentation of most targeted bioactive compounds (Tan et al., 2016). Nitrogen deficiency was responsible for the significant reduction in ethanol yield as reported by Malherbe et al. (2007). However, it is the most costly growth media among other components during fermentation (Li et al., 2017b; Andualem and Gessesse, 2013). *Saccharomyces cerevisiae* is commonly used for industrial production of bioethanol because of its ability to produce high ethanol concentrations, low cost and easy availability (Azhar et al., 2017; Li et al., 2017b). It requires nitrogen compounds to form its cellular protein and an adequate supply of nutrients to grow (Broach, 2012).

It has been reported that yeast performs better in media containing a mixture of amino acids and glutamic acids than in ammonium sulfate (Albers et al., 1996), although other sources of nitrogen such as yeast extract, beef extract, and malt extract have been reported to support microbial processes, the economic viability of these sources for bioethanol production on an industrial scale is in doubt due to their costs (Davis et al., 2006; Abbasiliasi et al., 2017). Corn steep liquor (CSL) has been identified as a cheap potential source of nitrogen in biochemical industries as an alternative to the costly nitrogen sources. It is a major side -product in the corn starch industry (Tan et al., 2016). It is also a cheap source of proteins, amino acids, minerals, vitamins, and trace elements and can be used as a rich and effective nutritional substitute for expensive complex media like beef extract, meat extract, yeast extract and peptone in fermentation studies (Lawford and Rousseau, 1997; Souza et al., 2016). Studies have shown that CSL can be used as a nutrition supplement and a cheap nitrogen source for cellular growth in the production of some fermentation products (Lawford and Rousseau, 1997; Seo et al., 2009). In another study, Edwinoliver et al. (2009) claimed that the utilization of CSL led to a reduction in the overall cost of lipase production medium by 55-60% of the total medium cost, thereby making the process economically attractive. CSL was also shown to be significant out of the ten low-cost media formulated for *Clostridium ragsdalei* in ethanol production (Gao et al., 2013). Davis et al. (2006) presented CSL as a good substitute for yeast

extract in the fermentation of hydrolyzed waste starch stream for ethanol production without altering the kinetics of the reaction.

Although some optimization studies have been reported (see Table 4.1) with respect to the use of CSL as nutritional enhancement in fermentation media (Saxena and Tanner, 2012; Maddipati et al., 2011; Kundiyana et al., 2010; Taiwo et al., 2018a), authors have used one variable at a time to establish their optimum conditions.

However, the fact that this method does not account for the interactive effects which may exist among the variables puts the reliability of such optimum conditions in doubt, especially if large-scale production is envisaged (Açıkel et al., 2010). The interactive effect among the fermentation parameters can be ascertained using effective optimization tools. Response Surface Methodology (RSM) is a statistical tool used for creating a relationship between a set of defined experimental variables and the observed results (Baş and Boyacı, 2007). RSM quantifies the effect of the control variables, alone or in combination, on a selected response. In addition to analyzing the effects of the independent variables, this method also generates a mathematical expression that describes the relationship between variables and a selected response (Osunkanmibi et al., 2015). Pereira et al. (2010) used a Placket-Burman design to initially select the critical nutrients for CSL and other low-cost nutrients for ethanol fermentation, followed by a Box-Behnken design for the optimization of the selected nutrients.

It is ascertained that most of the critical nutrients selected for optimization may be redundant, as they are already contained in adequate quantities in CSL (Hull et al., 1996). Hence, eliminating them may significantly improve the economics of the process, especially on an industrial scale. While the applicability of RSM to optimization studies has been successfully demonstrated (Osunkanmibi et al., 2015; Pereira et al., 2010), studies have reiterated that its predictive capability may be limited (Baş and Boyacı, 2007; Senanayake and Shahidi, 2002). Baş and Boyacı (2007) reported that the second-order equation, obtained from RSM did not show the interactive effects of pH and substrate concentration on the initial reaction rate of the enzymatic reaction in their study. Similar findings were reported in the lipase catalysis and optimization studies of borage oil, that there was no significant interaction among the variables considered using RSM (Senanayake and Shahidi, 2002).

Over the past years, data analysis tools such as artificial neural networks and evolutionary computing based on biological phenomena have evolved into well-established techniques for predicting and optimization of processes (Ebrahimpour et al., 2008). Globally, the applications of artificial neural networks (ANNs) have gained wider applications as a learning tool in bioprocessing, with focus areas such as pattern recognition, molecular sequences, data

forecasting, and fermentation optimization studies (Dumancas et al., 2017). ANN is a model that processes information using the knowledge of biological nervous systems. It is made up of a vastly interconnected network structure, consisting of many simple processing elements (the artificial neuron, unit or nodes) that are capable of performing the parallel computation for data processing. The theoretical basis has been described in detail elsewhere (Baş and Boyacı, 2007; Wesolowski and Suchacz, 2012).

ANN models have been reported to be preferred above RSM in accuracy and data prediction (Betiku et al., 2014; Ighose et al., 2017; Betiku and Taiwo, 2015). However, this may not be valid in all cases as the neural network is known for overfitting, which may cause overprediction and thus make validation of predicted data experimentally unrealistic (Tu, 1996; Schmidhuber, 2015). This chapter is focused on the use of corn steep liquor as a cheap replacement for yeast extract such that the CSL dosage and other fermentation controlling parameters could be optimized, using RSM and ANN techniques for ethanol production.

4.2   Objective

The objective of this chapter is to develop a low-cost fermentation medium that is definite and devoid of, redundant nutritional supplements, which may minimize the costs associated with the nutritional supplements and seed production that are essential for large-scale bioethanol production.

4.3   Methodology

The optimization studies were carried out in a shake flask and the experiment was designed using Box-Behnken. Details of the fermentation variables and methods used for this study are stated in sections 3.1 and 3.6 of Chapter 3.

4.3.1   RSM Analysis

Regression analysis was performed on the observed data obtained from the experimental design. The ethanol concentration was taken as the dependent variable for the two yeast strains; these values are the mean of triplicate data from fermentation studies. A second-order polynomial regression model was assumed for a predicted response, shown below in Equation 4.1. It gives the model proposed for each response of ethanol concentration on the two-yeast strain:

$$Y = b_0 + \sum_{i=1}^{k} b_i X_i + \sum_{i=1}^{k} b_{ii} X_i^2 + \sum_{i<1}^{k} b_i X_i X_j + \varepsilon \tag{4.1}$$

where Y is the independent variable (ethanol concentration), b_0 is the intercept value, b_i (i=1, 2…k) is the first-order model coefficient, b_{ij} is the interaction effect, b_{ii} represents the quadratic coefficients of X_i. X_i and X_j are the input variables that influence the response variable, and ε represents the random error. This data was analyzed using Design-Expert version 10.0. (Stat Ease Inc., Minneapolis USA).

4.3.2   ANN Analysis

Commercially available Neural Power®, version 2.5 (CPC-X software), was employed in this study. This software has a graphical user interface (GUI) that supports different types of training algorithms. This enables the user to load the data sets, design the network structure, select the training algorithm, and generate the different models for each output variable in a single operation (Ghaffari et al., 2006). The networks were trained with incremental backpropagation as it has been reported to be the most commonly used algorithm (Betiku and Taiwo, 2015). To determine the optimal network structure, only one hidden layer was used and the selected number for hidden and output layers (sigmoid, hyperbolic tangent, Gaussian, linear, threshold linear and bipolar linear) was iteratively determined by developing several networks. The effects of architecture and topology on neural network performance on ethanol fermentation have been reported elsewhere (Betiku and Taiwo, 2015). Therefore, four and five layers were selected as the best-hidden neurons for ST1 and AIY strains for ethanol production, as shown in Figure 4.1 (a) and (b).

The ANN model chosen in the present work is a multi-layer normal feedforward, with incremental back-propagation network and sigmoid as the transfer function for the ST1 strain, and hyperbolic tangent as transfer function for the AIY strain, which consist of a 5-4-1 and 5-5-1 topology respectively (Figure 4.1). The ANN data sets were trained using Root Mean Square Error (RMSE), average Correlation Coefficient (R) and average Determination Coefficients (DC) as the stopping criteria, values set at 0.0001,1,1 respectively. The default values were unaltered for the remaining ANN parameters in the software. The Box-Behnken design experimental data were divided into training and testing sets using the option available in the software: 30 of the data sets were used as training data and 16 were used as testing data (see Table 4.3). The training data was used to compute the network parameters. The testing data was used to ensure the robustness of the network parameters (Moghaddam et al., 2010).
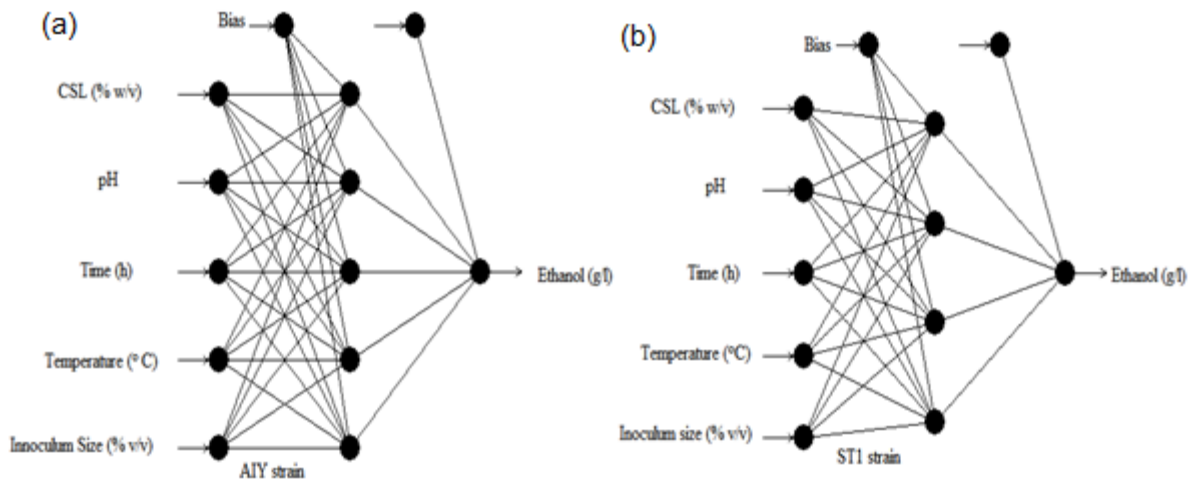
Figure 4.1: A multilayer feedforward perceptron (MLP) network consisting of (a) five inputs, one hidden layer with five neurons and one output for Anchor Instant Yeast (AIY) (b) five inputs, one hidden layer with four neurons and one output for ST1 (*Saccharomyces* Type 1)

4.3.2.1 Predictability of model evaluated in artificial neural network (ANN)

The predictive ability of the ANN methods is assessed based on the mean square error (MSE), root mean squared error (RMSE), the coefficient of determination (R^2), and the absolute average deviation (AAD) (Ghaffari et al., 2006), between the predicted values of the network and the actual values, which are calculated by Equations (4.2) – (4.4) as follows:

$$\text{MSE} = \frac{1}{n}\sum_{i=1}^{n}(y_i - y_{di})^2 \tag{4.2}$$

$$R^2 = \text{MSE}^{1/2} \tag{4.3}$$

where n is the number of points, y_i is the predicted value obtained from the neural network model, and y_{di} is the experimental value.

The coefficient of determination, R^2 reflects the degree of fit for the mathematical model. The closer the R-value to 1, the better the model fits the actual data (Moghaddam et al., 2010).

$$R^2 = 1 - \sum_{i=1}^{n}\frac{(y_i - y_{di})^2}{(y_{di} - y_m)^2} \tag{4.4}$$

where n is the number of points, y_i is the predicted value obtained from the neural network model, y_{di} is the experimental value, and y_m is the average of the experimental values.

Absolute Average Deviation (AAD) in Equation (4.5) is another important index to evaluate the ANN output error between the actual and the predicted output (Betiku and Taiwo, 2015).

$$AAD = \frac{100}{n} \left(\sum_{i=1}^{n} \left(\frac{y_i - y_{di}}{y_{di}} \right) \right)$$

(4.5)

where, y_i and y_{di} are the predicted and actual responses respectively, and n is the number of the experimental runs. The network having minimum MSE, RMSE, minimum AAD and maximum R^2 is considered as the best neural network model (Basri et al., 2007).

4.4 Results and discussion

4.4.1 Comparison of the fermentation performance based on Yeast Extract (YE) and Corn Steep Liquor (CSL) as inoculum component on ethanol fermentation of two yeast strains

The effects of inoculum, yeast extract and CSL on the fermentation performance of two yeast strains grown on glucose were investigated by examining the biomass growth, glucose consumption, and ethanol production. Figure 4.2(a) revealed that CSL supported the rapid utilization of the reducing sugar (from 100 to 5 g/L) within the first 30 h of fermentation, whereas the resultant ethanol was already maximal in the first 6 h of fermentation, and so was the growth of the biomass. Díaz-Montaño (2013) also reaffirmed that most *Saccharomyces cerevisiae* grew faster within the first 12 h of fermentation and sugar was depleted after 24 h of fermentation. The maximum biomass of 8 g/L was obtained, which was constant until 66 h when it started to decline. The maximum ethanol concentration of 41.92 g/L (which corresponds to 82% of the theoretical yield) was constant and started to decline after 66 h.

According to Gao et al. (2013), the decline in ethanol yield could be attributable to the reassimilation of produced ethanol by this yeast strain, which might occur as a result of an increase in oxygenation of the fermentation medium. The presence of CSL in the inoculum and fermentation media appeared to increase the consumption of glucose in the fermentation system for ethanol production, as displayed in Figure 4.2(a). It had been earlier stated that the addition of CSL in the fermentation broth increased substrate consumption and promoted high ethanol yield (Sarks et al., 2014). Although the ethanol concentration remained constant after 18 h of fermentation, it can be assumed that the energy derived from reducing sugar metabolism was channeled towards cell maintenance, since biomass growth remained constant from the 6 th to 66 th hours of fermentation (Rebnegger et al., 2016; Sarks et al., 2014).

On the other hand, when yeast extract was used as the nitrogen source for both the inoculum and the fermentation media, as shown in Figure 4.2(b), a relatively slow consumption of the reducing sugar compared to Figure 4.2 (a) was observed. When about 20% of the reducing sugar was converted to ethanol (during the first 18 h of fermentation), the ethanol concentration and biomass growth attained the optimum yield, 44.8 g/L (~ 88% theoretical value) and 8 g/L respectively. It should be noted that malt extract was used for plate media of *Saccharomyces* Type 1 yeast to investigate the effect of CSL while yeast peptone dextrose agar was later used to plate the same yeast strain. However, with regard to the effect of yeast extract, the idea is to rule out the possible transfer of the effect on the plate media extracts during the fermentation processes. However, the results showed that there was no significant difference when either malt extract or yeast peptone dextrose agar was used for plate media (data not shown). The results showed that the addition of CSL reduced the fermentation time and enhanced the growth of the organisms.

It can be seen from Figure 4.3 that fermentation progresses in a similar trend as shown in Figure 4.2 below. As shown in both figures, the peak biomass and ethanol concentration were observed at 8 g/L after 6 h of fermentation. It was evident from both figures (4.2 and 4.3) that there was an increase in cell mass at 12 h. On the other hand, a decrease in biomass and ethanol concentrations were observed at 18 and 42 h, and this could be as a result of glucose limitation which might be synonymous with the Crabtree effect, though this cannot be ascertained at this stage (Pfeiffer and Morley, 2014; Van Urk et al., 1989). The slow conversion of reducing sugar was also observed with yeast extract as a nutrient source. A similar observation had earlier been reported (Mezule and Dalecka, 2017). In general, these results show that CSL supported rapid consumption of the reducing sugar by both microbes. The ethanol yields were similar when using either CSL or yeast extract as a nutrient source for both microbes. The results also show that both microbes appeared to use the energy derived from the metabolism for cell maintenance rather than cell growth during fermentation. Table 4.1 compares the results obtainable in the present studies with the previous similar findings in literature with respect to the use of CSL as a nutritional enhancement in fermentation media.
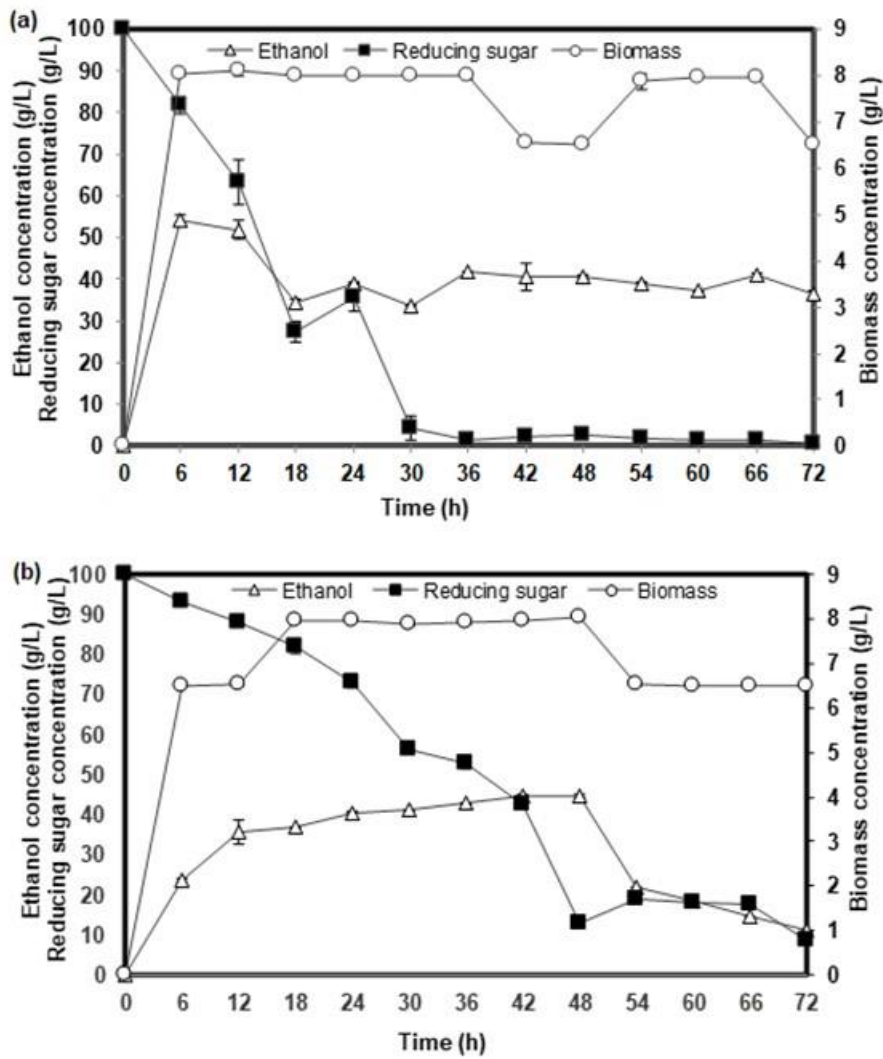
Figure 4.2: Profile of reducing sugar, ethanol and biomass concentration against fermentation time (a) with malt extract on plate media and corn steep liquor for inoculum and fermentation media using *Saccharomyces* Type 1 yeast (b) with yeast peptone dextrose on agar plate media and yeast extract as inoculum and fermentation media using *Saccharomyces* Type 1 yeast
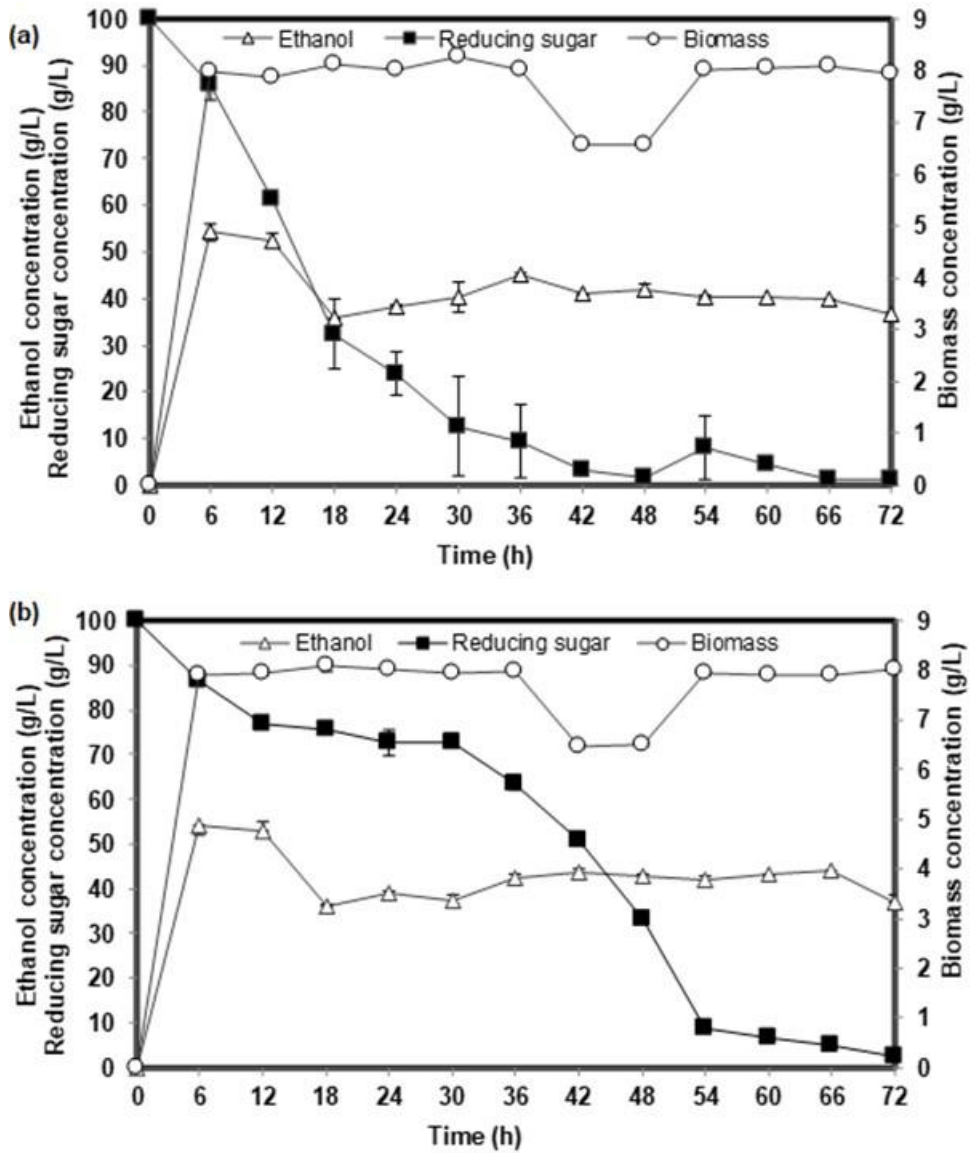
Figure 4.3: Profile of reducing sugar, ethanol and biomass concentration against fermentation time (a) with malt extract on plate media and corn steep liquor for inoculum and fermentation media using Anchor Instant Yeast (AIY). (b) With yeast peptone dextrose agar on plate media and yeast extract for inoculum and fermentation media using Anchor Instant Yeast (AIY)

Table 4.1: Comparing present work to previous studies

Glucose (g/L)	Type of yeast	Ethanol (g/L)	Yield (%)	CSL (% w/v)	Scale	Author
100	*Saccharomyces* Type 1	43.39	85	5.5	Shake Flask	This study
100	Anchor Instant Yeast	45.36	89	5.36	Shake Flask	This study
107	*Candida shehatae* FPL-702	40.1	73	5	Shake Flask	(Sreenath and Jeffries, 1996)
300	*Saccharomyces cerevisiae*	125	82	110	Shake Flask	(Pereira et al., 2010)

4.4.2 Ethanol production from glucose fermentation using CSL inoculum: Optimization studies

Optimization studies were carried out using appropriately designed experimental techniques. CSL was used as the only nitrogen source in the inoculum for glucose fermentation. 46 experiments were carried out. A quadratic regression equation was obtained. Thus, it was possible to obtain predictions from both the RSM and ANN models for the two yeast strains, Anchor Instant Yeast and *Saccharomyces* Type 1. It can be seen from Table 4.2 that for the AIY strain, an increase in the volume of CSL (5.5% w/v) enhanced the concentration of ethanol in the fermented broth, even at low inoculum size. On the other hand, low CSL (0.5% w/v) made ethanol concentration fall below 40 g/L (78% theoretical) until there was an increase in the volume of inoculum size. A similar trend was observed for the ST1 strain. It was observed that there was a relationship between the concentration of CSL and the inoculum size. That is, for an increase in the concentration of CSL, a reduction of inoculum size is necessary for an increased yield of ethanol, and vice versa. The results show that there was no significant difference in the ethanol yield and inoculum size when CSL was increased under the same fermentation condition.

Sreenath and Jeffries (1996) reported that the components of CSL supported biomass growth and fermentation activity, thus had a significant influence on the production of ethanol. It has been reported that yeast derives its cellular protein primarily from nitrogen compounds (Betiku and Alade, 2014). The mycelial growth of the biomass was rapid when more CSL was used rather than a lower amount of the nitrogen source.

4.4.3 RSM modeling results for ethanol production

The results of the experimental design along with the predicted responses are shown in Table 4.3 for AIY and ST1 yeast strains. For the ST1 yeast strain, the results show that only the synergistic effect of CSL with the inoculum size was significant (p<0.0001), while other fermentation parameters such as pH, temperature and time did not show any significant interaction, as presented in Table 4.4. It was noted that these operating parameters were carefully chosen to lie within the normal operating condition for this strain, as shown in Table 4.3, and may account for this observation (Jin et al., 2012). While *p*-value indicates the significance of the model parameters, the Fisher test (F-value) shows the level of significance of model parameters. However, it does not give a distinction between a positive or a negative significance of the model. The analysis of variance (ANOVA) of regression equation model shows that the model F-value of 4.07 implies the model is significant and there was only 0.44% chance that a model F-value this large could occur due to noise. Equation (4.6) shows the fit of the reduced equation for ST1 grown on CSL for ethanol production.

$$ST1(Ethanol) = 32.80 + 0.11X_1 + 1.23X_2 + 0.78X_5 - 0.31X_1X_5 + 0.17X_1^2 \qquad (4.6)$$

The response surface plot of the significant synergistic variable is depicted in Figure 4.4(a). A significant positive influence of corn steep liquor and inoculum size can be seen on ethanol concentration response. The desirability function (DF) graph was used to optimize the performance condition and the interaction effect of corn steep liquor and inoculum size, as in Figure 4.4 (a) The response surfaces of the desirability (*D*) between corn steep liquor and inoculum size was more than 0.92. This desirability of the optimized function obtained in this study was considered acceptable and ranged from 0.8 to 1 (Sarteshnizi et al., 2015). For the AIY yeast strain, the model Fisher test F-value of 2.74 with corresponding low probability (p<0.0001) implies that the model obtained is significant, as presented in Table 4.4. The results show that CSL, time, inoculum size, and the synergistic interaction of CSL and temperature with inoculum size were significant. Similarly, the double effect of CSL interaction recurred, as was observed in the ST1 strain reported earlier. The F-value of 2.82 implies the lack of fit is not significant relative to the pure error. There is a 12.66% chance that such a large " F-value" could occur due to noise. This implies that the model can suitably represent the actual relationship among selected factors. The final equation showing the terms of interaction in the second-order model is expressed in Equation (4.7).

$$AIY(Ethanol) = 39.19 + 0.82X_1 - 1.01X_3 - 0.84X_5 - 1.32X_1X_5 + 1.79X_4X_5 + 0.94X_1^2 \quad (4.7)$$

The graphical representation of the regression of Equation (4.7) in terms of a significant relationship between variables is depicted in Figure 4.4 (b). The independent variables CSL,

inoculum size and temperature affected the ethanol concentration significantly. The increase in the concentration of corn steep liquor influenced the concentration of ethanol and brought about a reduction in the use of inoculum size. Tesfaw and Assefa (2014) also confirmed the importance of the finding that lower inoculum size reduces the cost of production in ethanol fermentation. The mutually significant relationship between inoculum size and temperature was due to the use of a commercial strain (Anchor Instant Yeast) which operated within the specified temperature range (25-35 °C) in the design of the experiment.

Table 4.2: Responses of predictability of RSM and ANN on corn steep liquor effects for fermenting two yeast strains

Runs	CSL (%w/v)	pH	Time (h)	Temperature (°C)	Inoculum Size (% v/v)	Experimental values (g/L)	Predicted values (g/L)		Experimental values (g/L)	Predicted values (g/L)	
						STI	RSM	ANN	AIY	RSM	ANN
1	0.5	5.0	24	30	3.0	40.96	40.92	40.96	40.34	39.82	40.47
2	0.5	4.5	36	30	3.0	42.30	40.30	42.30	42.03	42.83	41.98
3	0.5	4.5	12	30	3.0	39.69	40.30	39.69	40.88	40.26	41.03
4	0.5	4.0	24	30	3.0	37.99	39.69	37.99	42.32	40.94	42.19
5	0.5	4.5	24	30	5.5	41.49	41.86	41.49	40.95	41.48	41.09
6	0.5	4.5	24	35	3.0	39.47	40.30	39.47	36.99	37.05	37.03
7	0.5	4.5	24	30	0.5	39.02	38.74	39.02	38.94	39.20	38.70
8	0.5	4.5	24	25	3.0	41.50	40.30	41.50	39.75	39.19	39.14
9	3.0	5.0	24	30	5.5	39.90	39.91	39.90	38.46	37.94	38.32
10	3.0	4.0	24	25	3.0	38.77	39.09	38.77	40.53	40.63	40.56
11	3.0	4.5	12	30	5.5	40.39	39.30	40.39	38.19	39.70	38.00
12	3.0	4.0	36	30	3.0	36.99	39.09	36.99	38.69	39.47	38.73
13	3.0	4.5	24	30	3.0	39.38	39.70	39.11	41.90	43.03	41.99
14	3.0	4.5	24	30	3.0	38.84	39.70	39.10	38.80	39.33	38.77
15	3.0	4.5	24	35	0.5	39.97	40.11	39.97	39.57	39.82	39.41
16	3.0	4.5	12	25	3.0	39.16	39.70	39.16	39.84	39.84	39.85
17	3.0	4.5	24	30	3.0	38.51	39.70	39.11	39.27	39.19	39.14
18	3.0	4.5	12	30	0.5	40.41	40.11	40.41	41.42	39.93	41.12
19	3.0	4.5	24	25	0.5	40.10	40.11	40.10	39.12	40.36	39.13
20	3.0	4.5	36	30	5.5	40.96	39.30	40.96	38.71	38.55	38.84
21	3.0	4.5	24	25	5.5	37.28	39.30	37.28	38.93	39.20	38.95
22	3.0	5.0	36	30	3.0	39.58	40.32	39.58	38.40	38.51	38.36
23	3.0	4.5	24	35	5.5	40.45	39.30	40.45	37.84	39.12	38.08

24	3.0	4.5	36	25	3.0	39.84	39.70	39.84	36.00	35.84	36.02
25	3.0	5.0	12	30	3.0	38.03	40.32	38.03	40.36	39.50	40.31
26	3.0	4.5	36	35	3.0	40.30	39.70	40.30	38.36	38.73	39.14
27	3.0	5.0	24	35	3.0	42.15	40.32	42.15	41.20	41.06	41.56
28	3.0	4.5	12	35	3.0	40.97	39.70	40.97	40.78	40.41	40.77
29	3.0	5.0	24	30	0.5	40.55	40.72	40.55	38.30	39.19	39.14
30	3.0	4.0	24	35	3.0	39.68	39.09	39.68	39.08	41.06	39.17
31	3.0	4.0	24	30	5.5	41.07	38.69	41.07	39.70	40.17	39.68
32	3.0	4.0	24	30	0.5	40.28	39.49	40.28	39.12	39.19	39.14
33	3.0	4.5	24	30	3.0	38.58	39.70	39.11	38.36	39.19	38.54
34	3.0	4.5	36	30	0.5	42.29	40.11	42.29	38.42	38.06	38.65
35	3.0	4.0	12	30	3.0	38.95	39.09	38.95	39.52	39.22	39.85
36	3.0	4.5	24	30	3.0	39.20	39.70	39.11	41.02	39.37	40.99
37	3.0	4.5	24	30	3.0	40.13	39.70	39.11	39.04	37.93	38.71
38	3.0	5.0	24	25	3.0	38.42	40.32	38.42	39.35	39.55	39.33
39	5.5	4.5	36	30	3.0	42.42	41.22	42.42	36.74	37.36	36.65
40	5.5	5.0	24	30	3.0	44.88	41.84	44.88	37.33	38.31	37.41
41	5.5	4.5	24	30	5.5	37.39	38.86	37.39	39.57	40.15	39.71
42	5.5	4.5	12	30	3.0	41.42	41.22	41.42	39.18	39.02	39.14
43	5.5	4.5	24	35	3.0	38.23	41.22	38.23	40.35	39.19	39.14
44	5.5	4.5	24	25	3.0	41.77	41.22	41.77	41.84	41.10	41.74
45	5.5	4.5	24	30	0.5	42.77	43.59	42.77	45.23	42.08	45.17
46	5.5	4.0	24	30	3.0	40.90	40.61	40.90	40.70	40.36	40.50

Table 4.3: Experimental design including the coded levels of each parameter and predicted values for ethanol production from STI and AIY using RSM and ANN

S/N	CSL (%w/v)	pH	Time (h)	Temperature (°C)	Inoculum size (%v/v)	*Saccharomyces* Type 1 (ST1)[α] Experimental (g/l)	Predicted RSM	Predicted ANN	Anchor Instant Yeast (AIY)[α] Experimental (g/l)	Predicted RSM	Predicted ANN
1	0	1	0	0	1	*39.9	39.91	39.9	*38.46	37.94	38.32
2	1	0	1	0	0	42.42	41.22	42.42	36.74	37.36	36.65
3	-1	1	0	0	0	40.96	40.92	40.96	40.34	39.82	40.47
4	0	-1	0	-1	0	*38.77	39.09	38.77	*40.53	40.63	40.56
5	0	0	-1	0	1	40.39	39.3	40.39	38.19	39.7	38
6	0	-1	1	0	0	36.99	39.09	36.99	38.69	39.47	38.73
7	0	0	0	0	0	*39.38	39.7	39.11	*41.9	43.03	41.99
8	0	0	0	0	0	*38.84	39.7	39.11	38.8	39.33	38.77
9	1	1	0	0	0	44.88	41.84	44.88	37.33	38.31	37.41
10	0	0	0	1	-1	39.97	40.11	39.97	39.57	39.82	39.41
11	0	0	-1	-1	0	39.16	39.7	39.16	39.84	39.84	39.85
12	1	0	0	0	1	37.39	38.86	37.39	*39.57	40.15	39.71
13	0	0	0	0	0	*38.51	39.7	39.11	*39.27	39.19	39.14
14	-1	0	1	0	0	42.3	40.3	42.3	42.03	42.83	41.98
15	0	0	-1	0	-1	40.41	40.11	40.41	41.42	39.93	41.12
16	0	0	0	-1	-1	*40.1	40.11	40.1	39.12	40.36	39.13
17	0	0	1	0	1	40.96	39.3	40.96	38.71	38.55	38.84
18	0	0	0	-1	1	37.28	39.3	37.28	38.93	39.2	38.95
19	-1	0	-1	0	0	*39.69	40.3	39.69	*40.88	40.26	41.03
20	0	1	1	0	0	39.58	40.32	39.58	38.4	38.51	38.36
21	-1	-1	0	0	0	37.99	39.69	37.99	42.32	40.94	42.19
22	0	0	0	1	1	*40.45	39.3	40.45	*37.84	39.12	38.08
23	1	0	-1	0	0	41.42	41.22	41.42	39.18	39.02	39.14

24	1	0	0	1	0	38.23	41.22	38.23	40.35	39.19	39.14
25	-1	0	0	0	1	*41.49	41.86	41.49	*40.95	41.48	41.09
26	-1	0	0	1	0	39.47	40.3	39.47	36.99	37.05	37.03
27	0	0	1	-1	0	39.84	39.7	39.84	36	35.84	36.02
28	0	1	-1	0	0	*38.03	40.32	38.03	*40.36	39.5	40.31
29	0	0	1	1	0	40.3	39.7	40.3	38.36	38.73	39.14
30	0	1	0	1	0	42.15	40.32	42.15	41.2	41.06	41.56
31	0	0	-1	1	0	*40.97	39.7	40.97	*40.78	40.41	40.77
32	0	1	0	0	-1	40.55	40.72	40.55	38.3	39.19	39.14
33	0	-1	0	1	0	39.68	39.09	39.68	39.08	41.06	39.17
34	0	-1	0	0	1	*41.07	38.69	41.07	*39.7	40.17	39.68
35	0	-1	0	0	-1	40.28	39.49	40.28	*39.12	39.19	39.14
36	1	0	0	-1	0	41.77	41.22	41.77	41.84	41.1	41.74
37	1	0	0	0	-1	*42.77	43.59	42.77	*45.23	42.08	45.17
38	1	-1	0	0	0	40.9	40.61	40.9	40.7	40.36	40.5
39	0	0	0	0	0	38.58	39.7	39.11	38.36	39.19	38.54
40	-1	0	0	0	-1	*39.02	38.74	39.02	*38.94	39.2	38.7
41	0	0	1	0	-1	42.29	40.11	42.29	38.42	38.06	38.65
42	0	-1	-1	0	0	38.95	39.09	38.95	39.52	39.22	39.85
43	-1	0	0	-1	0	*41.5	40.3	41.5	*39.75	39.19	39.14
44	0	0	0	0	0	39.2	39.7	39.11	41.02	39.37	40.99
45	0	0	0	0	0	40.13	39.7	39.11	39.04	37.93	38.71
46	0	1	0	-1	0	*38.42	40.32	38.42	*39.35	39.55	39.33

α Asterisked numbers represent ANN testing data set while unmarked numbers constitute the training data set

56
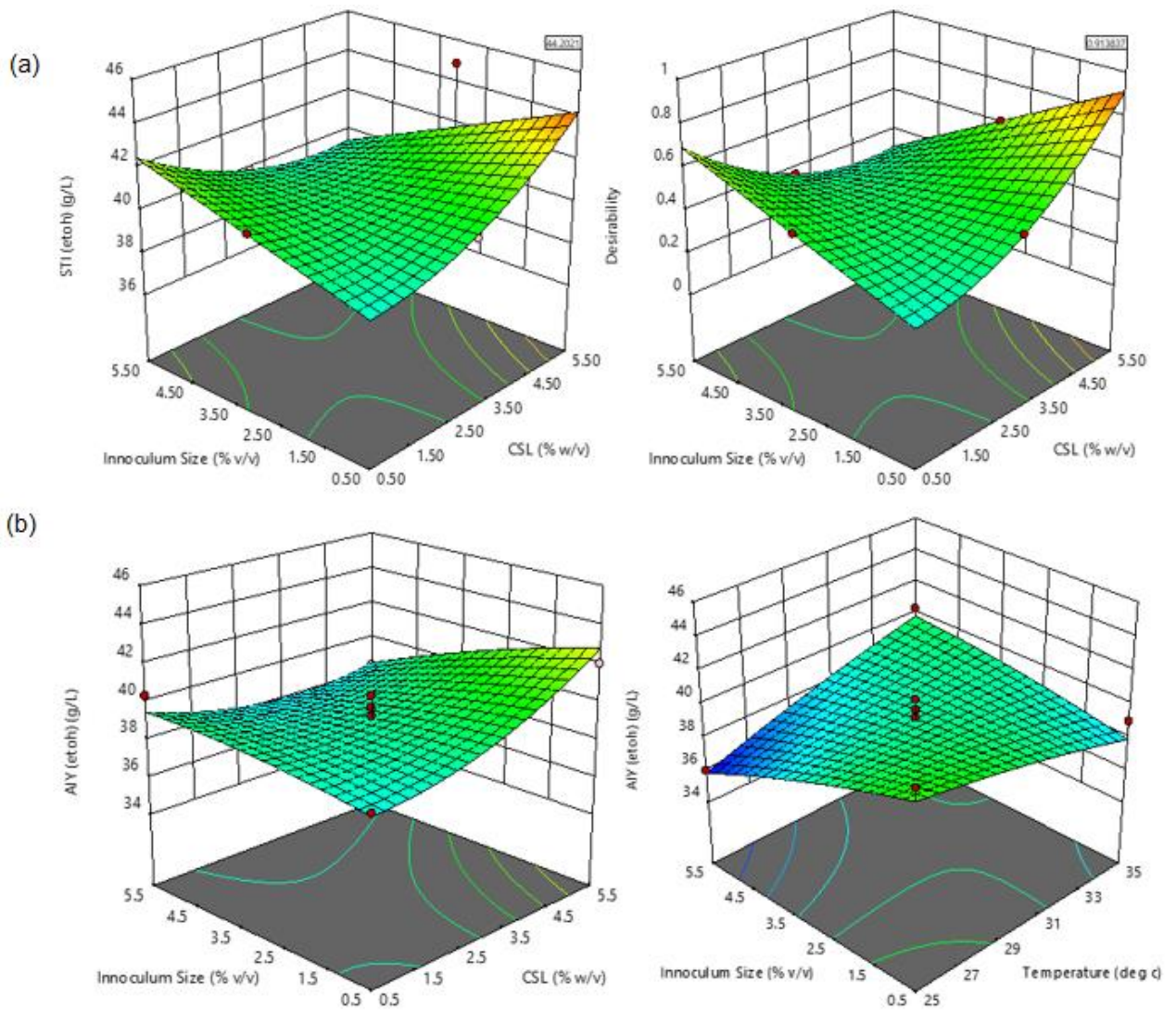
Figure 4.4: Response surface plot and desirability graph showing the relationship between inoculum size and CSL against ethanol concentration using *Saccharomyces* Type 1 strain (ST1) (b) Response surface plot describing the effect of inoculum size, CSL and temperature against ethanol concentration using Anchor Instant Yeast (AIY)

Table 4.4: Test of significance analysis of variance (ANOVA) for ethanol production using two yeast strains

Source	*Saccharomyces* Type 1 Yeast (ST1)					Anchor Instant Yeast (AIY)				
	SS	df	MS	F	*p*	SS	df	MS	F	*p*
Model	39.11	5	7.82	4.070	<0.0001	84.80	20	4.24	2.74	<0.0001
X_1	3.39	1	3.39	1.76	0.1916	10.84	1	10.84	6.99	<0.0001
X_2	6.05	1	6.05	3.15	0.0836	2.15	1	2.15	1.39	0.2503
X_3	-	-	-	-	-	16.28	1	16.28	10.51	<0.0001
X_4	-	-	-	-	-	0.65	1	0.65	0.42	0.5224
X_5	2.61	1	2.61	1.36	0.2505	11.32	1	11.32	7.31	<0.0001
$X_1 X_5$						6.97	1	6.97	4.50	<0.0001
$X_4 X_5$	15.39	1	15.39	8.00	<0.0001					
	-	-	-	-	-	12.75	1	12.75	8.23	<0.0001
X_1^2	11.67	1	11.67	6.07	<0.0001	7.65	1	7.65	4.94	<0.0001
X_5^2	-	-	-	-	-	0.68	1	0.68	0.44	0.5152
Residual	76.87	40	1.92			38.73	25	1.55		
Lack of fit	75.04	35	2.14	5.88	0.0280	35.58	20	1.78	2.82	0.1266
Pure Error	1.82	5	0.37			3.15	5	0.63		
Correlation						123.54	45			
Total	115.97	45								

SS **-** Sum of squares; df -degree of freedom; MS - Mean square, F - Fisher test, *p* - probability value

4.4.4   ANN modeling results for ethanol production

In this present study, a multilayer normal feed-forward with an incremental backpropagation network and sigmoid as transfer function is the preference ANN model used for the ST1 strain, and hyperbolic tangent (Tanh) as transfer function for the AIY strain, which consists of a 5-4-1 and 5-5-1 topology respectively, as shown in Table 4.5. The software default values of neural networks for learning rate and momentum were 0.15 and 0.8 respectively. In the case of the STI strain training data set, RMSE, MSE, R^2, AAD, were 0.2164, 0.04681, 0.9834 and 0.1613% respectively. Whereas for the testing data set, RMSE, MSE, R^2, AAD were 0.1641, 0.02693, 0.9834 and 0.3406% as seen in Table 4.5. Likewise, for the AIY strain training data set, RMSE, MSE, R^2, AAD, were 0.4333, 0.1878, 0.9219, and 0.8260 respectively, whereas for the testing data, RMSE, MSE, R^2, AAD were 0.1952, 0.0381, 0.986, 0.3406, as displayed in Table 4.5. In the comparison of the predicted and observed responses in both training and testing data sets, it is quite feasible that ANN did not only support the known data selected for the training but also had the ability for generalization of unknown data (the testing data set).

This indicates that statistical models resulting from ANN can be used adequately to describe the selected fermentation variables for ethanol production using the two yeast strains.

4.4.5 Comparing RSM and ANN optimization for ethanol production on the two yeast strains

The predicted and experimental ethanol output values of RSM and ANN are presented above in Table 4.3. The statistical measure comparing the whole data set for both RSM and ANN prediction and the experimental value was estimated in Table 4.6. Data obtained from the experiments using AIY appears to perform better than that obtained using ST1 in terms of the relationship between the fermentation control parameters and the ethanol produced. However, the ANN-based approach was better in fitting and estimation capabilities. Having established that, RSM optimization of the process parameters was more accurate when validated against experimental data, as presented in Table 4.7. The better prediction capabilities of ANN were also confirmed by the parity plots between the predicted values and experimental values for the two strains used in ethanol production, as displayed in Figures 4.1(a) and (b).

Table 4.5: Statistical measures and performance of incremental backpropagation algorithm for training, and testing data sets for ethanol production using STI and AIY

Strain	TF	TP	RMSE		MSE		R^2		AAD%	
			Training	Testing	Training	Testing	Training	Testing	Training	Testing
ST1	Sigmoid	5-4-1	0.2164	0.1641	0.04681	0.02693	0.9844	0.9834	0.1613	0.1402
AIY	Tanh	5-5-1	0.4333	0.1952	0.1878	0.0381	0.9219	0.9860	0.8260	0.3406

TF-transfer function; TP-Topology

Table 4.6: Statistical measure comparing the whole data set of RSM and ANN for ethanol production

Strain	RSM				ANN			
	R^2	AAD%	RMSE	MSE	R^2	AAD	RMSE	MSE
ST1	0.34	2.52	1.29	1.68	0.98	0.15	0.19	0.039
AIY	0.94	1.72	0.97	0.84	0.99	0.46	0.29	0.089

Table 4.7: Optimal parameters for glucose fermentation and the resulting ethanol concentration using RSM and ANN

Yeast strain	Tool	CSL (%w/v)	pH	Time (h)	Temperature (°C)	Inoculum (%v/v)	Predicted (g/L)	Validated (g/L)
AIY	RSM	5.36	4.06	12.99	28.14	0.72	45.48	45.36± 0.02
	ANN	0.50	4.99	35.99	34.17	5.49	45.73	40.60±0.01
ST1	RSM	5.50	5.00	26.83	32.68	0.50	44.20	43.38±0.0002
	ANN	0.50	4.99	36.00	34.17	5.49	45.73	41.30±0.001

4.5   Conclusion

CSL can be used as a lower-cost nutrient source in ethanol fermentation using ST1 and Anchor Instant Yeast (AIY) to replace more expensive yeast extract (YE). CSL can be used for nutrient-enhanced ethanol production in a shorter fermentation time interval compared with YE. These results show that the optimization methodologies described were effective in establishing the amount of CSL needed for scale-up of production. For ST1 strain, the optimal condition values established for the ethanol concentration using RSM were CSL of 5.5 (% w/v), pH of 5, time of 27 h, temperature of 33 °C, inoculum size of 0.5% v/v and with a predicted concentration of 44.20 g/L volume fraction, which was experimentally validated as 43.38 ± 0.0002 g/L. The optimal condition values established for ethanol concentration using ANN were CSL of 0.5 (% w/v), pH of 5, time of 36 h, temperature of 34 °C, inoculum size of 5.49% v/v, and predicted concentration of 45.73, which was also validated experimentally as 41.30 ± 0.001 g/L. For AIY strain, the optimal condition values established for the ethanol concentration using RSM were CSL of 5.36 (% w/v), pH of 4, time of 13 h, temperature of 28 °C, inoculum size of 0.72% v/v and with predicted concentration of 45.48 g/L, which was experimentally validated as 45.36 ± 0.02 g/L. The optimal condition values established for ethanol concentration using ANN were CSL of 0.5 (% w/v), pH of 5, time of 36 h, the temperature of 34 °C, inoculum size of 5.49% v/v, which was also validated experimentally as 40.6 ± 0.01 g/L. The training algorithm used in this study was multi-layer normal feed-forward incremental back-propagation. Comparing the efficacy of ANN with RSM in the ability to predict the ethanol concentration in this study, it was demonstrated that ANN was better than RSM in both data fittings and estimation capabilities but the RSM validation result was more accurate than ANN. This could be attributed to the small concentration of CSL predicted by ANN which could not favor the ethanol concentration. The study demonstrated that the commercial AIY yeast strain can perform almost the same as the industrial ST1 yeast strain, and CSL was a good nitrogen supplement for ethanol production.

Chapter 5 STATISTICAL OPTIMIZATION OF ACETOIN PRODUCTION USING CORN STEEP LIQUOR AS A LOW-COST NITROGEN SOURCE BY *BACILLUS SUBTILLIS CICC 10025*

5.1 Introduction

The increasing trend in the consumption of healthy food and the growing demand for natural products by consumers coupled with the price margin between synthetic and natural flavors has motivated the need for the bioproduction of natural flavors. Furthermore, the new United Nations sustainable development goals, particularly goals 3 and 12, state the need for the good health and well-being of the consumer and the need to ensure the sustainable production and consumption patterns of a food product (Bengtsson et al., 2018). Acetoin (3-hydroxy-2-butanone or acetyl methyl carbinol) is widely used in the food industry as a flavor enhancer, giving a buttery taste (Bae et al., 2016). It can also be used as a building block for various chemicals such as alkyl pyrazines, diacetyl, and acetyl butanediol (Bae et al., 2016; Zhang et al., 2012). Currently, most of the commercial acetoin is produced by chemical synthesis from 2, 3-butanedione and 2,3-butanediol. However, the use of such chemically-derived acetoin is restricted in food and cosmetic industries because of safety concerns (Li et al., 2017a).

The production of acetoin by microbial fermentation has attracted increasing interest due to its safety and environmental friendliness (Luo et al., 2014; Li et al., 2017a). Intensive efforts have been taken to enhance microbial production of acetoin, including screening of high-yield acetoin producing strains (Zhang et al., 2011; Xiao et al., 2012), metabolic engineering breeding (Nielsen et al., 2010; Xu et al., 2015; Sun et al., 2012) and fermentation optimization (Teixeira et al., 2002; Tian et al., 2014; Sun et al., 2012). The price of chemically generated acetoin (racemic) is over $16.2/kg, and biologically generated dietary acetoin can be twice as expensive (Liu et al., 2015). One of the chemicals that can use acetoin as a building block is 1,3-butadiene (BD), which has a worldwide market of 11 metric tonnes per year (Roncal et al., 2017). However, acetoin is only a minor by-product of 2,3-butanediol production, and its accumulation usually needs complex and expensive growth factors. Apart from carbon, which is the feedstock for most fermentation, nitrogen is another significant nutrition compositional element needed for active microbial growth necessary for the fermentation of most targeted bioactive compounds (Tian et al., 2016).

Previous studies have shown the potential of low-cost media and complex nitrogen sources like urea, peptone, yeast extract, beef extract, and soya bean (Yang et al., 2013; Tian et al., 2016; Xiao et al., 2007) for acetoin production, however, there is a dearth of information on the validation of its optimized condition. Nitrogen as a source is one of the main contributors

to the total material cost of commercial acetoin production. Therefore, replacing a costly nitrogen source with less expensive ones for acetoin production may improve the economics of the process. Corn steep liquor (CSL) has been identified as a potential nitrogen source in biochemical industries and as a good substitute for other expensive complex media. It is a major by-product found during corn starch processing, as well as a low-cost source of proteins, amino acids, minerals, vitamins and trace elements (Figure 5.1).

Also, it can be used as a rich and effective nutritional substitute for expensive complex media like yeast extract, beef extract and peptone in fermentations (Tang et al., 2006; Edwinoliver et al., 2009; Seo et al., 2009). Yang et al. (2013) reported that when a high concentration of CSL was supplemented in acetoin production, cell growth was improved and acetoin increased by threefold; his reports show that productivity of acetoin was improved by a high concentration of CSL (Yang et al., 2013). Another study reported that corn steep liquor had negative effects on the screening of medium components for acetoin production when set at lower concentrations using a newly isolated *Paenibacillus polymyxa* CS 107 (Zhang et al., 2012). The work by Xu et al. (2011) also affirmed that CSL and yeast extract had a positive influence on acetoin when screened among other nitrogen sources. All these authors reported differently on the optimized condition of CSL as a nitrogen source and mostly combined its usage with other costly nutrients like yeast extract and beef extract.

This necessitated the need for an optimization tool like response surface methodology (RSM). RSM is an empirical modeling technique used to establish the relationship between a set of controllable experimental factors and the observed results. It defines the effect of the independent variables, alone or in combination, on observable output (response) in chemical or biological processes. In addition to analyzing the effects of the independent variables, this methodology also generates a mathematical model. The applicability of RSM to optimization studies has been demonstrated successfully (Pereira et al., 2010). The view of this study is to develop a low-cost fermentation medium that precludes redundant nutritional supplements and minimizes the costs associated with industrial acetoin fermentation.
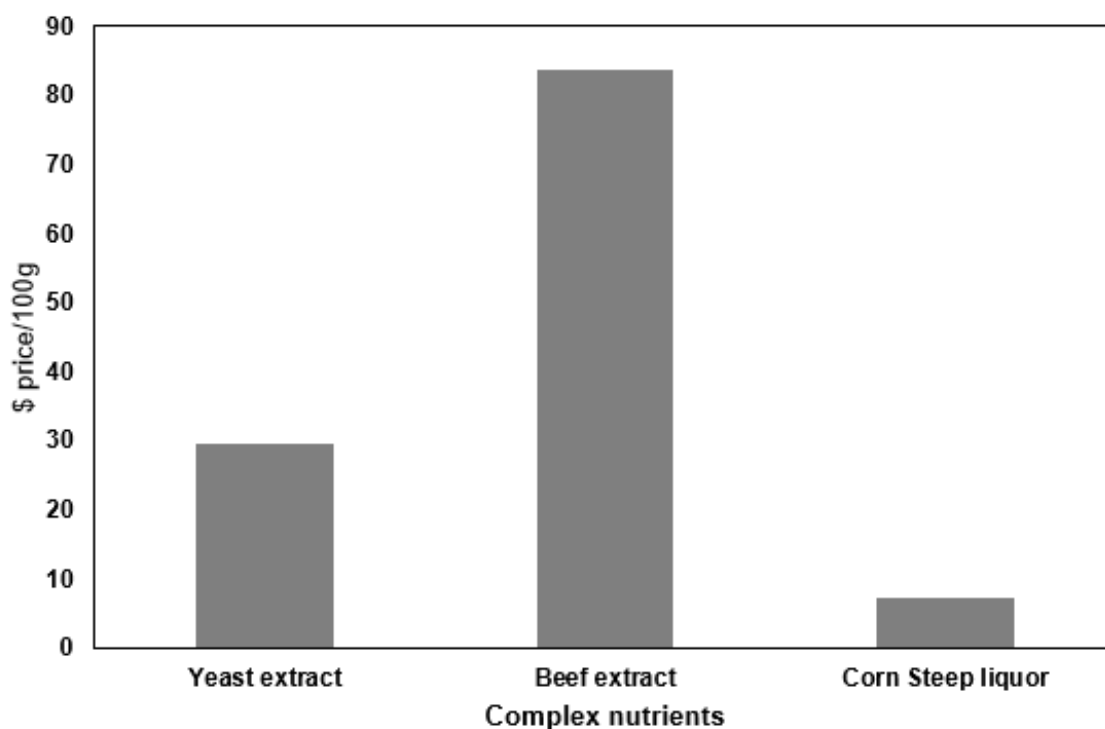
Figure 5.1: Graphical illustration of complex nitrogen nutrients and price margin

5.2 Objective

This chapter focuses on the use of corn steep liquor as a cheap replacement for other expensive complex nutrients by optimizing CSL and other fermentation controlling parameters, using the RSM technique for acetoin production.

5.3 Methodology

Studies were carried out on acetoin production by considering the influence of three controlling parameters which were; glucose as carbon source, corn steep liquor as nitrogen source and inoculum size of the synthesizing microbe. The experimental design and method of analysis have been described in detail in sections 3.2 and 3.6 as in Chapter 3.

A second-order mathematical equation, including all interaction terms, was used to calculate the predicted response of acetoin (see Equation 4.1).

5.4 Results and Discussion

5.4.1 Preliminary evaluation of complex nitrogen sources on AC production

Figure 5.2 (a) shows that CSL supported the rapid utilization of the reducing sugar (from 150 to 84 g/L) within the first 60 hours of fermentation (Sreenath and Jeffries, 1996) and the

resultant acetoin and biomass growth attained its maximum during this period. The maximum biomass growth was fairly constant at approximately 8 g/L until 144 hours before declining. Likewise, the maximum acetoin concentration of 7 g/L was attained after 60 hours of fermentation and later entered the decline phase. The decline in the acetoin concentration could be attributed to the glucose complete metabolism in the fermentation medium. Although the biomass concentration remained constant after 60 hours of fermentation, it can be assumed that the energy derived from reducing sugar metabolism was channeled towards cell maintenance since biomass growth remains constant and acetoin was still produced till the fermentation lapsed. The addition of CSL reduces the fermentation time and enhanced the growth and fermentation of the strain.

On the other hand, when yeast extract was used as the nitrogen source in the fermentation media as depicted in Figure 5.2(b), the results showed a rapid consumption of reducing sugar as compared to Figure 5.2(a) where CSL was used as a nitrogen source. More than 97% of the reducing sugar was converted (during the first 84 hours of fermentation), while the acetoin concentration was approaching a maximum and the biomass growth was constant, at 8.6 g/L and 7.7 g/L respectively. The wide range of amino acids, peptides, vitamins, inorganic salts, and carbon in growth media of yeast extract supported the biomass growth and rapid sugar utilization (Costa et al., 2002).

As shown in Figure 5.3(a), beef extract was used in place of CSL and yeast extract as discussed previously (Figure 5.2). Beef extract is a mixture of peptides and amino acids, nucleotide fractions, organic acids, minerals, and some vitamins. Its function can, therefore, be described as complementing the nutritive properties of peptone by contributing minerals, phosphates, energy sources and those essential factors missing from peptone (Prokofeva et al., 2000). The glucose almost depleted after 96 h until the elapsed fermentation time. It can, therefore, be deduced from the study that beef extract supported the utilization of glucose consumption, as more than 98% of the glucose was consumed within 96 h of fermentation. To see the clear difference of the complex media considered above, the acetoin fermentation was done without nitrogen sources (Figure 5.3 (b)) but with the simple salts in the fermentation media.

Maximum acetoin of about 5.2 g/L was obtained during the first 12 h of the fermentation, after which there was no significant change in the biomass growth from that period until the elapse time of fermentation. About 50% (72.53 g/L) of sugar concentration was not consumed till the fermentation time-lapse of 168 h, and the slow consumption of glucose can be traceable to the lack of rich nitrogen sources which were needed as a nutritional supplement (Betiku and Taiwo, 2015).
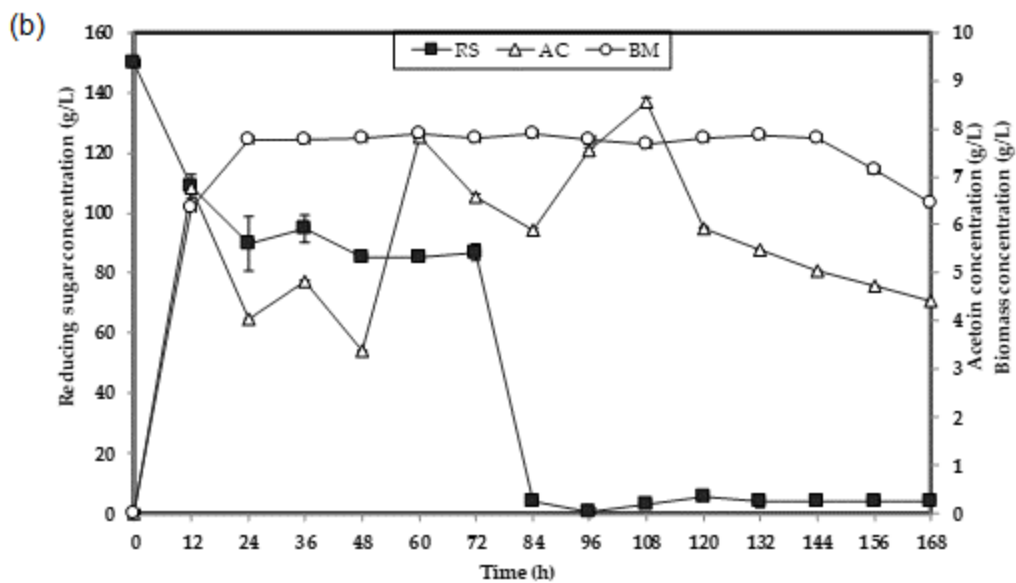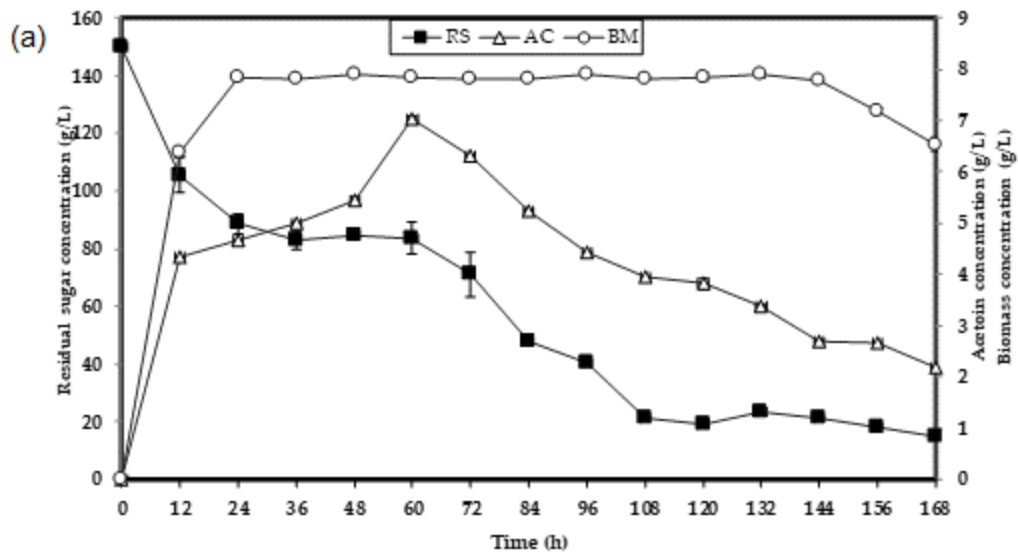
Figure 5.2: (a) Plots of AC fermentation using corn steep liquor as a nitrogen source (RS - Reducing sugar; AC - Acetoin; BM - biomass) (b) Plots of AC fermentation using yeast extract as a nitrogen source (RS - Reducing sugar; AC - Acetoin; BM - biomass)
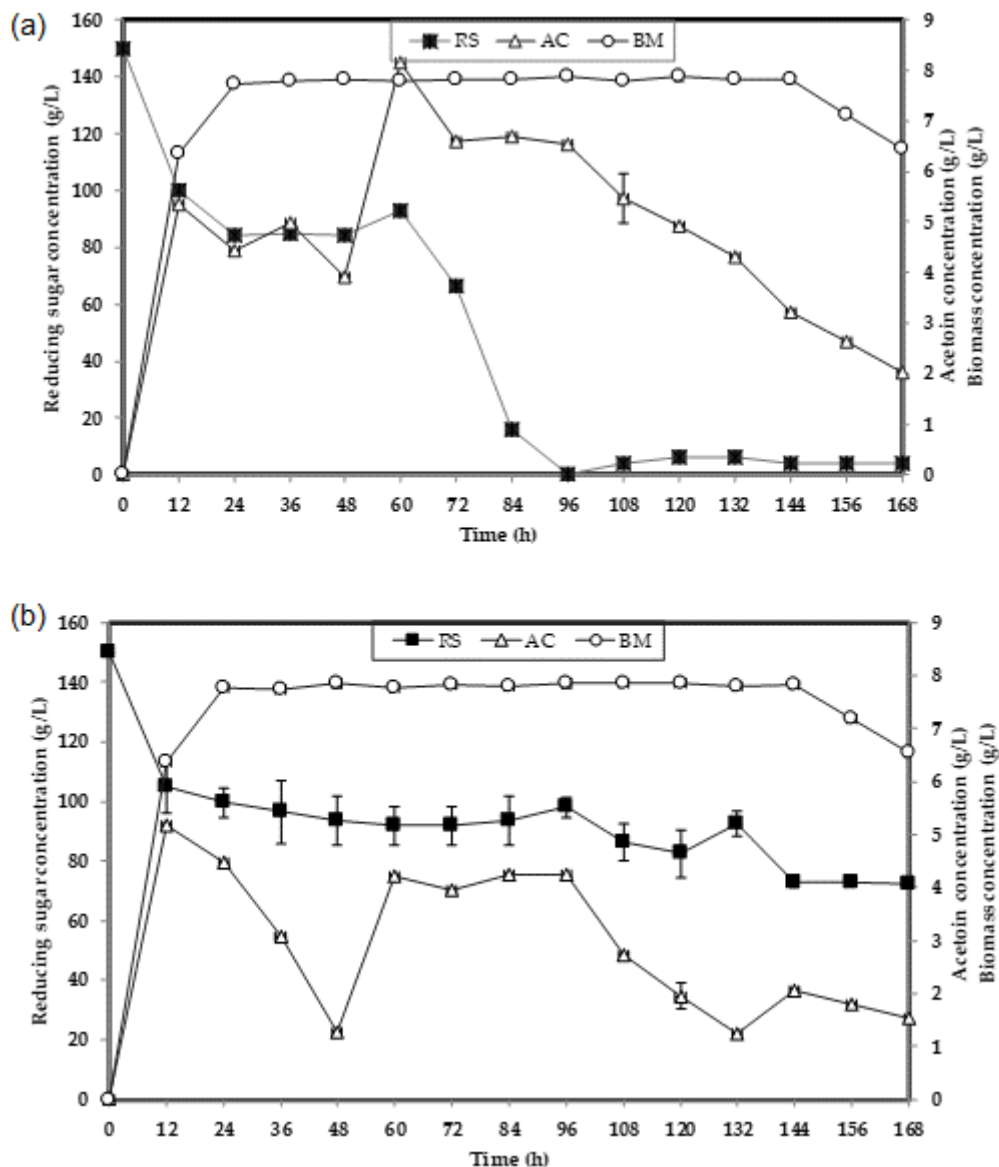
Figure 5.3: (a) Plots of AC fermentation using the beef extract as a nitrogen source (RS - Reducing sugar; AC - Acetoin; BM - biomass) (b) Plots of AC fermentation using none of the complex media as the nitrogen source (RS - Reducing sugar; AC - Acetoin; BM - biomass)

5.4.2 Metabolism of corn steep liquor on AC production

It was observed in the preliminary evaluation of nitrogen sources discussed earlier (Figure 5.2 and 5.3) that corn steep liquor support glucose in bioconversion to acetoin at a short time interval when compared to other nitrogen sources. Therefore, the fermentation time interval of acetoin was reduced from the initial 168 h (Figure 5.2 and 5.3) to 48 h (Figure 5.4) and a sample was taken at 2 h intervals, to investigate the biomass growth, utilization of glucose and the acetoin accumulation when corn steep liquor was used as a nitrogen source. The results show that the accumulated acetoin and the biomass growth were already at a peak of 7.03 and 7.83 g/L respectively, in the first 36 h of fermentation (Figure 5.4). The rapid utilization of

reducing sugar (from 150 to ~ 66 g/L) within 36 h of fermentation was due to high amino acids and polypeptides, which were excellent sources of nitrogen in corn steep liquor. This has been reported to support the growth of most microorganisms (Liggett and Koffler, 1948). CSL comprises a mixture of reducing sugars that contribute to the nutritional growth of the bacteria as confirmed from this study that the biomass growth increased steadily (7.7-7.8 g/L) from 18 h till 46 h and supported by Xiao et al. (2013). It can be affirmed from the findings that corn steep liquor hastens bioproduction of acetoin at a short time interval and this may enhance the cost-effectiveness of acetoin production to make the large-scale medium.
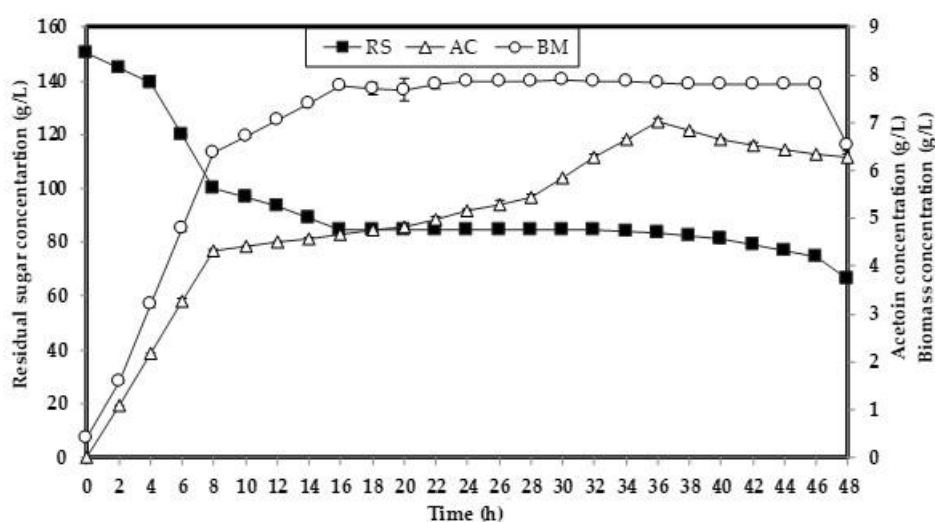
Figure 5.4: Acetoin growth profile using corn steep liquor at a short fermentation time interval (RS - Reducing sugar; AC - Acetoin; BM - biomass)

5.4.3 Optimization of AC production using response surface method

After the preliminary studies, response surface methodology coupled with Box-Behnken design was used for the optimization of the fermentation process with respect to glucose concentration, CSL concentration, and inoculum size with a view to maximizing the AC production. Table 5.1 shows the experimental conditions investigated together with the observed and predicted values. The data were fitted using the following second-order mathematical Equation (5.1):

$$Y = 7.81 - 1.37X_1 + 2.56X_2 - 0.61X_3 - 0.17X_1X_2 - 0.57X_1X_3 - 1.02X_2X_3 - 1.25X_1^2 - 0.50X_2^2 - 1.09X_3^2 \quad (5.1)$$

where Y is the acetoin (AC) produced in g/L and X_1 is glucose concentration, X_2 is corn steep liquor, X_3 is Inoculum size. The residuals between the observed and predicted (Table 5.1) values in this work revealed a good fit of the equation as shown by the parity graph which is a measure of agreement between the observed and predicted values (Figure 5.5). These

observations implied that the model developed for the fermentation process adequately described the actual relationship among the selected factors.

The three-dimensional graph and contour plot depicted in Figure 5.6 shows the relationship between corn steep liquor and glucose concentration when acetoin production attained its optimum (10.70 g/L). Further, the fact that the p-values of the model terms were significant at $p < 0.05$ (Table 5.2) and the observed low p-value of 0.0001, together with the corresponding F-value of 11.09 showed that the model obtained was significant. The F-value and p-value show that there were no significant effects of each term in the model (Dhillon et al., 2012). Table 5.2 displays the test of significance and ANOVA of the regression equation results. The coefficient of determination (R^2) was used to assess the goodness of fit of the regression equation. R^2 of 0.930 of the models demonstrated a good correlation between the observed and predicted values. It showed that 93% of sample variation for AC produced is attributed to the independent factors and 0.70% of the total variations were not described by the model (Betiku et al., 2014; Betiku and Taiwo, 2015). The adjusted R^2 of 0.90 proved that the model was significant. It has been suggested that R^2 should be less or equal to 80% for the good fit of a model (Joglekar and May, 1987).

Table 5.1: BBD of three independent factors for AC production including the coded levels of each parameter

Run	X_1 (g/L)	X_2 (% w/v)	X_3 (%v/v)	Observed AC (g/L)	Predicted AC (g/L)	Residuals
1	0	0	0	7.68	7.81	-0.13
2	1	0	-1	4.32	5.28	-0.96
3	1	-1	0	2.47	2.29	0.18
4	0	0	0	7.68	7.81	-0.13
5	0	-1	-1	3.66	3.24	0.42
6	0	1	-1	10.69	10.41	0.28
7	-1	0	-1	7.14	6.88	0.26
8	0	1	1	6.49	7.15	-0.66
9	-1	0	1	8.00	6.8	1.20
10	1	0	1	2.91	2.92	-0.01
11	0	0	0	7.68	7.81	-0.13
12	0	-1	-1	3.53	4.06	-0.53
13	1	1	0	7.88	7.08	0.80
14	-1	1	0	9.74	10.16	-0.42
15	0	0	0	7.70	7.80	-0.11
16	0	-1	0	5.72	4.74	0.98
17	-1	-1	0	3.65	4.69	-1.04

Table 5.2: Test of significance for every regression coefficient and ANOVA

Factor	Sum of squares	df	Mean square	F-value	*p*-value
X_1 (Glucose)	14.99	1	14.99	16.51	0.0048
X_1^2	6.52	1	6.52	7.18	0.0315
X_2 (CSL)	57.85	1	57.85	63.73	0.0001
X_2^2	1.06	1	1.06	1.17	0.3160
X_3 (Inoculum Size)	2.98	1	2.98	3.28	0.1131
X_3^2	4.98	1	4.98	5.48	0.0517
X_1X_2	0.12	1	0.12	0.13	0.7317
X_1X_3	1.29	1	1.29	1.42	0.2724
X_2X_3	4.14	1	4.14	4.56	0.0701
ANOVA					
Model	90.63	9	10.07	11.09	0.0022
Error	3.000E-004	3	1.000E-004		
Total sum of squares	96.99	16			
$R^2 = 0.93$,					
Adjusted $R^2 = 0.90$					
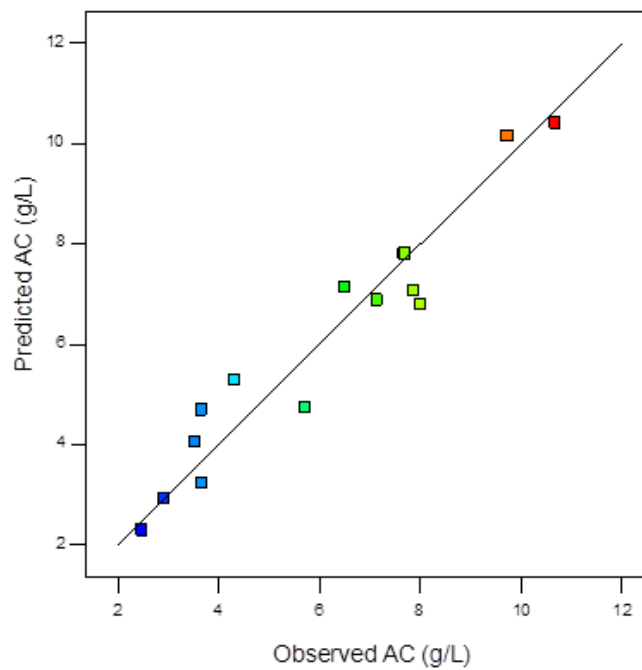
Figure 5.5: Parity plot of acetoin production
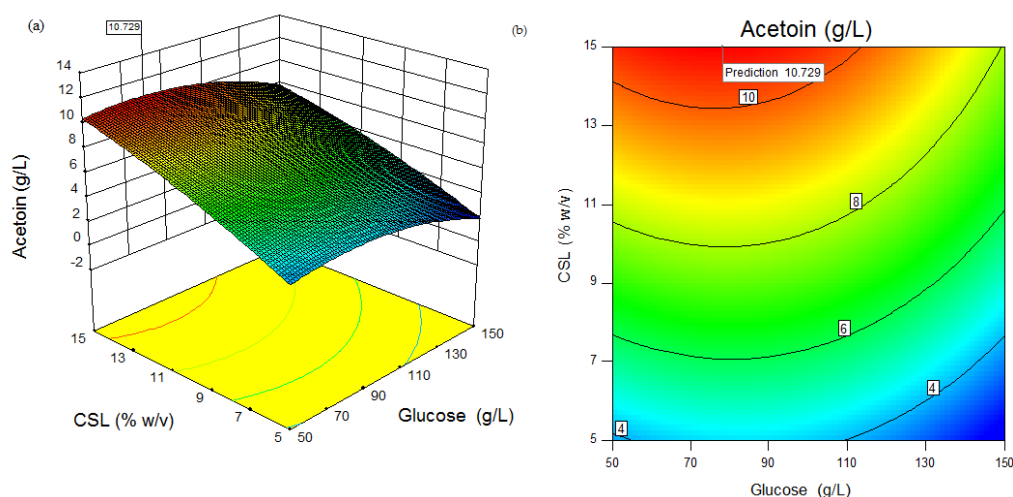
Figure 5.6: Contour and response surface plots (a) The response surface plot and (b) contour plot showing the effects of corn steep liquor and glucose on acetoin production

5.4.4 Model validation

The optimum values of the three factors selected for the fermentation process were obtained by solving Equation (5.1) using the Design-Expert software package (version 10.0). The optimal condition was statistically predicted as glucose concentration of 78.40 g/L, CSL of 15.00% w/v and inoculum size of 2.70% v/v. Under this condition, the AC concentration predicted was 10.73 g/L. To validate the model, the optimal condition values were applied to three independent experimental replicates and the average value of AC produced was 10.70 ± 0.1 g/L. The correlations between predicted and experimental values after optimization imply the validity of the response model and the existence of an optimum point (Ashengroph et al., 2013).

The bar chart (Figure 5.7) is a graphical view for each optimal solution showing the desirability of every dependent and independent factor with combined value. Independent factors were shown with red bars while the dependent response and combined values were displayed in blue. The desirability result was accurate as it falls within the acceptable value ranging between 0.8 to 1 (Sarteshnizi et al., 2015). The variance inflation factor (VIF) obtained in this work showed that the center points were orthogonal to all other factors in the model (Table 5.3).
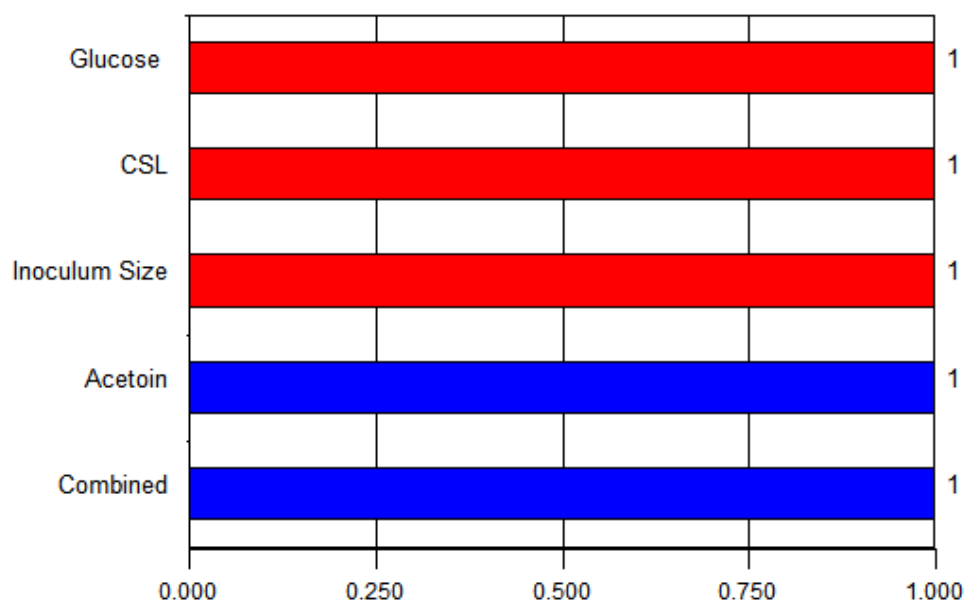
Figure 5.7: Numerical optimization bar graph of AC production

Table 5.3: Regression coefficients and significance of response surface quadratic

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	7.81	1	0.47	6.71	8.91	
X_1 (Glucose)	-1.37	1	0.34	-2.17	-0.57	1.00
X_2 (Corn steep liquor)	2.56	1	0.32	1.80	3.32	1.02
X_3 (Inoculum Size)	-0.61	1	0.34	-1.41	0.19	1.00
X_1X_2	-0.17	1	0.48	-1.30	0.96	1.00
X_1X_3	-0.57	1	0.48	-1.69	0.56	1.00
X_2X_3	-1.02	1	0.48	-2.14	0.11	1.00
X_1^2	-1.25	1	0.47	-2.35	-0.15	1.01
X_2^2	-0.50	1	0.47	-1.60	0.60	1.01
X_3^2	-1.09	1	0.47	-2.19	0.011	1.01

5.5   Conclusion

The feasibility of corn steep liquor to replace yeast extract and beef extract which are expensive nutrient sources in acetoin fermentation was investigated. Corn steep liquor, a low-cost nitrogen source, competes with other complex nutrients (yeast extract and beef extract) for acetoin production, and statistical optimization was carried out in batch fermentation. The model that best described the AC fermentation process was a quadratic model with R^2 of

0.930. The most significant positive factor for the process was glucose concentration and corn steep liquor, while inoculum size was an insignificant factor in the AC fermentation. The optimal condition predicted for the three independent factors was glucose concentration of 78.40 g/L, CSL of 15.00% w/v, and inoculum size of 2.70% v/v, which were validated experimentally with AC concentration of 10.70 ± 0.1 g/L. Based on these results, it can be concluded that the optimization methodologies developed were effective in ascertaining the amount of CSL required for commercial acetoin production, and that reduces the cost, time and effort associated with experimental techniques.

Chapter 6   BIOTRANSFORMATION OF FERULIC ACID INTO VANILLIN USING *BACILLUS AMYLOLIQUEFACIENS SUBSP* IN A LOW-COST MEDIA: MODELLING AND OPTIMIZATION

6.1   Introduction

Vanillin (4-hydroxy-3-methoxy benzaldehyde) is the main component of vanilla extract and is used universally as flavors in food and pharmaceuticals, in cosmetics, and the fine chemical industry (Chen et al., 2017b). Natural vanillin is obtained from *Vanilla planifolia* but the duration of cultivation, harvesting, and process of vanilla plants can be extended over a long time (Priefert et al., 2001). Although vanillin can be chemically synthesized using starting material like guaiacol and glyoxylic acid, the petrochemical origin of this substrate has been a great disadvantage (Carlquist et al., 2015). Further, the wide price variation between the natural and synthetic vanillin, and the health benefits and the limitation of meeting market demand through vanilla pods can be addressed through bioprocessing. Bioprocessing might, therefore, provide the basis for the establishment of flavoring industries that produce vanillin by bioconversion of natural sources (Zamzuri et al., 2014). Vanillin produced by the microbial biotransformation of ferulic acid has been labeled a natural product because it originates from biological sources that are transformed by living organisms and enzymes.

The bioproduction of vanillin has gained continuous research attention in areas such as reactor design (Chen et al., 2017b; Wu et al., 2008; Yan et al., 2016), choice of carbon sources (Tilay et al., 2010), isolation and screening of microorganisms (Zamzuri et al., 2014), genetic manipulation (Di Gioia et al., 2011), separation and recovery of products from the broth (Mota et al., 2016) and media manipulation (Kaur and Chakraborty, 2013; Tilay et al., 2010). Concerted efforts have been made to derive vanillin from renewable substance and common agricultural waste residue such as eugenol, isoeugenol, sugars, ferulic acid, sugar beet pulp, wheat straw, aromatic amino acid, vanillic acid, coconut husk, and groundnut shell (Yan et al., 2016; Kaur and Chakraborty, 2013). Among these substances, the potential of ferulic acid as a precursor for vanillin production has been reported (Paz et al., 2018; Yan et al., 2016). It is a low-cost feedstock for the production of high demand bioactive compounds when extracted from lignocellulose biomass waste. It is a phenolic compound found in the cell wall of lignocellulosic materials, where it is covalently linked with a variety of carbohydrates such as a glycoside conjugate, ester or amide, with inherent ability to release ferulic acid after pretreatment (Paz et al., 2018).

The biotransformation of ferulic acid into vanillin depends on several working parameters, which are temperature, incubation period, pH, substrate type and concentration, nitrogen

sources, choice of microbes, dissolved oxygen concentration and media manipulation (Barghini et al., 1998; Chen et al., 2017b; Kaur and Chakraborty, 2013). However, there are limited studies directed towards reducing the cost of nitrogen sources even though they might be significant in the production cost of vanillin. In this present study, *Bacillus amyloliquefaciens subsp* CICC 10025 was used for biotransformation of ferulic acid using corn steep liquor as the sole inorganic nitrogen source for vanillin production. More specifically, this study focuses on the media formulation of cheap microbial media to support and fulfill nutritional requirements for biotransformation of ferulic acid into vanillin, thus establishing the optimum conditions that might be relevant for the scale-up of the bioprocess.

6.2   Methodology

A preliminary experiment was carried out before optimization studies to establish the effect of pH, ferulic acid concentration and corn steep liquor on vanillin bioproduction. The detailed experimental method has been reported in Chapter 3, sections 3.3 and 3.6. A second-order polynomial equation, including all interaction terms, was used to calculate the predicted response of vanillin concentration (see Equation 4.1).

6.3   Results and discussion

6.3.1   Effect of media formulation on ferulic acid bioconversion to vanillin

The biotransformation was evaluated using two media to explore and compare vanillin production. The nutrient compositions (M1 and M2) were mentioned in chapter 3, section 3.3. The time course of substrate consumption (FA-ferulic acid), metabolite (vanillic acid) and product (vanillin) accumulation from the two media are illustrated in Figure 6.1 (a) and (b). The figures indicated a rapid depletion of FA and a significant increase in vanillin and vanillic acid concentration respectively. A maximum vanillin concentration of 396 mg/L was reached in M1 that contained yeast extract and peptone, while M2 that contained corn steep liquor performed better in bioconversion of FA into vanillin at a high yield of 668.39 mg/L. It can be seen in Figure 6.1(b) that less vanillic acid, which is a side product, was formed compared to Figure 6.1(a). The better yield obtained in the media containing CSL was probably due to the presence of certain nutrients that were absent in the other formulation (Amartey and Jeffries, 1994).

Chen et al. (2016) stated that medium formulation and composition have a significant effect on biotransformation as it can infuse energy into microorganisms and support growth with the substrate concentration for a high yield of the targeted product. Therefore, M2 that contains corn steep liquor was used for further studies.
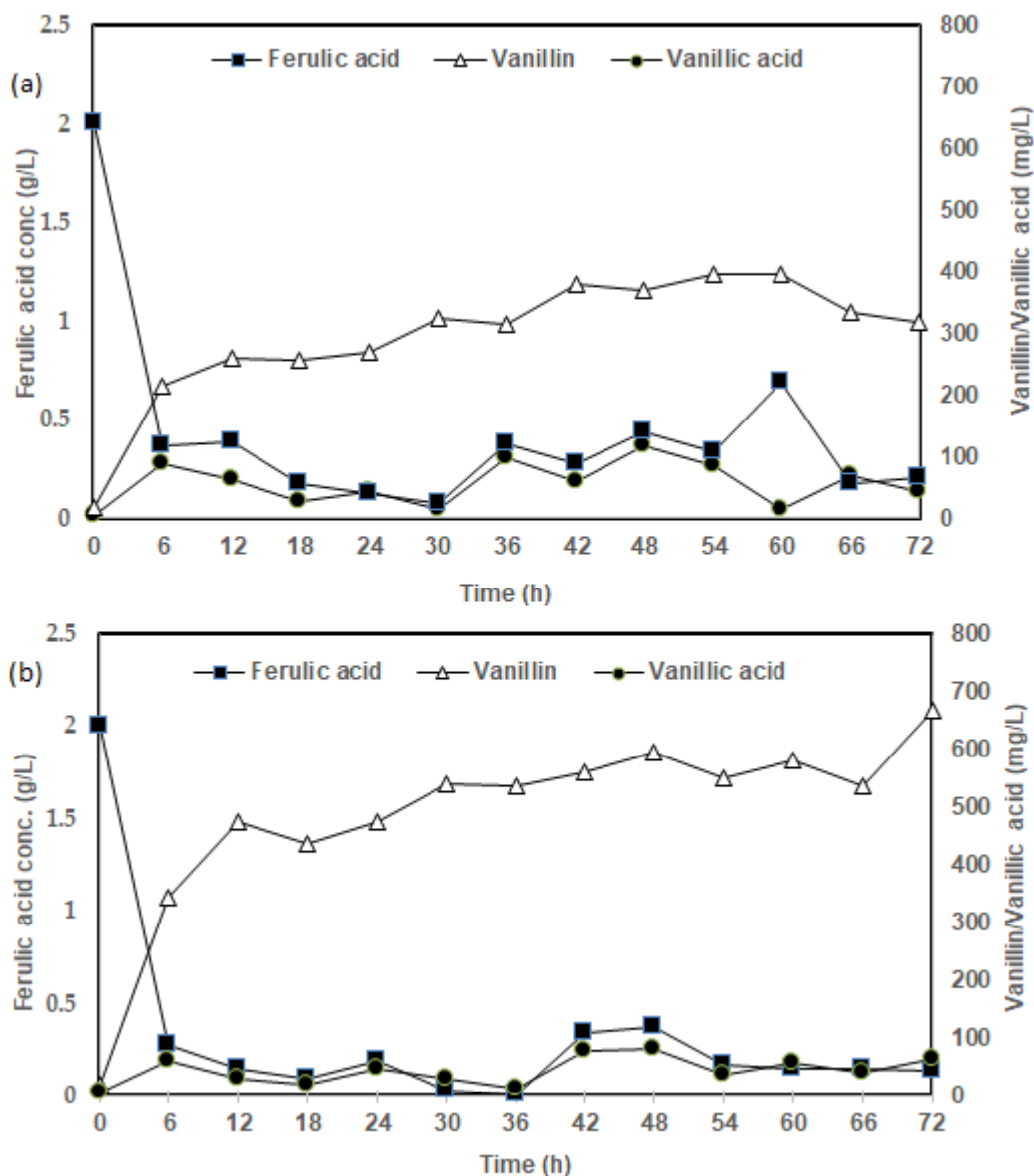
Figure 6.1: Biotransformation of ferulic acid into vanillin using two different fermentation media formulation (a) Media 1 (b) Media 2

6.3.2 Effect of corn steep liquor concentration on ferulic acid bioconversion to vanillin

The effect of different CSL concentrations (3,6,9,12,15) in g/L was investigated on biotransformation of FA into vanillin while other factors were kept constant (FA 2.0 g/L, pH 9.0, temperature 35 °C, agitation 150 rpm, time 72 h), as depicted in Figure 6.2 (a). It was observed that as the concentration of CSL increased, the vanillin concentration reached the maximum level. The peak concentration of vanillin (464.96 mg/L) was observed at 15 g/L of CSL. When the bioconversion was conducted without CSL, the vanillin produced was minimal (52.47 mg/L). These findings were confirmed by Tilay et al. (2010) who reported that CSL supported maximum vanillin production (30% molar

yield) more, compared with other nitrogen sources like yeast extract, beef extract, and peptone.

6.3.3 Effect of ferulic acid concentration on vanillin production

Experiments were carried out to evaluate the effect of initial ferulic acid concentration on its biotransformation to vanillin. Experiments were carried out at pH 9.0, temperature 35 °C, agitation 150 rpm, time 72 h, CSL 13 g/L and different ferulic acid concentration 0.5, 1.0, 1.5, 2.0 and 2.5 g/L as shown in Figure 6.2(b). Control of the experiment was also set without ferulic acid addition in the bioconversion. It can be observed in Figure 6.2(b) that as the concentration of initial FA increases, the vanillin concentration increases accordingly. Although, literature as reported that ferulic acid of high concentrations could be toxic for microbial cells and could inhibit the growth of microbes (Di Gioia et al., 2011). However, the media formulation enhanced the bioconversion process at increased ferulic acid concentration.

6.3.4 Effect of pH concentration on ferulic acid bioconversion to vanillin

The effect of pH (5, 8, 8.5, 9.0, 9.5) on the ferulic acid (FA) bioconversion to vanillin is shown in Figure 6.2(c) when the temperature was 35 °C, agitation 150 rpm, CSL 13 g/L, 2.0 g/L FA concentration and fermentation time of 72 h. The optimum pH was found to be 9.5, at which the vanillin concentration was maximum (499 mg/L). This corroborates previous findings (Paz et al., 2018; Yan et al., 2016) that acidic media did not favor transformation of FA into vanillin and that pH range of 8.0-9.5 favours maximum vanillin concentration. It is imperative to know the optimal pH for the activity of a chosen microorganism for a specific biochemical process as variation in pH can affect both the ionic state of substrate and enzymes involved in biotransformation (Yan et al., 2016).
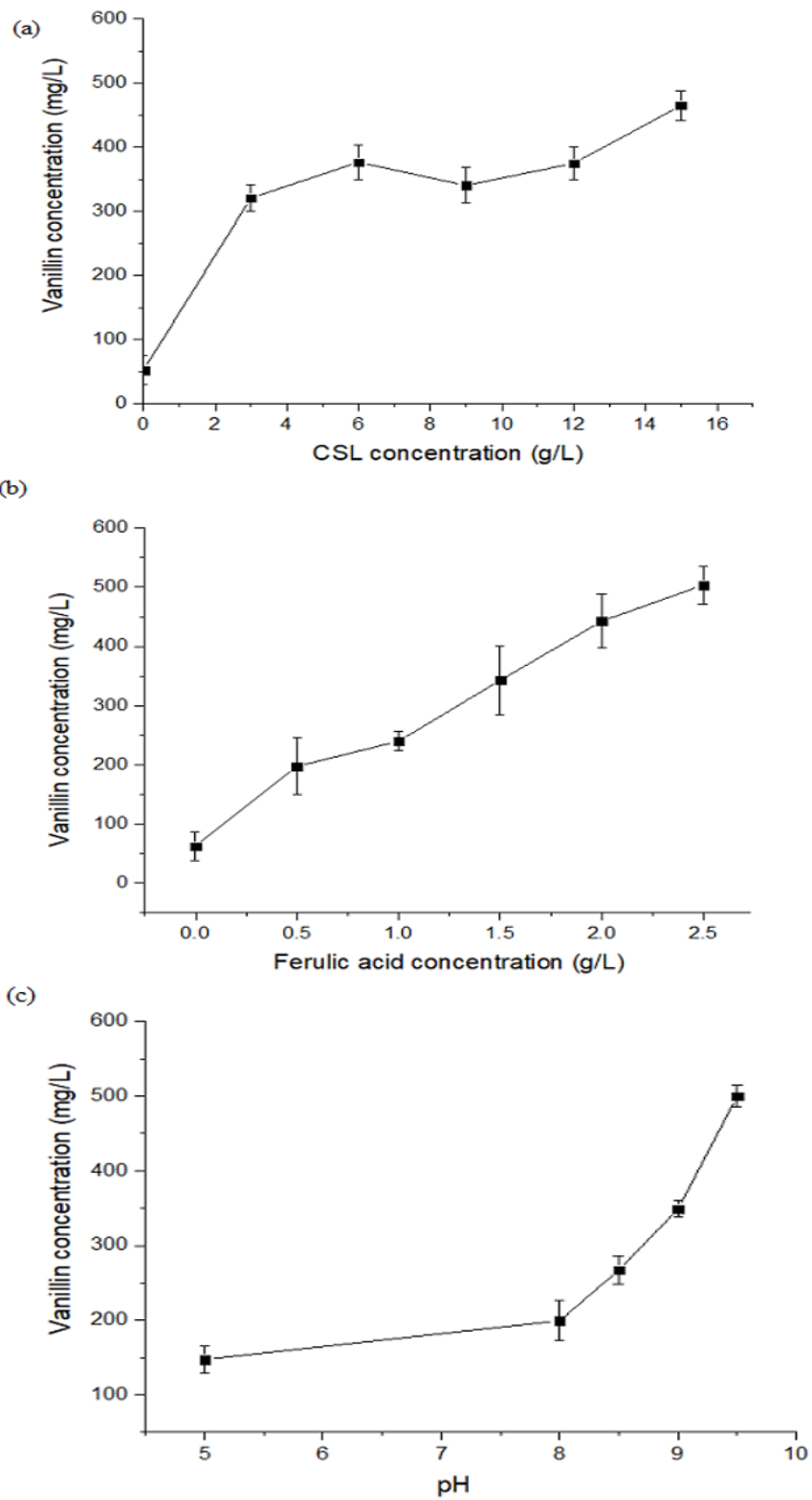
Figure 6.2: Plots showing (a) effect of corn steep liquor concentration, (b) effect of ferulic acid concentration (c) and pH on vanillin production

6.3.5 Modeling and optimization of vanillin production

Response surface methodology (RSM) with a Box-Behnken design (BBD) was used to statistically and mathematically model the bioconversion study to establish a relationship between the chosen variables for vanillin production. The various combinations of experimental conditions with their response values are given in Table 6.1. The observed data (Table 6.1) were subjected to an ANOVA and were fitted by Equation. 6.1 to evaluate these parameters on vanillin production. A quadratic polynomial regression equation was then obtained as:

$$\text{Vanillin concentration} = -8946.44 - 7.71X_1 - 76.13X_2 + 1937.25X_3 - 3.33X_1^2 - 32.50X_2^2 - 105X_3^2 + 4.17X_1X_2 + 5.83X_1X_3 + 23.50X_2X_3$$

(6.1)

The adequacy of the model was evaluated by several indicators, viz. model significance (F and P-value), coefficient of determination (R^2), the coefficient of variation (CV), and adequate precision (Chen et al., 2017b), as depicted in Table 6.2. The F-value, i.e., the ratio of the noise to response, for the regression model was 179.89, which implies that the model was highly significant (p<0.0001). There was only a 0.01% chance that for a Model F-value, such magnitude could occur due to noise. R^2 is the proportion of variation in the response that is explained by the model. It falls between 0 and 1, and a value above 0.90 suggests a good fit for a model. A higher value of R^2 (0.995) indicated that the second-order polynomial could be used for prediction with reasonable precision, and only 0.5% of the total variation was not explained by the model. Adjusted R^2 (adj-R^2), derived from the sample size and the number of terms in the model, is also a measure of goodness of fit. It has been reported that it is preferable to assess the fit of multiple regression models with adj-R^2 than the R^2 model (Daneshi et al., 2010). If the sample size is not very large and there are many terms in the model, adj-R^2 may be noticeably smaller than R^2 (Chen et al., 2017b).

In this study, the adj-R^2 value (0.990) demonstrated a high significance of the model. In general, predicted R^2 (pred-R^2) can be used to evaluate how well the model predicts responses for new observations. The value of pred-R^2 (0.931) also suggests a high correlation between the predicted and observed values. In addition, the difference between values of adj-R^2 and pred-R^2 was smaller than 0.2, which indicated no large block effect or no possible problem with the model and/or data (Mourabet et al., 2012).

The coefficient of variance (CV) is the ratio of the standard error of estimated data to the average value of the observed response. Usually, the CV value should be less than 10% with

the lower value indicating higher reliability of the experiment (Betiku et al., 2016). The low CV value of 2.75% showed that the response of the developed model was dependable. Adequate precision is a measure of the range in predicted response relative to its associated error. Its value of 44.58, greater than 4, demonstrated an adequate signal, and thus the predicted model could be used for navigating the design space. Furthermore, a standard deviation (SD) of 7.11 indicated the model was compliant with the predicted response. It can be seen from Table 6.2 and Equation 6.1 that linear terms X_1, X_2, and X_3 and quadratic terms X_1^2, X_2^2, and X_3^2 had a significant positive effect on vanillin concentration. This indicated that selected variables had a positive influence on vanillin concentration. The significant positive effect of these quadratic terms implied that they tend to increase vanillin yield markedly at high levels. Thus, this study shows that the selected ranges of variables fitted well for the optimization of vanillin production.

From Table 6.2, it can be observed that the interaction terms X_1X_2, X_1X_3, and X_2X_3 were significant ($p>0.05$), which indicated that they influenced vanillin yield. This means that the effect of CSL concentration (X_1), initial ferulic acid concentration (X_2) and pH (X_3) are controlling factors for the maximum production of vanillin. To further explained the interactive effect of the variables followed by optimization of each variable for maximization of vanillin, contour plots of the RSM were drawn as a function of two variables at a time, holding the remaining variable at the zero levels. Figure 6.3 shows the parity plot of predicted values versus observed values, which proved the accuracy of the model as the values align on a straight line with little deviation from the parity line. A total of three response plots and three corresponding contour plots were produced for the responses (Figure 6.4). Figure 6.4(a) shows the three-dimensional plot and contour interactive effect of initial ferulic acid concentration and CSL on vanillin at a fixed pH of 9. The yield of vanillin increased (150-350 mg/L) as initial ferulic acid concentration increased from 0.5 -2.5 g/L when CSL equally increased from 3 g/L to 9 g/L. However, when the concentration of CSL decreased to 3 g/L and FA was at the peak (2.5 g/L), the vanillin concentration fell to 250 mg/L. This confirms that the constituents in CSL aid the biotransformation of FA into vanillin (Rodríguez-López et al., 2016).

Figure (6.4b) displays a 3D surface diagram of contour interactive effect in corn steep liquor concentration and pH at a fixed FA concentration of 1.5 g/L. It can be clearly seen that as the concentration of CSL and pH increased, a corresponding increase in vanillin yield was observed. The yield of vanillin increased from 170 to 335 mg/L but decreased as the concentration of CSL decreased, even when pH increased. Thus, it can be affirmed that

increasing the concentration of CSL while keeping the pH of the medium basic enhanced the concentration of the vanillin (Paz et al., 2018).

Figure (6.4c) depicts a relationship between FA and pH concentration at a fixed 6 g/L CSL concentration. As the ferulic acid and pH increased, there was a corresponding increase in vanillin. A small concentration of FA (0.5 g/L) at pH 8.5 did not favor more of vanillin yield but when the FA concentration increased from (0.5-2.5 g/L) and the pH peak at 9.5, a maximum vanillin yield of 380 mg/L was observed. Therefore, there is a need to control both pH and ferulic acid concentration to maximize the vanillin yield. Chen et al. (2017b) reported that microbial growth inhibition may occur at high initial ferulic acid concentration, which in turn leads to a decrease in substrate utilization.

Table 6.1: The experimental conditions and results of RSM Box-Behnken design for vanillin production

Run	CSL conc. (g/L)	FA conc. (g/L)	pH	Observed Vanillin conc. (mg/L)	Predicted Vanillin conc. (mg/L)	Residual
1	-1	0	1	260.00	260.62	-0.62
2	0	0	0	300.00	300.00	0.00
3	-1	-1	0	150.00	154.00	-4.00
4	0	-1	1	230.00	225.37	4.63
5	-1	0	-1	170.00	160.62	9.38
6	0	0	0	300.00	300.00	0.00
7	0	1	1	380.00	374.63	5.38
8	1	1	0	350.00	346.00	4.00
9	0	0	0	300.00	300.00	0.00
10	1	0	-1	210.00	209.37	0.63
11	-1	1	0	250.00	254.75	-4.75
12	1	-1	0	200.00	195.25	4.75
13	0	-1	-1	126.00	131.37	-5.37
14	0	1	-1	229.00	233.63	-4.63
15	1	0	1	335.00	344.37	-9.37
16	0	0	0	300.00	300.00	0.00
17	0	0	0	300.00	300.00	0.00

Table 6.2: Analysis of variance table (ANOVA) for vanillin production

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	81934.81	9	9103.87	179.89	< 0.0001
X_1-CSL	8778.12	1	8778.12	173.46	< 0.0001
X_2-FA	31626.13	1	31626.13	624.93	< 0.0001
X_3-pH	27612.50	1	27612.50	545.62	< 0.0001
X_1X_2	625.00	1	625.00	12.35	0.0098
X_1X_3	306.25	1	306.25	6.05	0.0435
X_2X_3	552.25	1	552.25	10.91	0.0131
X_1^2	3789.47	1	3789.47	74.88	< 0.0001
X_2^2	4447.37	1	4447.37	87.88	< 0.0001
X_3^2	2901.32	1	2901.32	57.33	0.0001
Residual	354.25	7	50.61		
Lack of Fit	354.25	3	118.08		
Pure Error	0.000	4	0.000	Pred-R^2	0.931
Cor Total	82289.06	16		Adeq precision	44.583
SD	7.11	R^2	0.995		
% CV	2.75	adj-R^2	0.990		

Adeq - Adequate; adj - adjusted; Pred - prediction
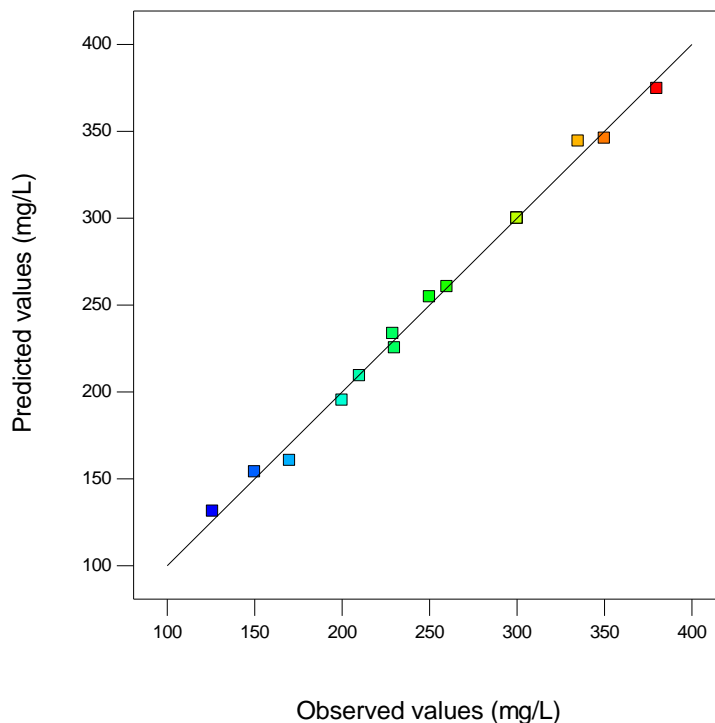
Figure 6.3: Plot of predicted value versus observed values
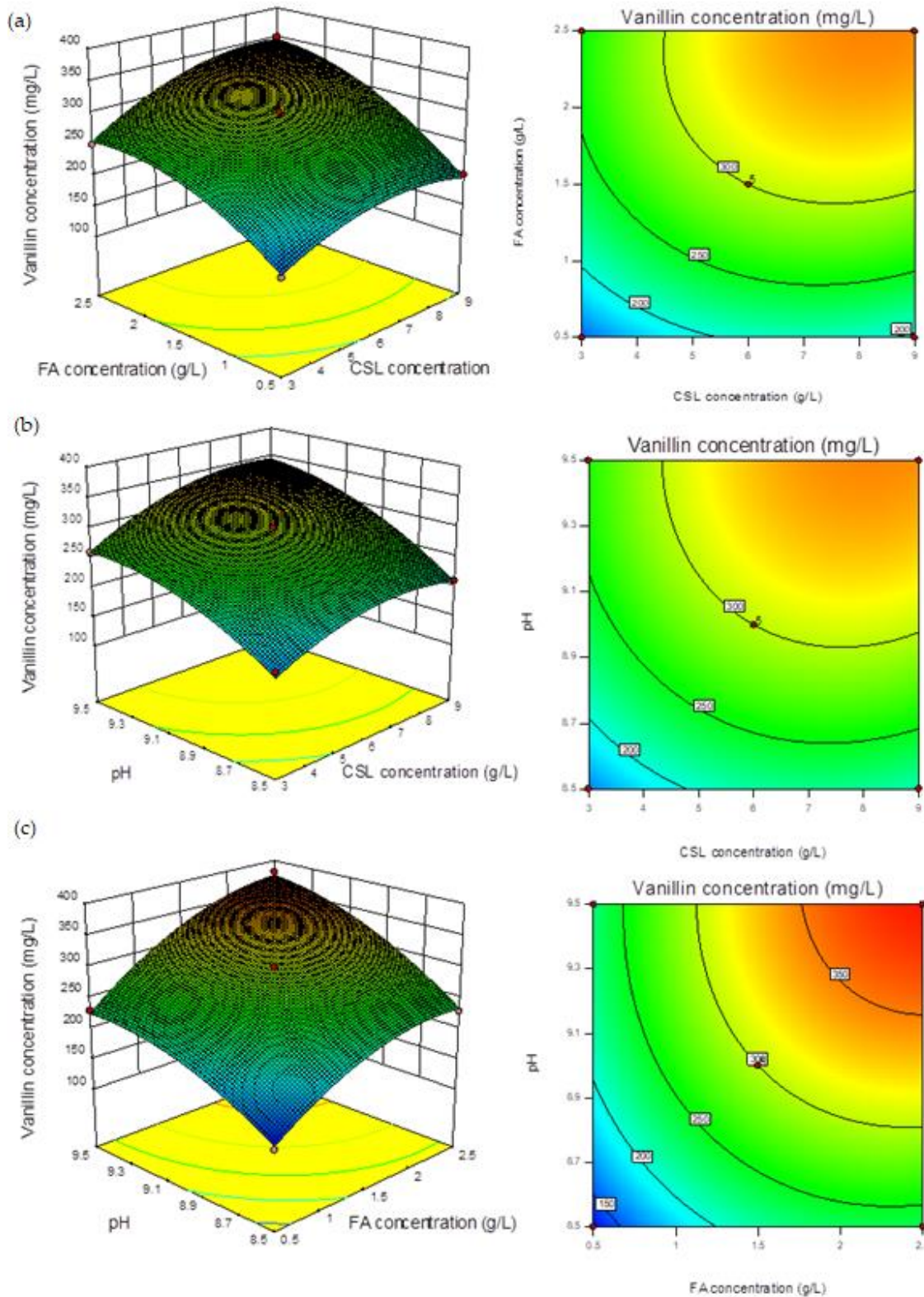
Figure 6.4: Surface regression model plots and their corresponding contours on the interaction between variables on vanillin production (a) interaction between ferulic acid (g/L) and corn steep liquor (b) interaction between pH and corn steep liquor (c) interaction between pH and ferulic acid

6.3.6   Numerical optimization of vanillin bioproduction using desirability function

The desirability function is a suitable technique for the simultaneous determination of optimum performance levels of input variables. Desirability ranges from zero to one for any given response, and a desirability value of one represents the ideal case, while that of zero indicates that one or more responses fall outside desirable limits. The whole response desirability value can be obtained from the geometric average of all the individual response desirability values (Equation (6.2)).

$$D = \left(d_1 \times d_2 \times d_3 \times .... \times d_n\right)^{\frac{1}{n}} = \left(\prod_{i=1}^{n} d_i\right)^{\frac{1}{n}}$$
(6.2)

where, d_i means the individual desirability value of the ith response and n represents the number of responses in the measure, i = 1. . . n. That is, when all responses achieve the target, the D value (i.e., the overall desirability), is equal to 1. Numerical optimization using Derringer's desired function methodology was used in order to determine the optimum parameters for the maximum production of vanillin. In Design-Expert software, the CSL concentration, initial FA concentration, and pH were selected to be "in range" and the amount of vanillin was set to maximize its yield. The weights of all factors were equal to 1, and the response was chosen to a point that maximized the desirability function (desirability set to 1) (Derringer and Suich, 1980).

Figure 6.5a represents the individual desirability function (d_i) for each of the responses, and the calculated geometric mean (D = 1). Figure 6.5b shows a desirability ramp for numerical optimization of the three selected goals. Using the desirability function, all response factors were recalculated to maximize the vanillin yield, and the optimum operating conditions for the maximum vanillin production were found to be 7.72 g/L for CSL concentration, 2.33 g/L for FA concentration and pH of 9.36 with the overall desirability of 1.
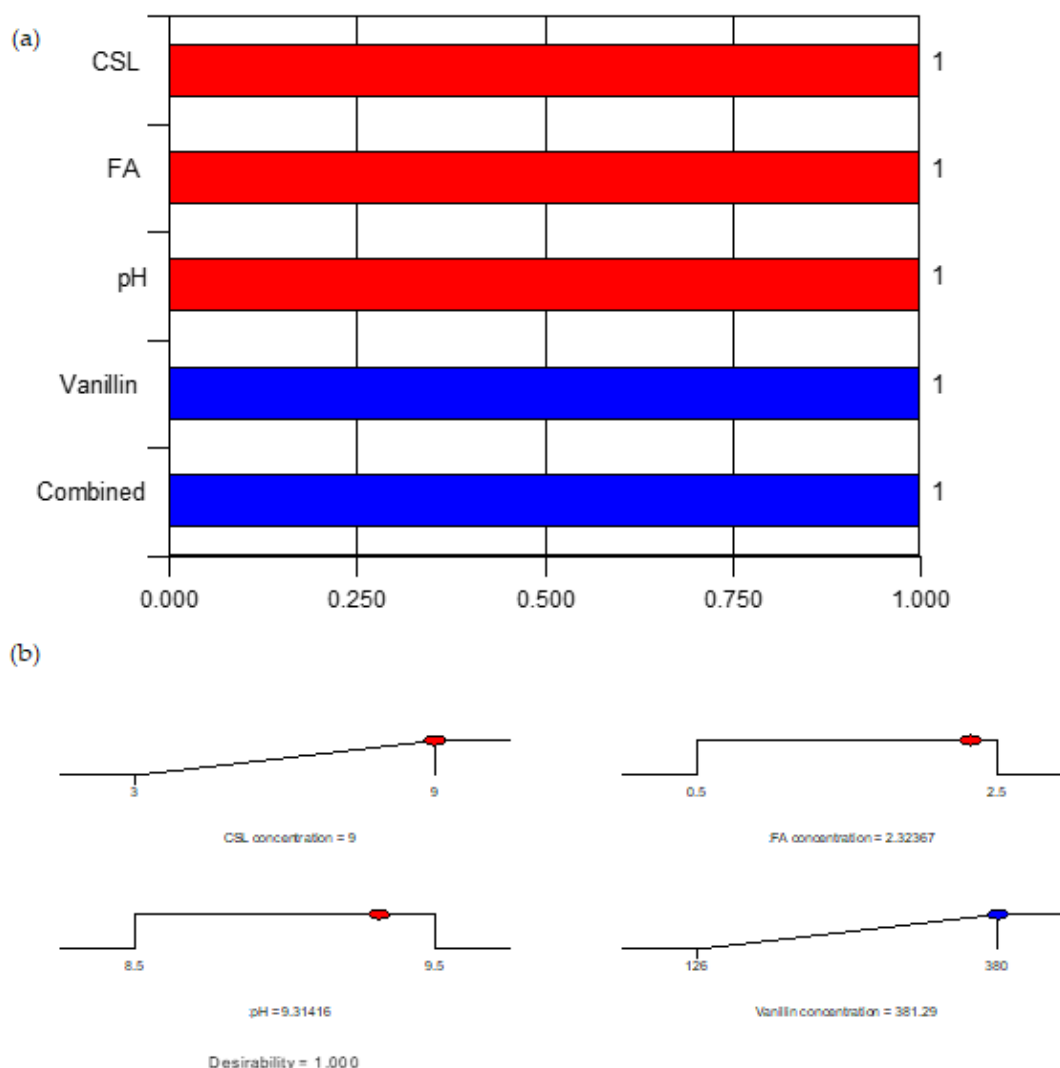
Figure 6.5: (a) Desirability bar graph on numerical optimization for vanillin production (b) Ramp function graph

6.3.7 Validation test

Under the optimum conditions acquired from response surface methodology analysis, vanillin concentration was predicted to be 384.40 mg/L. The verification experiments were performed in triplicate and the validated yield was 386 mg/L. The difference between the validated and predicted values was 0.42%, implying that the optimal conditions could be practically used for bioconversion of ferulic acid to vanillin. The optimum values of three selected factors were acquired by solving Equation (6.2) using the Design Expert Software package (version 10.0).

The biotransformation process for vanillin production from ferulic acid using different microorganisms and inorganic nitrogen sources has been reported in the literature and the results obtained are comparable to the optimum vanillin yield obtained from this study (Table

6.3). Chen et al. (2016) obtained 0.16 mg/L amount of vanillin when *Bacillus subtilis* B7-S was used as the bioconversion microbe, and Karmakar et al. (2000) decarboxylated ferulic acid to 4-vinyl guaiacol, which was immediately converted to vanillin of 16.8 mg/L using *Bacillus coagulans*. Paz et al. (2018) postulated that *Bacillus aryabhattai* BA03 carried out a decarboxylation of ferulic acid to the maximal vanillin concentration of 147.1 mg/L.

Tilay et al. (2010) is the only recent study that adopted corn steep liquor as an inorganic nitrogen source for the production of vanillin using *Pycnoporus cinnabarinus.* The authors statistically optimized vanillin concentration to 126 mg/L. Nitrogen sources have been reported to be directly proportional to the cost of raw material in commercial fermentation, and the use of cheaper sources of nitrogen may directly influence the cost of producing vanillin (Tan et al., 2016). This study shows the significance of using low cost-nitrogen sources like corn steep liquor as a substitute for extracts of yeast and beef (Table 6.3).

Table 6.3: Biotransformation of vanillin from ferulic acid using nitrogen sources

Ferulic acid (g/L)	Organism	Vanillin (mg/L)	Vanillin Molar yield (%)	Inorganic nitrogen source	Author
0.6	*B. subtilis B7-S*	0.16	0.03	Peptone &Yeast extract	(Chen et al., 2016)
1.0	*Bacillus coagulans*	16.8	2.14	Yeast extract	(Karmakar et al., 2000)
1.0	B. aryabhattai BA03	147.1	18.81	Yeast & beef extract	(Paz et al., 2018)
1.0	*Pycnoporus cinnabarinus*	126	16.08	Corn steep liquor	(Tilay et al., 2010)
2.33	***Bacillus amyloliquefaciens subsp***	**386**	**21.14**	**Corn steep liquor**	**This study**

6.4   Conclusion

Corn steep liquor can be considered as a suitable alternative to other expensive nitrogen nutrients for the bioproduction of ferulic acid to vanillin. As evident in the result, vanillin concentration was affected significantly by ferulic acid concentration, CSL concentration, and the media pH. A second-order mathematical model with R^2 of 0.995 can be used to predict the vanillin concentration using the response surface method ($p < 0.05$). The optimum conditions

required to achieve 384.40 mg/L of vanillin from bioconversion of ferulic acid were 7.72 g/L of CSL, 2.33 g/L of ferulic acid concentration and pH of 9.34. The concentration of vanillin was validated experimentally to an accuracy of 0.42%.

Chapter 7   OPTIMIZATION OF FERMENTATION PROCESS VARIABLES BY TAGUCHI ORTHOGONAL ARRAY DESIGN FOR IMPROVED ACETOIN PRODUCTION

7.1   Introduction

The fermentation process is more effective in the bioreactor system compared to the shake flask since the bioreactor offers control of some process variables. Variables such as dissolved oxygen concentration, pH, foaming level, agitation, temperature, gas mixture (nitrogen, oxygen, air, carbon dioxide) can be controlled in the bioreactor system. In addition, the advantage of using a bioreactor over a shake flask has been reported comprehensively by Obom et al. (2013). These authors attribute the superior performance of bioreactor studies compared with shake flask studies to the effective growth environment at the precise temperature they provide, the fixed speed of agitation, and pH regulation within the bioreactor. Some of the shortcomings in shake flask studies do not apply to bioreactor studies

Studies have reported improved yield of acetoin using different strategies such as *Bacillus subtilis* encoding (Bae et al., 2016), isolating *Bacillus* species (Xiao et al., 2007), and shake flask optimization (Tian et al., 2014). Among several strategies used in improving the yield of bioprocess, process optimization using statistical tools is one such approach. See Chapter 5 for discussion of shake flask optimization of acetoin. However, due to shortcomings in shake flask studies and the need to improve the yield of acetoin, this chapter describes further studies in a bioreactor using Taguchi optimization technique.

Taguchi experimental design is a fast method of optimization, and achieves remarkable outcomes in the simultaneous study of many factors, making its mark in quality products supplemented with better process performance, and rendering high yield and better stability (Chen et al., 2017a). Taguchi design established the importance of statistically aligned experiments in predicting the settings of product (and/or processes) on various parameters (Das et al., 2014). The Taguchi design method applies fractional factorial test designs called orthogonal arrays (OAs) that serve to reduce the number of experiments. The selection of a suitable OA depends on the number of control factors and their levels. Using OA design can estimate multiple process variables which simultaneously affecting the performance while minimizing the number of experiment performed (Shahavi et al., 2016).

Thus, statistical optimization of fermentation variables was investigated in the bioproduction of acetoin in a bioreactor to improve the yield parameter when compared to a shake flask study.

7.2 Methodology

Acetoin optimization studies were first carried out in a shake flask study (Chapter 5). The scale-up optimization of the optimum variables from shake flask study into a bioreactor was done using Taguchi orthogonal design. The experimental design details and method of analysis can be found in Chapter 3, section 3.4 and 3.6. The Minitab Statistical Software (package 17) was used to determine the outcomes of the fermentation experiment since the choice variables fitted better in the Minitab experimental design package than Design-Expert software for this study. The signal-to-noise ratio (*S/N*), which is the logarithmic function of the desired output, served as an objective function for optimization. For each run, *S/N* ratio corresponding to the-larger-the-better objective function was computed using relation in the Equation (7.1) expressed below:

$$\frac{S}{N} = -10\log\left(\frac{1}{n}\sum_{i=1}^{n}\frac{1}{y_i^2}\right) \tag{7.1}$$

The terms in Equation (7.1) are defined as; *S/N*, the signal to noise ratio; n, the number of experiments carried out; *i* is the experimental run number, and y is the output result of the experiment.

7.3 Results and discussion

7.3.1 Effects of aeration and agitation on acetoin production in the bioreactor

The production of acetoin was studied at aeration rates of 0.5, 1.0 and 1.5 slpm (Figure 7.1). During the fermentation, the agitation speed of the impeller was kept between 150-400 rpm and fermentative temperature of 37 °C. In the study, it was observed that high agitation (400 rpm) was not favorable even when aeration was increased from 0.5 -1.0 slpm. This could be due to the size of the bioreactor working volume (1L) which could not favor aeration and agitation as it caused foaming. Acetoin concentration was already maximum (2.57g/L and 3.69 g/L) as at day two of the study (batch 3 and 8). However, when the agitation (batch 4 and 5,) was maintained at 300 rpm and aeration increased from 0.5 to 1.5 slpm, acetoin concentration attained its maximum (63.43 g/L) on day two and three of fermentation studies. Bajaj and Singhal (2010) support these findings that agitation and aeration are key drivers in the determination of the total mass and oxygen transfer in fermentation processes. The authors further stressed that agitation plays the role in the movement of nutrients and flow of air and oxygen, while aeration supplies oxygen to the culture medium and support in the mixture of the fermentation broth, especially when the agitating speed is very slow
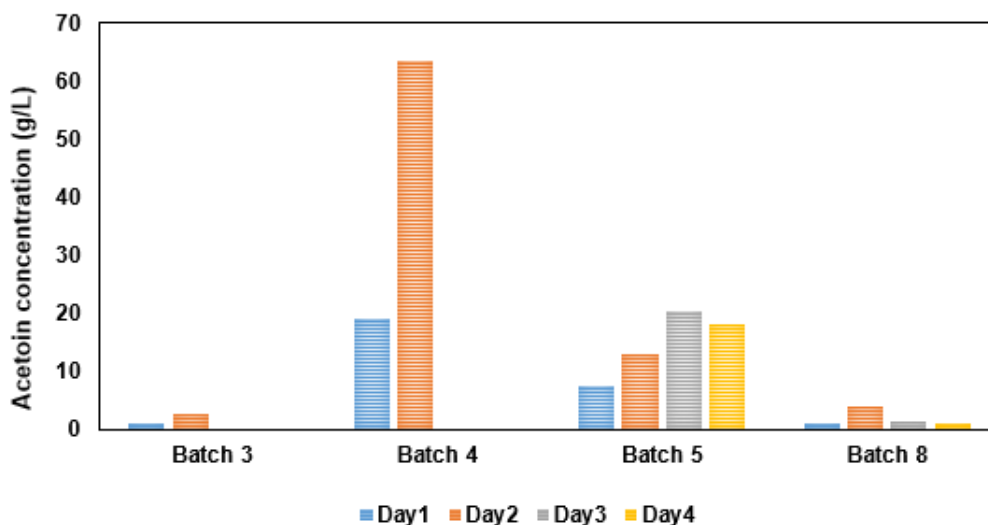
Figure 7.1: Batch production of acetoin production showing the effects of aeration and agitation

7.3.2 Effects of pH (controlled and uncontrolled) on acetoin batch production:

Experiments (batch 1 and 2) were carried out for 5 days. It was observed that when pH was left uncontrolled (batch 2) and pH-controlled at 7 (batch 1), there was a continuous increase in acetoin production in both studies, while the uncontrolled pH study attained optimum level at day 2 and decreased drastically till fermentation elapses in day 5 (Figure 7.2). The peak of acetoin on day two was reached when the fermentation broth was within the surviving the pH for bacillus growth and bioconversion (Dai et al., 2015a). Likewise, in a similar batch study of controlled and uncontrolled pH (batches 7 and 9), it was observed that pH 6.5 brought about a continuous increase until the fermentation elapsed. When pH was uncontrolled (batches 9 and 2), a continuous decrease in the production of acetoin was shown, and the batch processes could be terminated on day 4. In the study by Dai et al. (2015a), the author's findings revealed that *Bacillus* strains can survive at low pH but the most suitable range for acetoin production is pH 6.5 to 7.0.
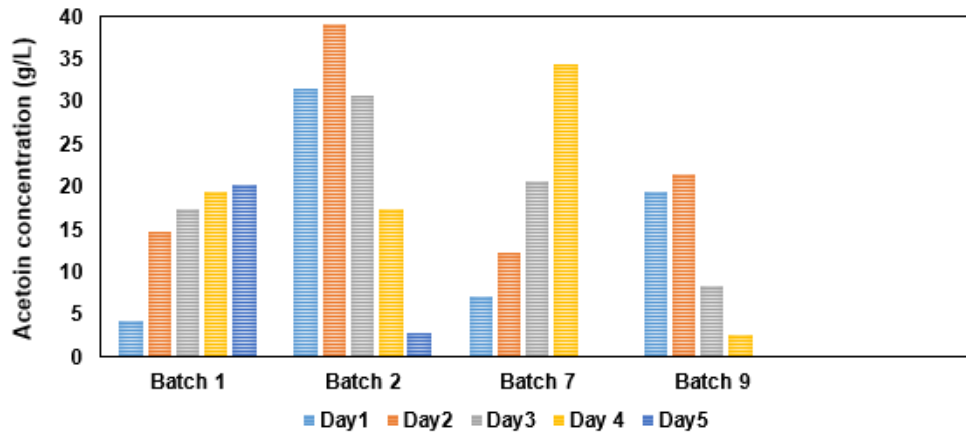
Figure 7.2: Batch production of acetoin production showing the effects of controlled and uncontrolled pH

7.3.3 Signal to noise ratio

Taguchi design uses the signal to noise (S/N) ratio to measure the value of significance in the choice of a variable when experiments are performed. S/N is characterized into three categories: nominal is better, the-smaller-the-better and the-larger-the-better. In this work, the-larger-the-better was applied with intention of achieving a high yield of acetoin in the batch fermentation study. Therefore, the greater the delta value of S/N, the better the output yield yielded output. The optimum conditions in this approach were obtained by sorting the delta value in increasing order of significance. A higher delta value signifies a greater effect of the variable on the whole experimental study.

According to Taguchi, variability in product performance with reference to noise factors should be minimized while variability with reference to signal factors must be maximized. Table 7.1 shows the result of S/N ratio analysis against the fermentation variables and acetoin concentration (g/L). In Table 7.2, S/N ratio analysis revealed that the optimum condition for acetoin production was similar to that obtained using main effects plot (300 rpm, 1.5 slpm, 2 days and pH 6.5) of the fermentation variable. The results show that agitation was the highest influencing parameter in improving acetoin yield (Figure 7.3).

Table 7.1: Acetoin production variables, concentration, and S/N ratio

Batch number	Agitation (rpm) (A)	Aeration rate (slpm) (B)	Time (days) (C)	pH (D)	Acetoin conc. (g/L)	S/N ratio
1	150	1.5	5	7.0	15.25	23.67
2	300	1.0	5	Uncontrolled	24.28	27.71

3	400	1.0	2	7.0	2.44	7.75
4	300	1.5	2	6.5	41.20	32.30
5	300	0.5	4	7.0	14.73	23.36
6	150	0.5	2	Uncontrolled	13.80	22.80
7	150	1.0	4	6.5	18.60	25.39
8	400	0.5	5	6.5	1.57	3.92
9	400	1.5	4	Uncontrolled	2.57	8.20

Table 7.2: Optimum conditions by utilizing signal to noise ratio

Level	Agitation (A)	Aeration (B)	Time (C)	pH (D)
Units	rpm	slpm	days	-
1	23.951	16.693	20.948	19.567
2	27.789	20.281	18.984	20.535
3	6.621	21.387	18.429	18.259
Delta	21.168	4.694	2.518	2.276
Rank	1	2	3	4
Optimum	A2	B3	C1	D1

The highest plots of each parameter were chosen as depicted in Figure 7.3, as evidence that the ratio was the-larger-the-better, a term used for optimization in Taguchi orthogonal design. The same optimum conditions could be seen in Table 7.2 by the delta values. Therefore, the optimum conditions obtained by this approach were as follows: A2, B3, C1, and D1.
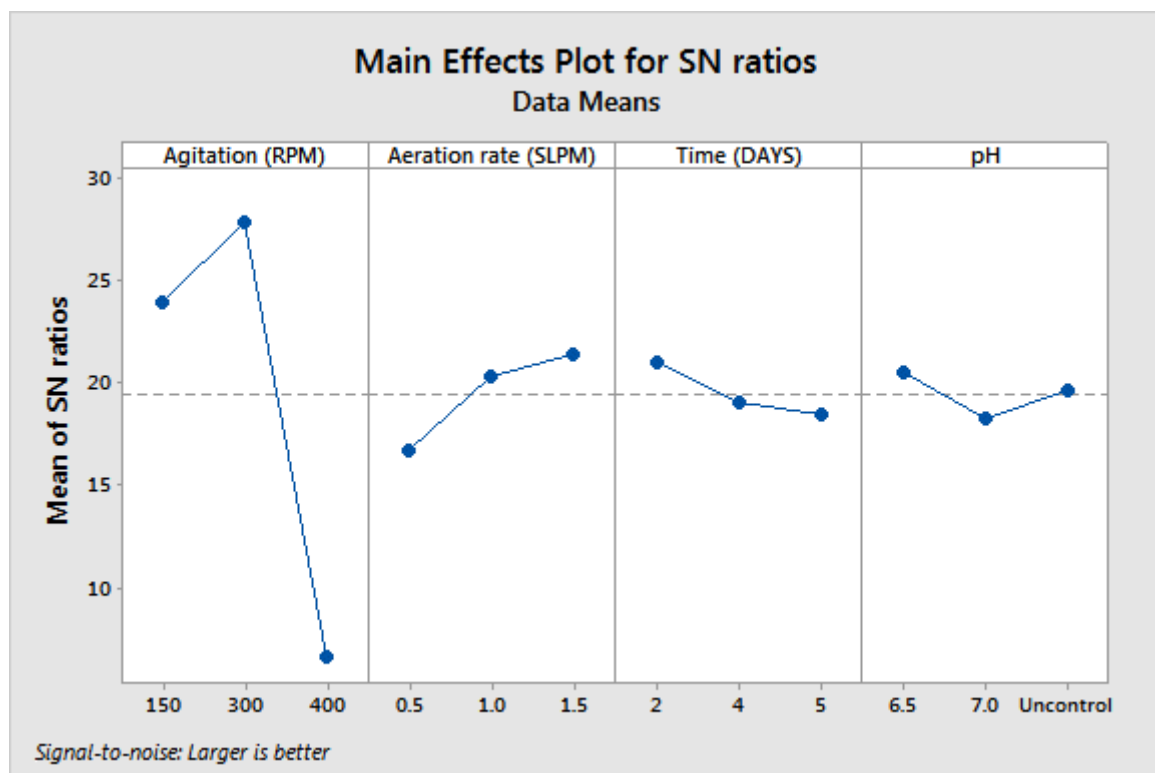
Figure 7.3: Main effects plot for S/N ratios

7.3.4 Analysis of mean

Table 5 shows a response table for analysis of mean (ANOM) and it demonstrates the equality of sample means. The main objective of the ANOM is to test the effects of a designed

experiment in which all the variables were fixed (Dar and Anuradha, 2018). The ANOM table 5 is estimated using the Equation (7.2) below:

$$m = \frac{1}{9} \sum_{j=1}^{9} \beta_j$$

(7.2)

where j represents the number of experimental runs from 1 to 9; m is the overall mean level result; β is the dependent variables (acetoin concentration). In Table 7.3, A, B, C, D were independent fermentation variables, the selected bold numbers were the minimum in every column of the three-step level, as per range, and used in setting the rank for all the variables. Based on the main effects plot for a mean of fermentation variables as shown in Figure 7.4, the optimum combination was agitation of 300 rpm, aeration of 1.5 slpm, fermentation time of 2 days and pH of 6.5. The same optimum was coded as A2, B3, C1, and D2 in Table 7.3.

Table 7.3: ANOM response table for means

Level	Agitation A	Aeration rate B	Time C	pH D
Units	rpm	slpm	days	-
1	15.883	**10.033**	19.147	20.457
2	26.737	15.107	**11.967**	**10.807**
3	**2.193**	19.673	13.700	13.550
Delta	24.543	9.640	7.180	9.650
Rank	1	3	4	2
Optimum	A2	B3	C1	D2

Delta = maximum level of mean - minimum level of mean
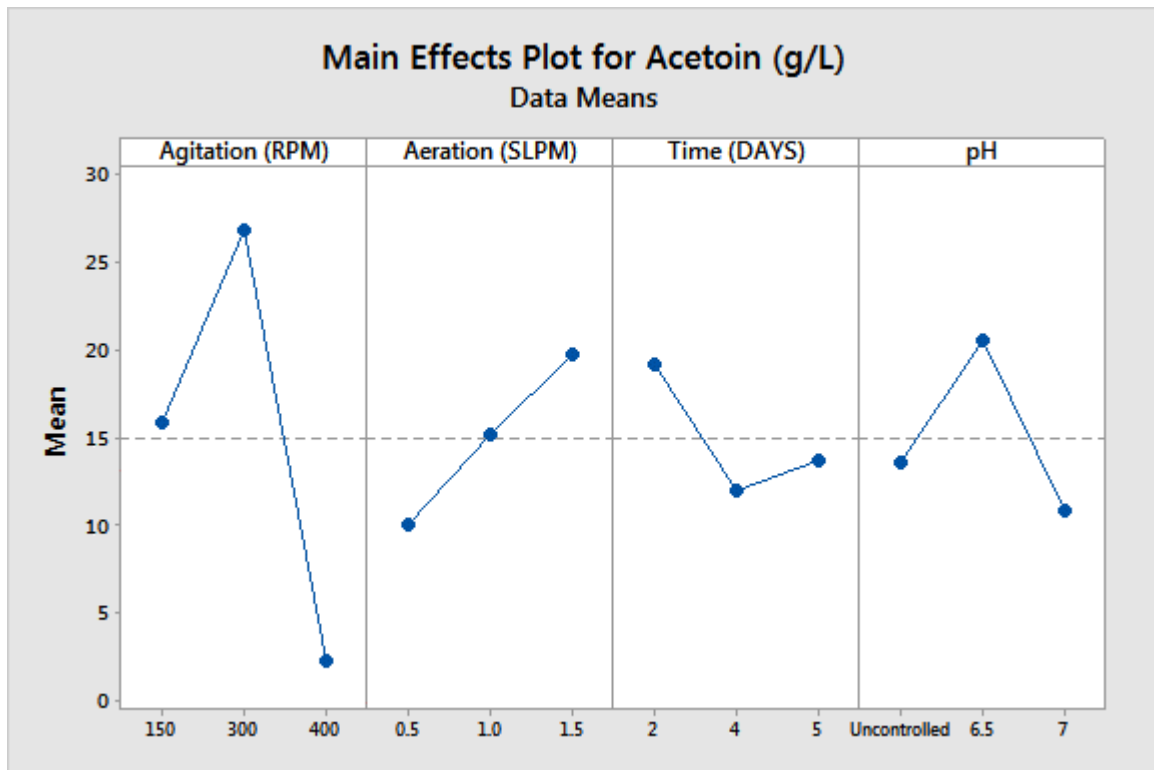
Figure 7.4: Main effects plot for the mean of fermentation variables

7.3.5 Analysis of variance

Analysis of Variance (ANOVA) is used to investigate the relationship between a response and predictor variables. In Table 7.4, ANOVA was used to measure the percentage contribution of each fermentation variable in the batch study of acetoin.

The percentage contribution of the variable shown is represented in Equation 7.3 below:

$$\% \, contribution = \frac{sum \, of \, square \, of \, a \, variable}{total \, sum \, of \, squares} \qquad (7.3)$$

Table 7.4: Contribution of fermentation variables

Variable	Units	Degree of freedom	Adj SS	Percentage contribution	Rank
Agitation	rpm	2	907.59	70.92	1
pH	-	2	148.35	11.59	2
Aeration	slpm	2	139.52	10.90	3
Time	days	2	84.22	6.58	4
Total		8	1279.68		

df - Degree of freedom; Adj SS - Adjusted Sum of Squares

Figure 7.5 shows that the plotted lines were not parallel to each other, indicating the existence of certain relationships between the variables and the acetoin concentration. In Table 7.4, the

percentage contribution of each fermentation variable on acetoin yield was shown in this order: agitation, pH, aeration and time by 70.92, 11.59, 10.90 and 6.58% respectively.
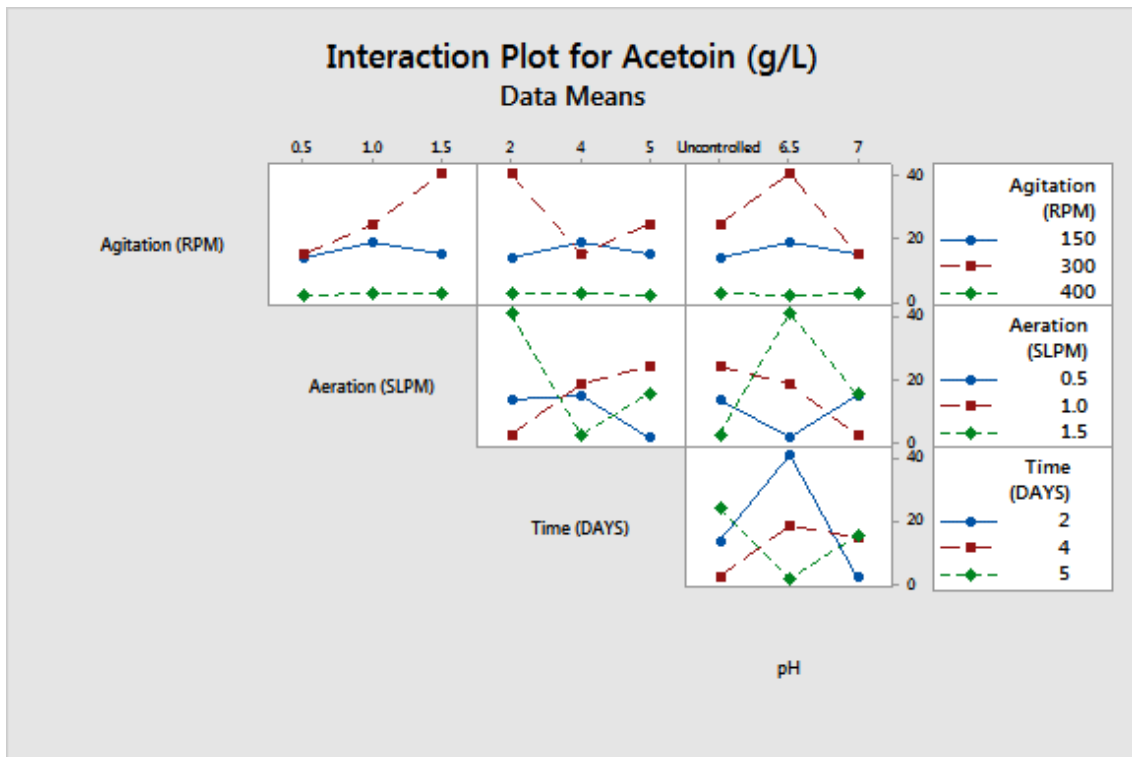
Figure 7.5: Interaction plot for acetoin concentration (g/L)

7.3.6 Validation of the experiment and performance of the shake flask to the bioreactor study

The optimum conditions established from the acetoin experimental studies using Taguchi design are shown in Table 7.5. The optimum variables with experimental acetoin concentration of 41.20 g/L were validated as 41.72 g/L. Table 7.6 shows the difference in performance between the shake flask and bioreactor based on the process variable. A fourfold gain (42.30 g/L) of acetoin concentration was obtained in the bioreactor studies with 59.25% molar yield.

Table 7.5: Experimental and validated optimal conditions for acetoin production

Optimum Variables				Experimented	Validated
Agitation	Aeration	Time	pH	Acetoin conc.	Acetoin conc.
rpm	slpm	days		(g/L)	(g/L)
300	1.5	2	6.5	41.20	41.72

Table 7.6: Comparing the shake flask and bioreactor studies performance

Process Variables	Shake Flask	Bioreactor
Vessel Size	100 mL	1.3 L
Agitation	150 rpm	300 rpm
Aeration	-	1.5 slpm
Fermentation time	2 days	2 days
pH	7.0	6.5
Temperature	37 °C	37 °C
Initial glucose concentration	78.40 g/L	156.80 g/L
Acetoin Concentration	10.70 g/L	42.30 g/L
Percentage molar yield	28.00%	59.25%

7.4   Conclusion

This study reported the statistical optimization and validation of fermentation process variables for improved acetoin production from shake flask to batch study in a bioreactor using Taguchi orthogonal array design. The influence of agitation, aeration, time and pH respectively was analyzed by Taguchi orthogonal array design. The statistical model was successfully validated with agitation as the most controlling variable among other fermentation variables. Optimized process variables of 300 rpm agitation, 1.5 slpm aeration, 2 days fermentation time and pH 6.5 were scaled up at bioreactor level with a fourfold gain (42.30 g/L) of acetoin concentration when compared to shake flask optimization study. The results demonstrated that the commercial production of acetoin can be envisaged using a biological approach since there is a significant yield from the scale-up study.

Chapter 8   RECOVERY OF ACETOIN FROM *BACILLUS SUBTILIS*
FERMENTATION BROTH BY SUPERCRITICAL CO₂ EXTRACTION

8.1   Introduction

The recovery of acetoin from the fermentation broth is usually challenging due to a low concentration of acetoin in the fermented broth (Liu et al., 2011; Nielsen et al., 2010; Xiao and Lu, 2014). Apart from the acetoin production pathway, the separation and purification processes play a major role in obtaining acetoin of high quality for industrial purposes. The affinity of acetoin with water is strong, compared to that of alcohol and water mixture (Figure 8.1a). This interaction imposes difficulties in recovering acetoin from the fermentation broth. However, the separation of 2,3-butanediol which is an analog of acetoin (Figure 8.1b) from aqueous solution has been reported widely (Ji et al., 2011; Xiu and Zeng, 2008). The high yield of 2, 3-butanediol obtained from its aqueous solution caused its separation technique to be studied by many authors (Jeon et al., 2014; Koutinas et al., 2016; Celińska and Grajek, 2009).

Separation techniques for 2,3-butanediol could be used as references for acetoin separation since they share some common properties (Sun et al., 2012). These techniques include stream stripping (Qureshi and Maddox, 1995), solvent extraction (Birajdar et al., 2014), pervaporation (Shao and Kumar, 2009), sugaring-out extraction (Dai et al., 2015b) and vacuum membrane distillation (Jeon et al., 2014). The main drawback of liquid-liquid extraction is that the organic solvents used in acetoin recovery cause a carcinogenic effect on consumers, especially in industrial food applications (Khosravi-Darani and Vasheghani-Farahani, 2005; Grosse et al., 2011). Likewise, the distillation technique applied for the recovery of acetoin from the broth imposes some problems due to loss of reagent through entrainment and the difficulties in phase separation (Xiao and Lu, 2014). Although some techniques have been investigated for acetoin extraction and purification, these methods are often operated at an elevated temperature which causes the product quality to deteriorate.

Besides, such processes may not be environmentally friendly, because of the high energy consumption and the use of toxic solvents, coupled with the elaborate purification steps to remove the solvent residues from the final food products (Welti-Chanes and Velez-Ruiz, 2016). As a consequence, separation processes operating at a moderate temperature and avoiding harmful extraction aids have been investigated in food and industrial production processes (Perrut, 2000; Hrnčič et al., 2018).

Currently, the supercritical carbon dioxide (scCO₂) extraction technique has been shown as an alternative method useful for the safe recovery of aroma compounds (Dawiec-Liśniewska

et al., 2018; Saffarionpour and Ottens, 2018). This fact is important because of its unique properties which alter in response to the regulation of pressure and temperature, the solvent power of sc-CO_2 and the selectivity of the extraction (Khaw et al., 2017). In addition to being non-toxic, non-flammable, and chemically stable, the low critical temperature of CO_2 allows performing the process at near-ambient temperatures by avoiding or minimizing the thermal degradation of the final product (Macedo et al., 2008; Oliveira et al., 2009). The supercritical fluid extraction process has been used for numerous applications and its usage in the fractionation of bioactive compounds from fermentation broth is still of growing interest (Catchpole et al., 2012; Lukin et al., 2018).

Downstream processing of acetoin, therefore, needs to be addressed for the holistic improvement of the process economy in fermentation technology. The objective of this chapter is to investigate the feasibility of recovering acetoin from a typical fermentation broth containing acetoin using supercritical carbon dioxide. Operation parameters for maximum recovery were through validation of the established operating parameters obtained using a simulated broth in a supercritical pilot plant.
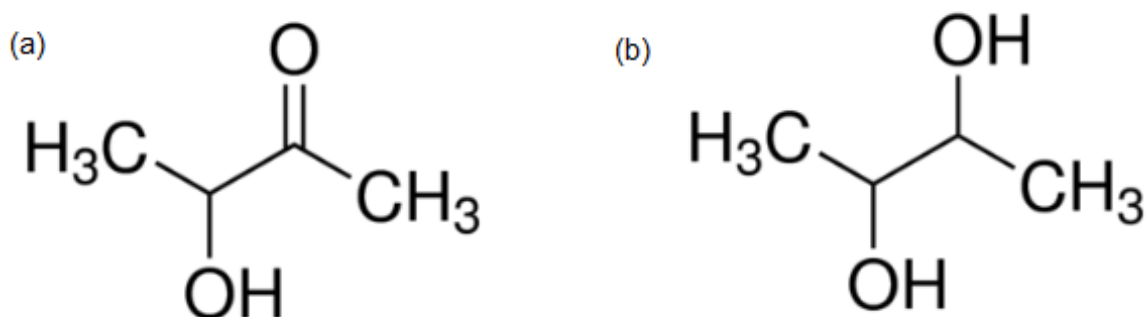
Figure 8.1: (a) the molecular structure of acetoin and (b) the molecular structure of 2,3 butanediol

8.2   Methodology

A model solution of acetoin (simulated broth) was prepared to mimic a typical concentration of acetoin in fermented broth (see details in section 3.5.2.1 of Chapter 3). An aliquot of the broth was used as feed in the pilot plant that was operated in a batch system. Acetoin was concentrated and recovered from the broth using supercritical CO_2 in a fractionating pilot plant (Figure 8.2). The following equations were used in estimating the concentration factor and percentage recovery in the study (equation 8.1-8.2).

The concentration factor was the concentration of the extracts (g/L) divided by the concentration of the initial feed solution (g/L) (equation 8.1).

$$\text{Concentration Factor} = \frac{\text{concentration of extract}}{\text{concentration of initial feed}} \qquad (8.1)$$

The percentage recovery was obtained by the mass (g) in either extract or raffinate, divided by its mass (g) in the initial feed solution.

$$\text{Recovery (\%)} = \frac{\text{mass of the extract or raffinate}}{\text{mass of the initial feed}} \times 100\% \qquad (8.2)$$

The pilot plant used for this experiment is shown in Figure 8.2 (details of its operation and its process flow diagram is shown in section 3.5 and Figure 3.4 of Chapter 3). The experimental approach and analysis of broth preparation can be found in sections 3.5 and 3.6 of Chapter 3.

A sampling of the raffinate was done at 5 min interval while extract was collected after 30 minutes. The reason for the sampling time differences is to create enough contact time for the solubility of acetoin in sCO_2. The samples collected at the raffinate and extracts were analyzed, and the concentration of acetoin was determined using the spectrophotometric method (details in Appendix A).

Figure 8.2: Supercritical fluid extraction pilot plant set up for acetoin recovery

8.3 Results and discussion

A graphical representation of acetoin concentration at the raffinate against sampling time is shown in the scattered plot graphs while the acetoin concentration of the extracts in the separation vessels (separator 1, 2, 3) against different fractionating time is depicted using a bar chart (Figure 8.3-8.10).

8.3.1 Acetoin concentration and recovery from the simulated broth

Fractionation time, temperature, pressure and CO_2 feed rate have been reported as controlled parameters affecting supercritical fluid extraction of natural products (Khaw et al., 2017). The effects of these factors were investigated in this study of acetoin recovery from the simulated broth. The concentration of acetoin with respect to the feed solution at the raffinate and extract was estimated. Concentration factors and percentage recovery was estimated for this study (details of experimental data are shown in Appendix F).

8.3.1.1 Effect of contact time on acetoin concentration and recovery from the simulated broth

The first experimental run (Run 1) was performed by selecting the operating parameters for this study within the equilibrium solubility data of acetoin in supercritical CO_2 as reported in Effendi et al. (2013) and solubility data of water in supercritical CO_2 as shown by Wang et al. (2018).On this basis, the experimental conditions were set at a temperature of 80 °C, pressure of 200 bar and CO_2 feed rate of 10 kg/h in a batch system. Figure 8.3 (a) and (b) show the effect of contact time on the concentration of acetoin with respect to the feed solution at the raffinate and extract respectively. The separator conditions and the corresponding densities of CO_2 in separator 1 and 2 are shown in Table 8.1 below.

Table 8.1: Separator conditions and corresponding densities

Separator	Temperature (°C)	Pressure (bar)	Density (kg/m³)
1	18	54	794.8
2	29	45	147.3

The results show a steady decrease in the organic content of the raffinate with time. It was also observed that there was a consistent trend in every 5 min interval from 150 min to the elapsed time of the experiment (Figure 8.3a). The concentration of the extract in separators 1 and 2 was one- and fivefold to that of the feed between 90 to 270 minutes (Figure 8.3b). A concentration factor of 6 and 83% recovery was estimated after the fractionation. It can also be deduced from Figure 8.3b that the concentration of acetoin was higher in separator 1

compared to separator 2 but the reverse was the case for the amount of mass extract obtainable from the two separators (data detailed in Appendix F).

The fractionating time is a significant factor that needs to be considered for the efficient recovery of aroma since it has an influence on the composition of the extracted product (Capuzzo et al., 2013). The result of the experiment (Run 1) shows the feasibility of the broth concentration and recovery at a shorter time of 30 to 90 min. Thus, an extension of the fractionating time for acetoin concentration and recovery is not important beyond this point.

Figure 8.3: (a) Percentage recovery of simulated broth at the raffinate against sampling time at 80 °C, 200 bar, 10 kg/h (b) acetoin concentration against sampling time at the extract (separator 1 and 2) for experimental run 1

8.3.1.2 Effect of temperature on acetoin concentration and recovery from the simulated broth

Operating conditions of the second experimental run (Run 2) were selected on the basis discussed above for Run 1 (see section 8.3.1.1) with only a change in the temperature that was set at 37 °C for Run 2. The reason for this change was to keep the concentration and recovery of acetoin at its fermentation temperature (37 °C). In practice, especially in food-related industries, it is usually desirable that the critical temperature of the solvent be kept very low (Laitinen, 2000). Likewise, Eller et al. (2008) reported that capital costs and energy inputs are proportional to the pressure and temperature requirements of critical fluid separation, so it is advisable to keep it low as possible. On this basis, the column operating conditions in Run 2 were set at a temperature of 37 °C, pressure of 200 bar, and CO_2 feed rate of 10 kg/h in a batch system. In addition, since Run 1 provided informed about the feasibility of extraction in a shorter time, the extraction time was reduced from 270 to 30 min (Figure 8.4 a).

The graphical overview of the acetoin concentration obtained from the raffinate against the fractionating time of 30 min is depicted in Figure 8.4 (a). Likewise, the acetoin concentration of the extracts at the separation vessels (separator 1 and 2) was drawn against the sampling interval of 60 to 75 min as displayed in Figure 8.4 (b). For this study, a graphical overview of Run 1 and Run 2 was used to investigate the effect of operating at high temperature (80 °C) and acetoin fermentation temperature (37 °C) at constant pressure of 200 bar and CO_2 feed rate of 10 kg/h (Figure 8.3a and 8.4a).

When temperature changed from 80 to 37 °C, the concentration factor of acetoin reduced from 6 to 2 with respect to the feed solution at the raffinate within the first 30 min contact time and there was a decrease in percentage recovery (83 to 82%) (Run 1 and Run 2).This could be due to an increase in the solvent density whenever there was a decrease in temperature (Pansuriya and Singhal, 2009; Khaw et al., 2017). The concentration of the extract in separators 1 and 2 was approximately twofold to that in the feed between 60 to 75 minutes (Figure 8.4b). The experiment was further carried out at the fermentation temperature basically to avoid the possible thermal damage of the product and to save energy costs, bearing in mind the process of integrating bioprocess with high-pressure technology (Khosravi-Darani and Vasheghani-Farahani, 2005).
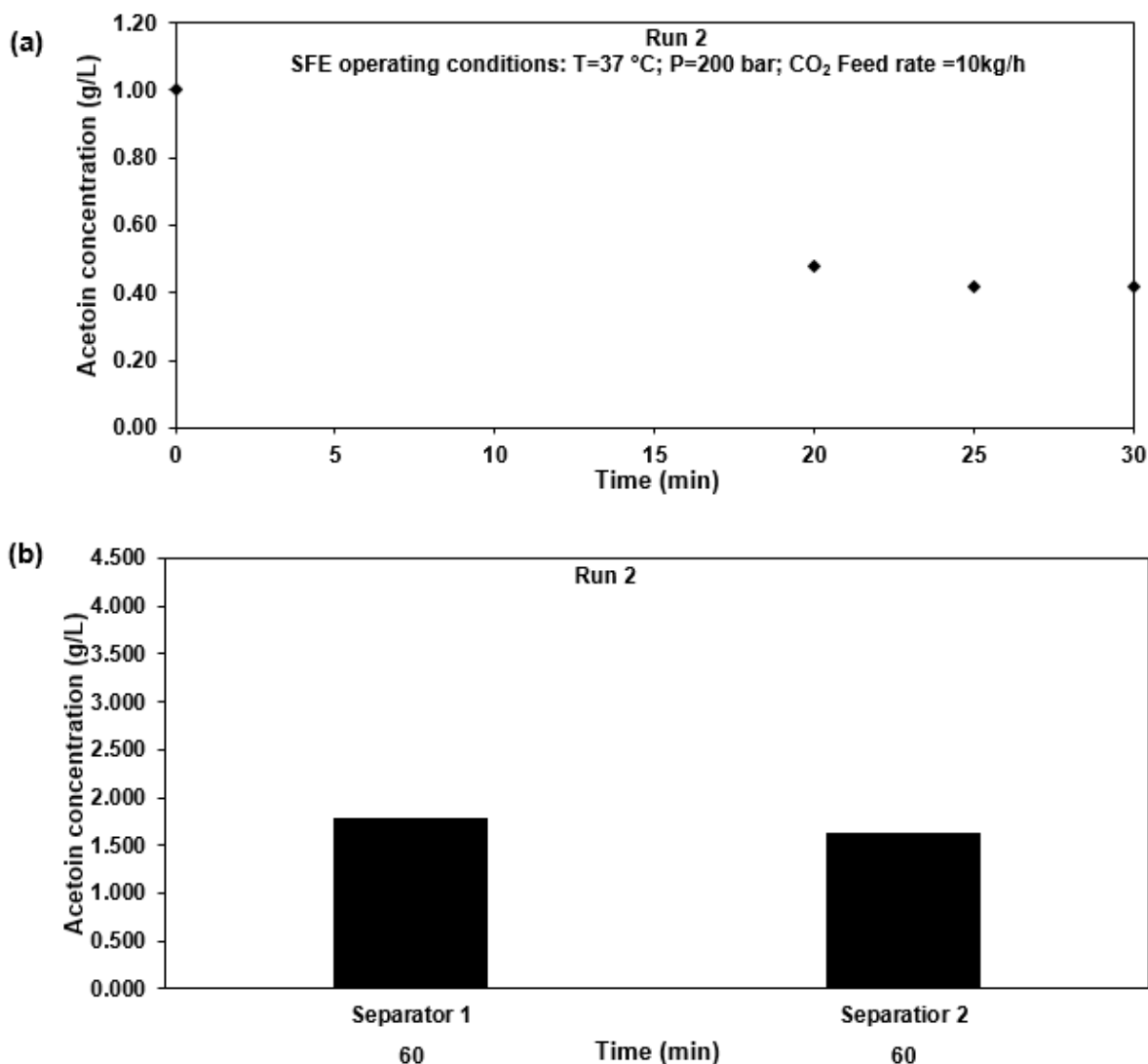
Figure 8.4; (a) Percentage recovery of simulated broth at the raffinate against sampling time at 37 °C, 200 bar, 10 kg/h (b) acetoin concentration against sampling time at the extract (separator 1 and 2) for experimental run 2

8.3.1.3 Effect of pressure on acetoin concentration and recovery from the simulated broth

The third experimental run (Run 3) replicated the column operating conditions of Run 2 with a modification in the change in pressure from 200 to 300 bar. The basis for this change was to study the effect of pressure change on acetoin concentration and recovery.

The concentration of acetoin obtained from the raffinate against the sampling time is shown graphically in Figure 8.5(a). Further, the concentration of the acetoin extracts at the separation vessels (separators 1 and 2) were drawn against the sampling time interval of 50 to 105 min, as displayed in Figure 8.5(b). The effects of increasing pressure from 200 to 300 bar while

maintaining temperature and CO_2 feed rate at 37 °C and 10 kg/h were investigated in Run 2 and Run 3, as depicted in Figure 8.4a and 8.5a respectively. The results showed a ten percent decrease (from 82 to 72%) in recovery of acetoin and a corresponding increase in a concentration factor of acetoin in the extract from 2 to 4 at 25 min contact time. This is explained by the principle that an increase in pressure increases the density of supercritical CO_2. This therefore resulted in an increase in solvation power (Pansuriya and Singhal, 2009).

Therefore, increased pressure at 300 bar decreased the percentage recovery of acetoin at the raffinate but increased the concentration factor of acetoin in the extract. The concentration of the extract in separators 1 and 2 were two and fourfold to that on the feed after 50 min contact time (Figure 8.5b).
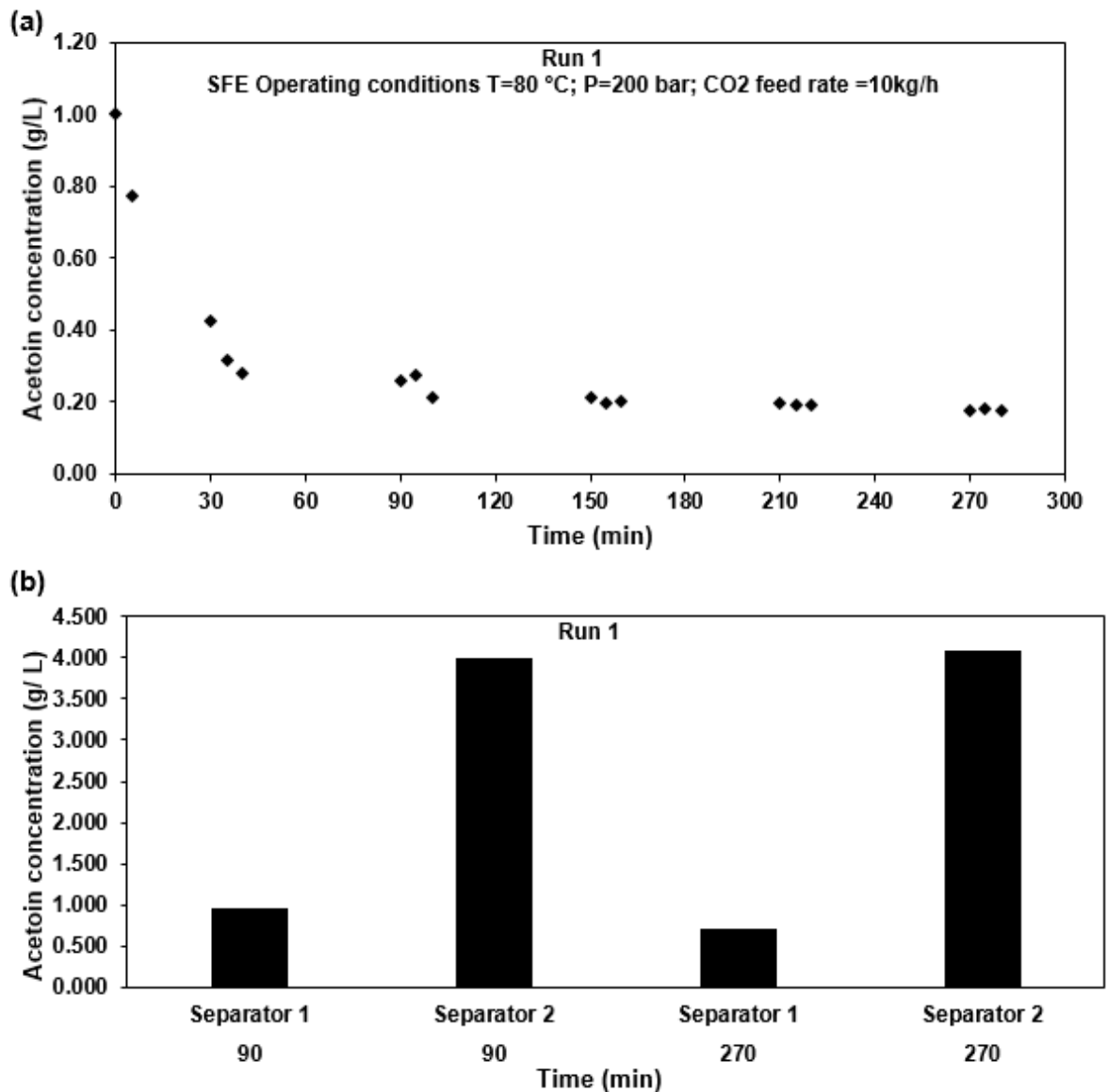
(a)
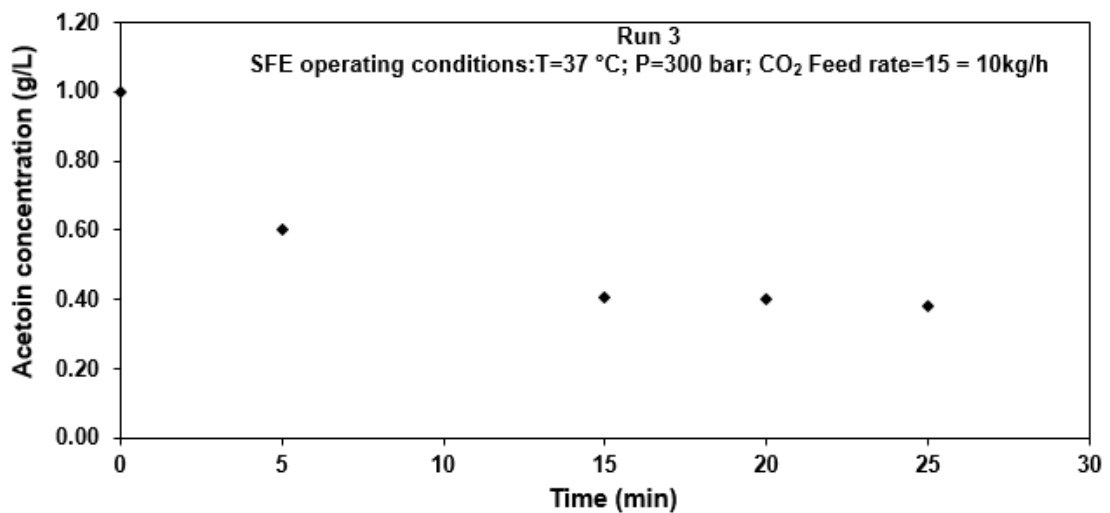
(b)

Figure 8.5: (a) Percentage recovery of simulated broth at the raffinate against sampling time at 37 °C, 300 bar, 10 kg/h (b) acetoin concentration against sampling time at the extract (separator 1 and 2) for experimental run 3

8.3.1.4 Effect of supercritical CO_2 feed rate on acetoin concentration and recovery from the simulated broth

The fourth experimental run (Run 4) was comparable to the column operating conditions of Run 3 with a modification in CO_2 feed rate changed from 10 to 15 kg/h. The reason for this modification was to examine the effects of supercritical CO_2 feed rate on the concentration and recovery of acetoin from the simulated broth.

The graphical display of the acetoin concentration obtained from the raffinate against the sampling time is shown in Figure 8.6(a). Further, the concentration of the acetoin extracts at the separation vessels (separator 1 and 2) was drawn against the sampling interval of 60 min as displayed in the bar chart of Figure 8.6 (b). For this study, the effects of changing supercritical fluid extraction feed rate from 10 to 15 kg/h while maintaining temperature at 37 °C and pressure of 300 bar is represented in Figures 8.5(a) and 8.6(a) of Run 3 and four (Run 4), consecutively. Figures 8.5(a) and 8.6(a) show the effect of $scCO_2$ feed rate on the concentration of acetoin with respect to the feed solution at the raffinate at a contact time of 5 to 30 min.

The increased $scCO_2$ feed rate from 10 to 15 kg/h increased the percentage recovery of acetoin in the raffinate with respect to the feed from 72 to 78% and a one percent increase in concentration factor (from four- to fivefold). This could be due to the fact that at low flow rates of the solvent, the mass transfer resistance limits the amount of solute transported into the bulk of the solvent, and the $scCO_2$ exits the column unsaturated. Further, as the flow rate increases, the mass transfer resistance continues to decrease until the exiting solvent is saturated, and thus equilibrium was achieved and increased recovery might be attained (Kumoro and Hasan, 2007). There was a fivefold increase in the concentration of acetoin extract in separator 1 and 2 after 60 minutes (Figure 8.6b).

Figure 8.6: (a) Percentage recovery of actual broth at the raffinate against sampling time at 37 °C, 300 bar, 15 kg/h (b) acetoin concentration against sampling time at the extract (separators 1 and 2) for experimental run 4

8.3.2 Acetoin concentration and recovery from the fermentation broth

The details of the fermentation broth preparation used as feed for acetoin recovery studies in supercritical fluid extraction were reported in section 3.5.2.2 of Chapter 3. This fermentation broth was centrifuged and used directly as feed in the supercritical fluid extraction pilot plant. The results obtained from the acetoin recovery studies in the simulated broth experiments were used with relevant literature data in related studies (Solana et al., 2016; Qureshi and Eller, 2018) as the basic operating conditions for validating the feasibility of acetoin recovery from fermentation broth. Therefore, three validation experiments were performed at a constant fermentation temperature of 37 °C while varying the pressure from 100 to 300 bar and CO_2 feed rate from 5 to 15 kg/h (Figure 8.7 to 8.10).

8.3.2.1.1　Validation of feasibility recovery using actual fermentation broth

The fifth experimental run (Run 5) was carried out as the first validation experiment. The column operating conditions were comparable to the literature condition sets by Solana et al. (2016) and Qureshi and Eller (2018). Acetoin concentration obtained from the raffinate and the estimated percentage recovery was drawn against the sampling interval time of 5 to 30 min as depicted in Figure 8.7. For this study, the column condition was set at a temperature of 37 °C, the pressure at 100 bar and CO_2 feed rate at 5 kg/h. It can be inferred from the results that the percentage recovery of acetoin with respect to the feed solution at the raffinate was one to twofold from 5 to 30 min contact time (Figure 8.7). The reason why the literature optimum fractionation conditions could not favor the concentration of acetoin more than one-to twofold in the extracts and raffinate may be due to factors such as difference in feed composition and the mass transfer mechanism which might have occurred since the authors referred to (Solana et al. (2016) and Qureshi and Eller (2018)) used bench-scale equipment as compared to pilot plant used in the present study (Prado et al., 2011).
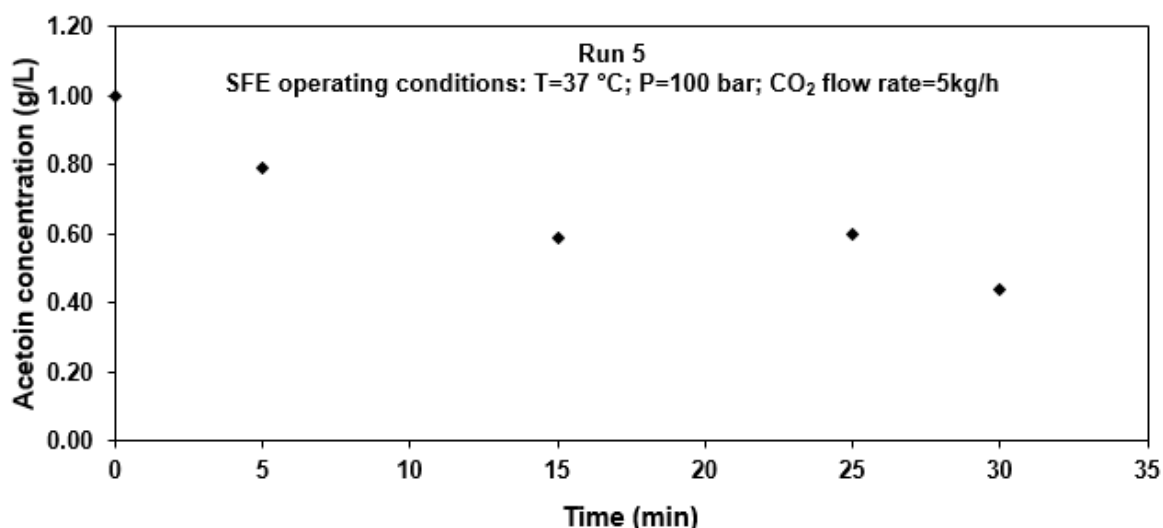
Figure 8.7: Percentage recovery of actual broth at the raffinate against sampling time at 37 °C, 100 bar, 5 kg/h

The sixth experimental run (Run 6) was the second validation test carried out. The column operating conditions in Run 2 with the simulated broth as feed were repeated for the fermentation broth validation test. The investigation was carried out at a temperature of 37 °C, pressure of 200 bar and 10 kg/h CO_2 feed rate. The concentration of acetoin obtained from the raffinate was plotted against the sampling time of 5 to 25 min as described in Figure 8.8. The concentration of acetoin with respect to the feed solution at the raffinate, at a contact time of 5 to 25 min was less than twofold that of the feed as shown in Figure 8.8.

The concentration of the extracts (separator 1) from the first and second validated experimental studies (Run 5 and Run 6) was plotted against the sampling interval of 30 min as displayed in Figure 8.9. There were no extracts obtained in separator two and three within the sampling time interval.

The concentrations of the acetoin extract in separator one were 1.41 and 0.91 g/L for validated Run 5 and Run 6 as depicted in Figure 8.9.
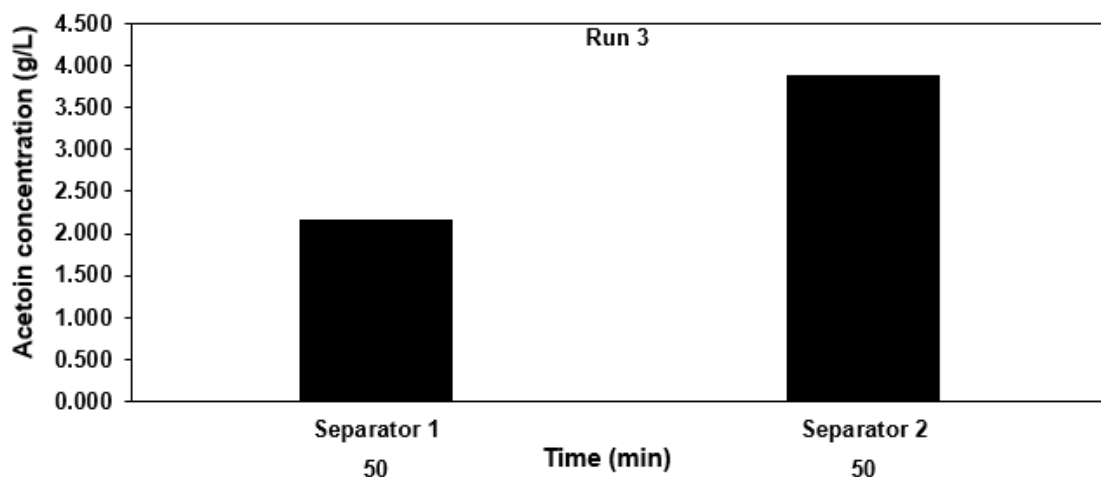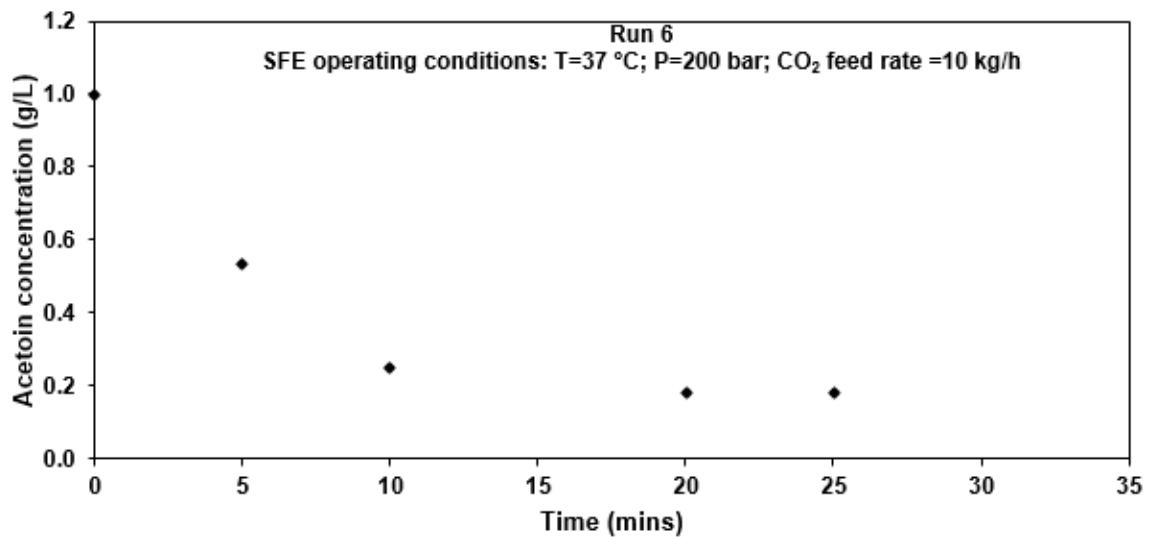

Figure 8.8: Percentage recovery of actual broth at the raffinate against sampling time at 37 °C, 200 bar, 10 kg/h
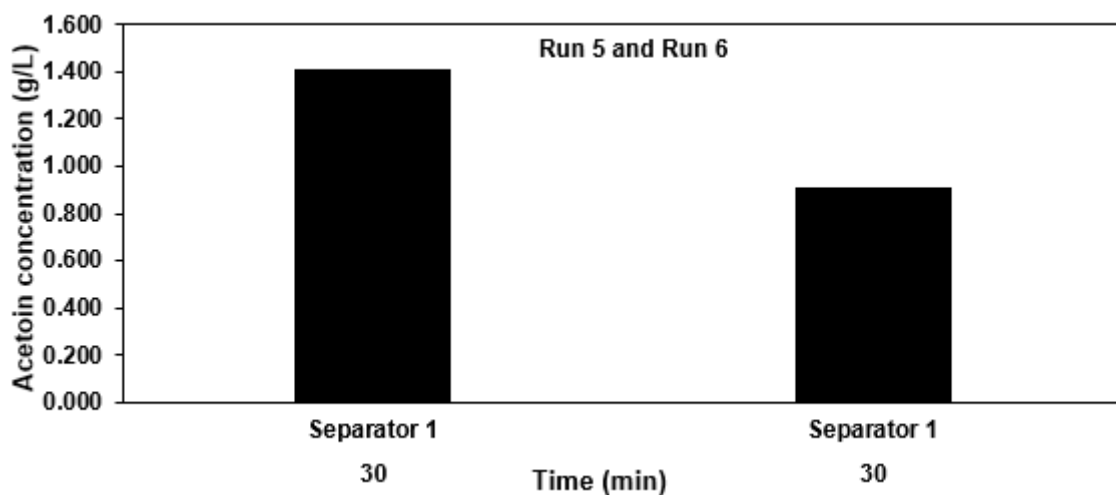
Figure 8.9: Acetoin concentration against sampling time (min) at the extract (separator 1) for experimental run 5 and 6

The seventh experimental run (Run 7) was the last validation test carried out on the actual fermentation broth. The same column operating conditions in Run 4 for simulation broth

investigation studies was chosen for the final validation test (Table 8.1). The basic reason for the choice of these conditions was the increased concentration factor recorded in the simulation broth studies (Figure 8.6a). For these studies, column operating conditions were set at a temperature of 37 °C, pressure of 300 bar and CO_2 feed rate of 15 kg/h. A graphic representation of the acetoin concentration obtained from the raffinate and the estimated percentage recovery was drawn against the sampling interval time of 5 to 30 min as shown in Figure 8.10(a). Further, the concentration of the acetoin extracts at the separation vessels (S1-separator 1, S2-separator 2 and S3-separator 3) was plotted against the sampling interval of 30 min as displayed in the bar chart of Figure 8.10(b).

In Figure 8.10(a), it can be deduced that the feasibility study compared well as the concentration of acetoin with respect to the feed solution at the raffinate, at a contact time of 5 to 30 min were (0.20 g/L) and (0.15 g/L) for the simulated and actual fermentation broth respectively (Figure 8.6a and 8.10a).

The concentrations of the extract in the two separators (1 and 2) were 0.41 and 0.65 g/L. About 2 g/L of acetoin was obtained after 30 minutes in separator three (Figure 8.10b). The result shows the feasibility of acetoin recovery using simulated broth as a demonstration for the actual fermentation broth recovery studies (Table 8.2). A concentration factor of 3 was estimated for the extract against the feed concentration and 77% recovery was obtainable at the raffinate.

Table 8.2: Experimental conditions of counter-current column of acetoin fractionation

Runs	Feed	Temperature (°C)	Pressure (bar)	CO_2 Feed rate (kg/h)	Concentration Factor	Recovery (%)
1	SB	80	200	10	6.41	83
2	SB	37	200	10	2.30	82
3	SB	37	300	10	4.00	72
4	SB	37	300	15	5.04	78
5	**FB**	**37**	**100**	**5**	**2.00**	**56**
6	**FB**	**37**	**200**	**10**	**1.20**	**68**
7	**FB**	**37**	**300**	**15**	**3.00**	**77**

SB-simulated broth; FB-fermentation broth; Bold-validated column conditions
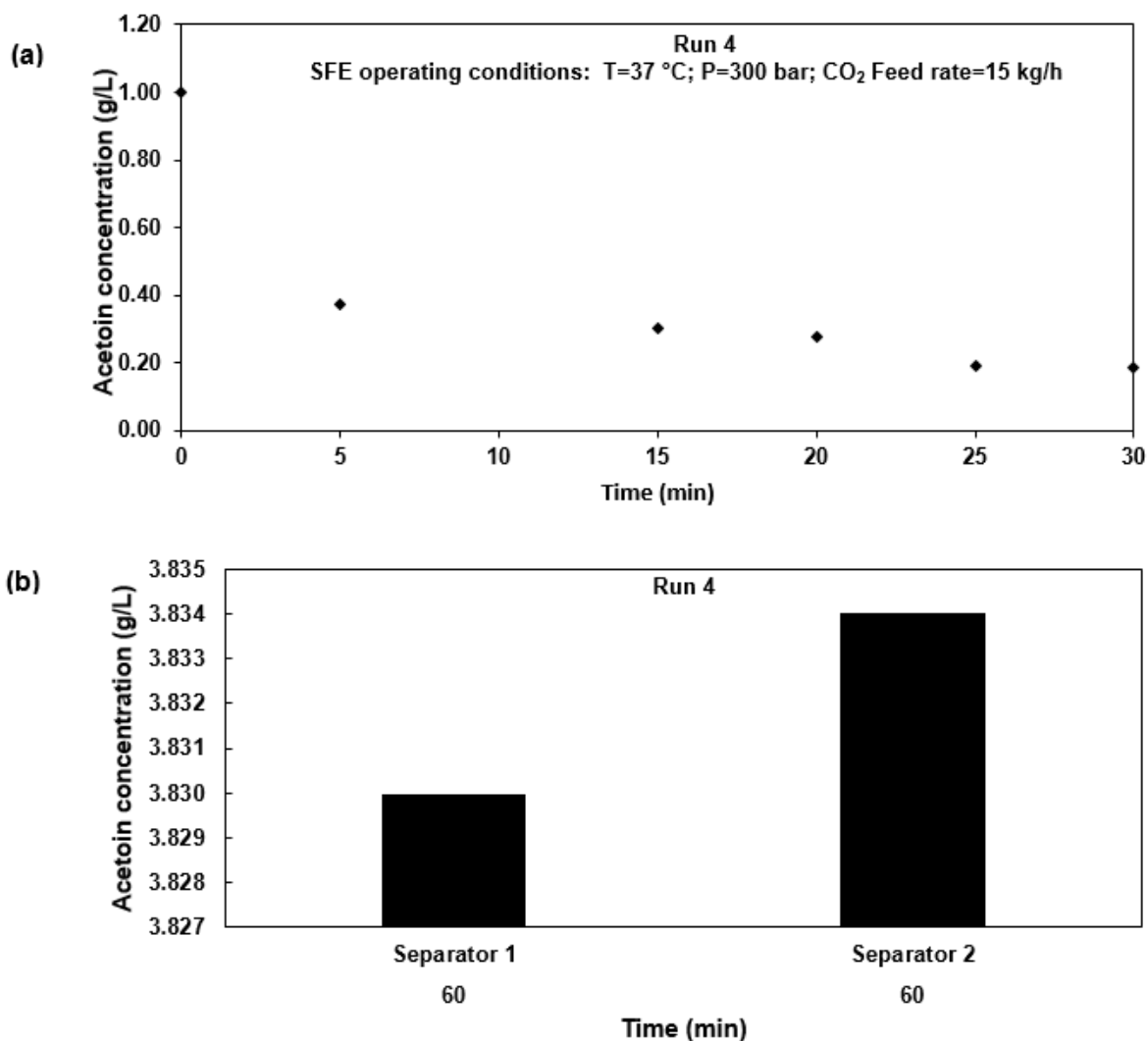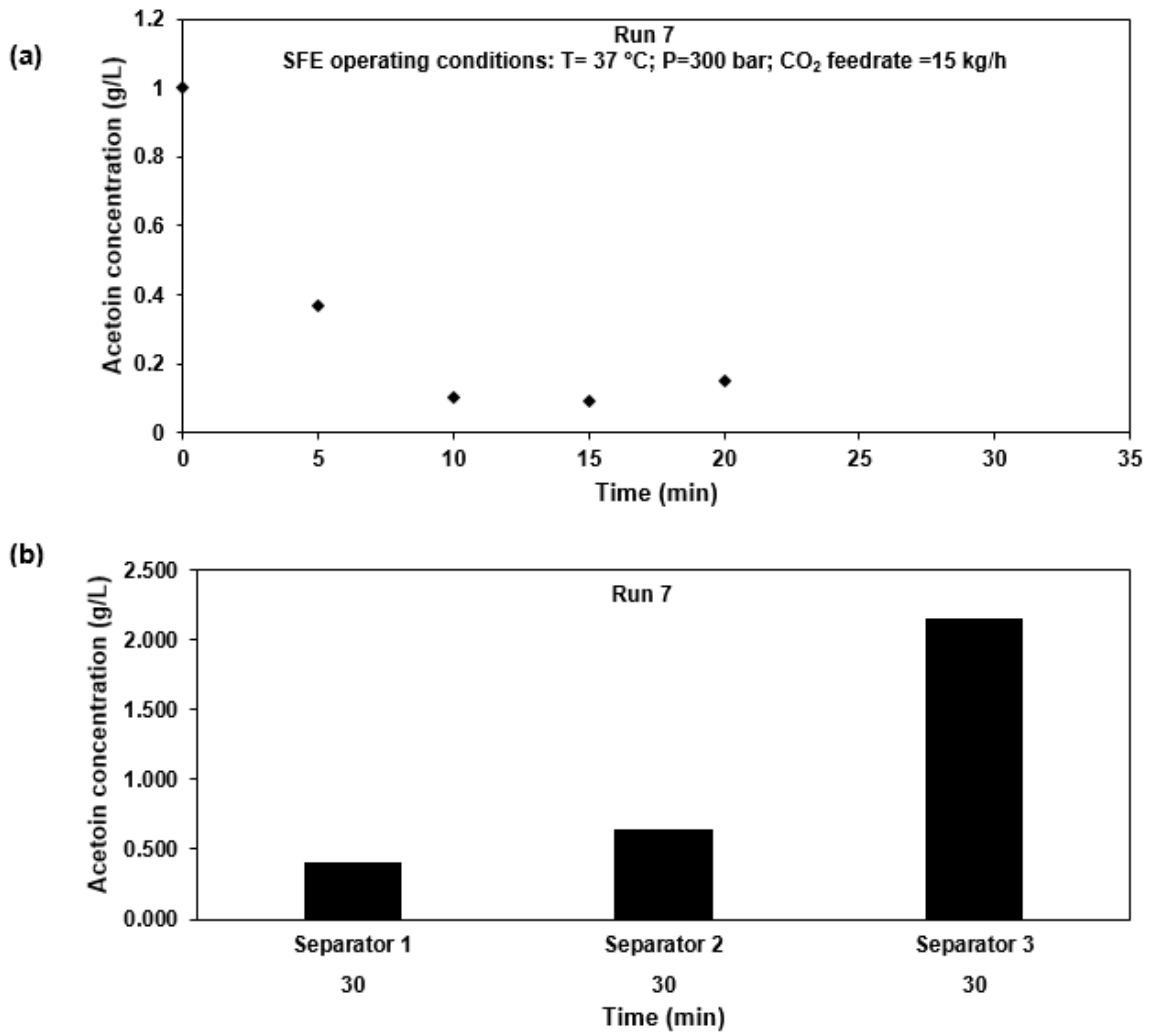
Figure 8.10: (a) Percentage recovery of actual broth at the raffinate against sampling time at 37 °C, 300 bar, 15 kg/h (b) acetoin concentration (g/L) against sampling time (min) at the extract (separator 1, 2 and 3) for experimental run 7

8.3.3 Acetoin concentration and recovery from fermentation and simulated broth by different separation techniques

Table 8.3 shows different reported techniques used in the recovery of acetoin from the broth. Dai et al. (2017a) extracted acetoin from *B. subtilis* DL01 fermentation broth with six solvents. The authors considered the influence of six solvents on the growth of bacteria and the dispersion of acetoin and glucose in the sugaring-out extraction technique. About 60% of acetoin was recovered from the broth. Further purification of acetoin broth was done using vacuum distillation.

In another study, the same authors investigated the recovery of acetoin from fermentation broth using the salting-out extraction (SOE) technique. Single-stage salting out extraction and two-stage counter current technique were used for separation. Ethyl acetate and dipotassium phosphate were explored in the extraction and purification process. An acetoin recovery of 95.3% was obtained through a single SOE process using mixed solvent of ethanol and 20% ethyl acetate; recovery of 91.3% by two-stage counter current salting-out extraction was obtained with ethyl acetate as solvent and dipotassium phosphate concentration 50% (m/v) in fermentation broth (Dai et al., 2017b).

Sun et al. (2012) An aqueous two-phase system (ATPS) containing acetone and phosphate was used to extract acetoin from fermentation broth. A partition and recovery coefficient of 22.3% and 96.4% of acetoin were attained by a system made up of 30% acetone and 35% dipotassium hydrogen phosphate. Further purification steps were conducted through pH adjustment and dilution crystallization. Although these reported techniques seem promising, the chance of solvent residue remaining in the final product is still a certainty. The results of the present study show that the supercritical CO_2 extraction technique compares well with the reported methods found in the literature for acetoin downstream processing.

The results of the present study show that the supercritical CO_2 extraction technique compares well with the reported method found in the literature for acetoin downstream processing.

Table 8.3: Separation techniques used in acetoin recovery

S/N	Separation technique	Extractant	Recovery (%)	K	Broth source	Reference
1	Sugaring-out extraction	Different organic solvent/glucose systems	61.2	0.61*	*B. subtilis DL01*	(Dai et al., 2017a)
2	Single-stage salting-out extraction	Ethanol	95.3	5.56	*B. subtilis DL01*	(Dai et al., 2017b)
3	Two-stage countercurrent salting-out extraction	Ethyl acetate and Dipotassium phosphate	91.3	18.7	*B. subtilis DL01*	Dai et al., 2017b)
4	Salting-out extraction	Acetone/phosphate	96.4	22.3	*Serratia marcescens H32*	(Sun et al., 2012)
5	**Supercritical fluid extraction**	**Carbon dioxide**	**78**	**20.61**	**Simulated broth**	**Present study**
6	**Supercritical fluid extraction**	**Carbon dioxide**	**77**	**14.33**	***B. subtilis CICC 10025***	**Present study**

*Calculated; $K = C_E/C_R$; K- distribution coefficient; C_E = concentration of solute at extract and C_R = concentration of solute at raffinate respectively

8.4   Conclusion

Although different techniques are being tested for acetoin extraction with reasonable percentage recovery yield, nevertheless, the question of toxic residues and environmental friendliness related to the solvent used in earlier extraction studies remains unanswered. The results from this study show that it is possible to recover acetoin at 78% and 77% from the raffinate in both simulated and fermentation broth using supercritical CO_2. The supercritical fluid extraction technique used in this study performs at a comparable percentage to the previous method reported in the literature (Table 8.2). It is worth nothing that this study is the first to use a method of supercritical fluid extraction (SFE) to recover acetoin from fermentation broth at the time of the investigation.

Chapter 9   CONCLUSIONS AND RECOMMENDATIONS

9.1   Summary and conclusions

The aim of this work was to investigate the application of integrated bioprocess-supercritical fluid extraction techniques for the production and recovery of selected bioproducts (ethanol, acetoin and vanillin) with a view to developing a low-cost process that might be of economic importance. It was hypothesized that supercritical fluid fractionation is a technically and economically viable alternative to existing separation methods for the commercial-scale production of the selected bioproducts. The spectrophotometric method was developed and used in determining the concentration of the targeted product in the broth, while the product recovery was carried out using a supercritical fluid extraction and fractionation system. The microbes chosen for the biotransformation of the selected bioproducts were *Saccharomyces* and *Bacillus* species.

The study began with ethanol being the first bioproduct, while corn steep liquor (CSL) was identified as a good nitrogen supplement for ethanol production. Response surface methodology (RSM) and artificial neural network (ANN) were used as statistical tools in optimizing the ethanol production variables. The optimal condition values established for the ethanol concentration using RSM were CSL of 5.36 (% w/v), pH of 4, time of 13 h, temperature of 28 °C, inoculum size of 0.72% v/v, and with a predicted concentration of 45.48 g/L. This was experimentally validated as 45.36 g/L. The optimal condition values established for ethanol concentration using ANN were CSL of 0.5 (% w/v), pH of 5, time of 36 h, temperature of 34 °C, and inoculum size of 5.49% v/v, with predicted concentration of 45.73 g/L. This was also validated experimentally as 40.6 g/L. The study demonstrated that Anchor Instant Yeast strain could perform almost the same as the industrial *Saccharomyces* Type 1 yeast strain in ethanol fermentation. The variables needed to develop a low-cost fermentation medium that was definite and devoid of redundant nutritional supplements was established. The results may provide a basis for establishing the factors needed for scaling up of a typical process.

Acetoin production and statistical optimization were carried out in batch fermentation. The feasibility of corn steep liquor to replace yeast extract and beef extract which were expensive nutrient sources in acetoin fermentation was investigated. The model that best described the acetoin fermentation process was a quadratic model with R^2 of 0.930. The most significant positive factors for the process were glucose concentration and corn steep liquor while inoculum size was an insignificant factor in acetoin fermentation. The optimal condition predicted for the three independent factors was glucose concentration of 78.40 g/L, CSL of 15.00% w/v, inoculum size of 2.70% v/v, which were validated experimentally with acetoin concentration of 10.70 g/L. The results of this study justify the need for the development of

cost-effective nutritional use of corn steep liquor for the bioproduction of acetoin on an industrial scale.

The bioconversion of ferulic acid into vanillin was investigated considering the effect of certain controlling variables. The prospect of corn steep liquor as a low-cost nitrogen source was explored for the bioconversion of ferulic acid to vanillin production in this work. Results showed that corn steep liquor, initial ferulic acid concentration and pH of the culture medium had profound effects on the amount of vanillin produced using *Bacillus amyloliquefaciens subsp*. The best model that described the biotransformation process was a second-order mathematical model with R^2 of 0.995. The optimal condition predicted for the three independent factors was CSL of 7.72 g/L, the initial ferulic acid concentration of 2.33 g/L and pH of 9.34, which were validated experimentally with vanillin concentration of 386 mg/L.

Statistical optimization and validation of fermentation process variables for improved acetoin production from shake flask into a bioreactor using Taguchi orthogonal array design was further studied. The influence of agitation, aeration, time and pH was analyzed by Taguchi orthogonal array design. The statistical model was successfully validated with agitation as the most controlling factor. Optimized process parameters of 300 rpm agitation, 1.5 slpm aerations, time 2 days and pH of 6.5 were scaled up at bioreactor level with a fourfold gain (42.30 g/L) of acetoin titre when compared to the shake flask optimization study. The results show that the commercial production of acetoin can be envisaged since there is an increasing yield in the scale-up studies of acetoin in the bioreactor.

The feasibility of acetoin recovery from simulated and fermentation broth was studied in the supercritical CO_2 pilot plant. The effect of supercritical parameters such as pressure, CO_2 feed rate, temperature and contact time were investigated. The results from this study show that it is possible to recover acetoin at 78 and 77% in both simulated and fermentation broth respectively. The supercritical fluid extraction technique used in this study performs at a comparable percentage to the previous methods reported in the literature.

In conclusion, this study provides the fundamental basis needed for the development and design of processes for the production and recovery of the three selected bioproducts (bioethanol, acetoin, and vanillin). The lessons gathered from this study could be applicable to process development for the production and recovery of other bioproducts.

Therefore, scientific improvements presented by this study are as follows:
- ❖ Optimization variables that may be used for the commercial production of the selected bioproducts (ethanol, acetoin, and vanillin) were established using a low-cost fermentation medium.

- ❖ Acetoin concentration and recovery from fermentation broth using supercritical techniques were tested for the first time.
- ❖ The technical feasibility of the bioprocess-supercritical CO_2 extraction technique was established.

9.2 Recommendations

This study did not include an economic evaluation of the process investigated. Thus, the cost-benefit analysis of the bioprocess-supercritical CO_2 extraction technique needs to be addressed in order to evaluate the operational cost of the process and make an informed decision about its commercialization. Further, there is a call for more efficient process development, separation and recovery of the selected products from dilute aqueous systems for easy integration with high pressure technology.

References

Abbasiliasi, S., Tan, J. S., Ibrahim, T. a. T., Bashokouh, F., Ramakrishnan, N. R., Mustafa, S. & Ariff, A. B. 2017. Fermentation factors influencing the production of bacteriocins by lactic acid bacteria: A review. *RSC Advances,* 7(47)**:**29395-29420.

Açıkel, Ü., Erşan, M. & Açıkel, Y. S. 2010. Optimization of critical medium components using response surface methodology for lipase production by rhizopus delemar. *Food and Bioproducts Processing,* 88(1)**:**31-39.

Aden, A. & Foust, T. 2009. Technoeconomic analysis of the dilute sulfuric acid and enzymatic hydrolysis process for the conversion of corn stover to ethanol. *Cellulose,* 16(4)**:**535-545.

Águeda, V. I., Delgado, J. A., Uguina, M. A., Sotelo, J. L. & García, Á. 2013. Column dynamics of an adsorption–drying–desorption process for butanol recovery from aqueous solutions with silicalite pellets. *Separation and Purification Technology,* 104**:**307-321.

Alade, O. & Betiku, E. 2010. Design of a continuous bioreactor for laboratory scale production of ethanol from cassava starch hydrolysate using saccharomyces cerevisiae. *Ife Journal of Technology,* 19(2)**:**15-20.

Albers, E., Larsson, C., Lidén, G., Niklasson, C. & Gustafsson, L. 1996. Influence of the nitrogen source on saccharomyces cerevisiae anaerobic growth and product formation. *Applied and environmental microbiology,* 62(9)**:**3187-3195.

Amartey, S. & Jeffries, T. W. 1994. Comparison of corn steep liquor with other nutrients in the fermentation of d-xylose by pichia stipitis cbs 6054. *Biotechnology Letters,* 16(2)**:**211-214.

Andualem, B. & Gessesse, A. 2013. Production of microbial medium from defatted brebra (milletia ferruginea) seed flour to substitute commercial peptone agar. *Asian Pacific Journal of Tropical Biomedicine,* 3(10)**:**790-797.

Ashengroph, M., Nahvi, I. & Amini, J. 2013. Application of taguchi design and response surface methodology for improving conversion of isoeugenol into vanillin by resting cells of psychrobacter sp. Csw4. *Iranian Journal of Pharmaceutical Research,* 12(3)**:**411-421.

Attard, T., Mcelroy, C. & Hunt, A. 2015. Economic assessment of supercritical co_2 extraction of waxes as part of a maize stover biorefinery. *International Journal of Molecular Sciences,* 16(8)**:**17546-17564.

Azhar, S. H. M., Abdulla, R., Jambo, S. A., Marbawi, H., Gansau, J. A., Faik, A. a. M. & Rodrigues, K. F. 2017. Yeasts in sustainable bioethanol production: A review. *Biochemistry and Biophysics Reports,* 10**:**52-61.

Bae, S.-J., Kim, S. & Hahn, J.-S. 2016. Efficient production of acetoin in saccharomyces cerevisiae by disruption of 2, 3-butanediol dehydrogenase and expression of nadh oxidase. *Scientific reports,* 6**:**27667.

Bajaj, I. B. & Singhal, R. S. 2010. Effect of aeration and agitation on synthesis of poly (γ-glutamic acid) in batch cultures of bacillus licheniformis ncim 2324. *Biotechnology and Bioprocess Engineering,* 15(4)**:**635-640.

Banchero, M. 2013. Supercritical fluid dyeing of synthetic and natural textiles–a review. *Coloration Technology,* 129(1)**:**2-17.

Baral, N. R. & Shah, A. 2016. Techno-economic analysis of cellulose dissolving ionic liquid pretreatment of lignocellulosic biomass for fermentable sugars production. *Biofuels, Bioproducts and Biorefining,* 10(1)**:**70-88.

Barghini, P., Di Gioia, D., Fava, F. & Ruzzi, M. 2007. Vanillin production using metabolically engineered escherichia coli under non-growing conditions. *Microbial Cell Factories,* 6(1)**:**13.

Barghini, P., Montebove, F., Ruzzi, M. & Schiesser, A. 1998. Optimal conditions for bioconversion of ferulic acid into vanillic acid by pseudomonas fluorescens bf13 cells. *Applied Microbiology and Biotechnology,* 49(3)**:**309-314.

116

Baş, D. & Boyacı, İ. H. 2007. Modeling and optimization ii: Comparison of estimation capabilities of response surface methodology with artificial neural networks in a biochemical reaction. *Journal of Food Engineering,* 78(3)**:**846-854.

Basri, M., Rahman, R. N. Z. R. A., Ebrahimpour, A., Salleh, A. B., Gunawan, E. R. & Rahman, M. B. A. 2007. Comparison of estimation capabilities of response surface methodology (rsm) with artificial neural network (ann) in lipase-catalyzed synthesis of palm-based wax ester. *BMC Biotechnology,* 7(1)**:**53.

Bengtsson, M., Alfredsson, E., Cohen, M., Lorek, S. & Schroeder, P. 2018. Transforming systems of consumption and production for achieving the sustainable development goals: Moving beyond efficiency. *Sustainability science,* 13(6)**:**1533-1547.

Benner Jr, B. A. 2015. Supercritical fluid extraction of organics in environmental analysis. *Encyclopedia of Analytical Chemistry***:**1-15.

Bernad, L., Keller, A., Barth, D. & Perrut, M. 1993. Separation of ethanol from aqueous solutions by supercritical carbon dioxide—comparison between simulations and experiments. *The Journal of supercritical fluids,* 6(1)**:**9-14.

Bers, J. A., Dismukes, J. P., Miller, L. K. & Dubrovensky, A. 2009. Accelerated radical innovation: Theory and application. *Technological Forecasting and Social Change,* 76(1)**:**165-177.

Betiku, E. & Alade, O. 2014. Media evaluation of bioethanol production from cassava starch hydrolysate using saccharomyces cerevisiae. *Energy Sources, Part A,* 36(18)**:**1990-1998.

Betiku, E., Odude, V. O., Ishola, N. B., Bamimore, A., Osunleke, A. S. & Okeleye, A. A. 2016. Predictive capability evaluation of rsm, anfis and ann: A case of reduction of high free fatty acid of palm kernel oil via esterification process. *Energy conversion and management,* 124**:**219-230.

Betiku, E., Omilakin, O. R., Ajala, S. O., Okeleye, A. A., Taiwo, A. E. & Solomon, B. O. 2014. Mathematical modeling and process parameters optimization studies by artificial neural network and response surface methodology: A case of non-edible neem (azadirachta indica) seed oil biodiesel synthesis. *Energy,* 72**:**266-273.

Betiku, E. & Taiwo, A. E. 2015. Modeling and optimization of bioethanol production from breadfruit starch hydrolyzate vis-à-vis response surface methodology and artificial neural network. *Renewable Energy,* 74**:**87-94.

Bhamidipati, M., Scurto, A. M. & Detamore, M. S. 2013. The future of carbon dioxide for polymer processing in tissue engineering. *Tissue Engineering Part B: Reviews,* 19(3)**:**221-232.

Bhatia, S., Sharma, K., Dahiya, R. & Bera, T. 2015. History and scope of plant biotechnology. *In:* BHATIA, S., SHARMA, K., DAHIYA, R. & BERA, T. (eds.) *Modern applications of plant biotechnology in pharmaceutical sciences.* Elsevier Inc: Academic Press.

Birajdar, S. D., Padmanabhan, S. & Rajagopalan, S. 2014. Rapid solvent screening using thermodynamic models for recovery of 2, 3-butanediol from fermentation by liquid–liquid extraction. *Journal of Chemical & Engineering Data,* 59(8)**:**2456-2463.

Boock, J. T., Freedman, A. J., Tompsett, G. A., Muse, S. K., Allen, A. J., Jackson, L. A., Castro-Dominguez, B., Timko, M. T., Prather, K. L. & Thompson, J. R. 2019. Engineered microbial biofuel production and recovery under supercritical carbon dioxide. *Nature Communications,* 10(1)**:**587.

Broach, J. R. 2012. Nutritional control of growth and development in yeast. *Genetics,* 192(1)**:**73-105.

Brochado, A. R., Matos, C., Møller, B. L., Hansen, J., Mortensen, U. H. & Patil, K. R. 2010. Improved vanillin production in baker's yeast through in silico design. *Microbial cell factories,* 9(1)**:**1.

Brunner, G. 2013. *Gas extraction: An introduction to fundamentals of supercritical fluids and the application to separation processes*, Springer Science & Business Media.

Budich, M. & Brunner, G. 2003. Supercritical fluid extraction of ethanol from aqueous solutions. *The Journal of supercritical fluids,* 25(1)**:**45-55.

Bull, A. T. 2001. Biotechnology for industrial sustainability. *Korean Journal of Chemical Engineering,* 18(2)**:**137-148.

Campo, G. & Carmenálajo, M. 1992. Spectrophotometric determination of biacetyl in distillates of wine by flow injection. *Analyst,* 117(8)**:**1343-1346.

Capuzzo, A., Maffei, M. & Occhipinti, A. 2013. Supercritical fluid extraction of plant flavors and fragrances. *Molecules,* 18(6)**:**7194-7238.

Carlquist, M., Gibson, B., Karagul Yuceer, Y., Paraskevopoulou, A., Sandell, M., Angelov, A. I., Gotcheva, V., Angelov, A. D., Etschmann, M. & Billerbeck, G. M. 2015. Process engineering for bioflavour production with metabolically active yeasts–a mini-review. *Yeast,* 32(1)**:**123-143.

Carroll, A. L., Desai, S. H. & Atsumi, S. 2016. Microbial production of scent and flavor compounds. *Current opinion in biotechnology,* 37**:**8-15.

Catchpole, O., Tallon, S., Dyer, P., Montanes, F., Moreno, T., Vagi, E., Eltringham, W. & Billakanti, J. 2012. Integrated supercritical fluid extraction and bioprocessing. *American Journal of Biochemistry and Biotechnology,* 8**:**263-287.

Catchpole, O. J., Tallon, S. J., Eltringham, W. E., Grey, J. B., Fenton, K. A., Vagi, E. M., Vyssotski, M. V., Mackenzie, A. N., Ryan, J. & Zhu, Y. 2009. The extraction and fractionation of specialty lipids using near critical fluids. *The Journal of Supercritical Fluids,* 47(3)**:**591-597.

Celińska, E. & Grajek, W. 2009. Biotechnological production of 2, 3-butanediol—current state and prospects. *Biotechnology advances,* 27(6)**:**715-725.

Chandrashekhar, H. & Rao, J. V. 2010. An overview of fermenter and the design considerations to enhance its productivity. *Pharmacologyonline,* 1**:**261-301.

Chen, H.-J., Chang, S.-N. & Tang, C.-W. 2017a. Application of the taguchi method for optimizing the process parameters of producing lightweight aggregates by incorporating tile grinding sludge with reservoir sediments. *Materials,* 10(11)**:**1294.

Chen, P., Yan, L., Wu, Z., Li, S., Bai, Z., Yan, X., Wang, N., Liang, N. & Li, H. 2016. A microbial transformation using bacillus subtilis b7-s to produce natural vanillin from ferulic acid. *Scientific reports,* 6**:**20400.

Chen, P., Yan, L., Zhang, S., Wu, Z., Li, S., Yan, X., Wang, N., Liang, N. & Li, H. 2017b. Optimizing bioconversion of ferulic acid to vanillin by bacillus subtilis in the stirred packed reactor using box-behnken design and desirability function. *Food Science and Biotechnology,* 26(1)**:**143-152.

Choudhury, A. R., Bhattacharjee, P. & Prasad, G. S. 2013. Development of suitable solvent system for downstream processing of biopolymer pullulan using response surface methodology. *PloS one,* 8(10)**:**e77071.

Clark, J. H. & Macquarrie, D. J. 2008. *Handbook of green chemistry and technology*, John Wiley & Sons.

Clark, S. & Winter, C. K. 2015. Diacetyl in foods: A review of safety and sensory characteristics. *Comprehensive Reviews in Food Science and Food Safety,* 14(5)**:**634-643.

Converti, A., Aliakbarian, B., Domínguez, J., Vázquez, G. B. & Perego, P. 2010. Microbial production of biovanillin. *Brazilian Journal of Microbiology,* 41(3)**:**519-530.

Costa, E., Teixidó, N., Usall, J., Atarés, E. & Viñas, I. 2002. The effect of nitrogen and carbon sources on growth of the biocontrol agent pantoea agglomerans strain cpa-2. *Letters in applied microbiology,* 35(2)**:**117-120.

Cvjetko Bubalo, M., Vidović, S., Radojčić Redovniković, I. & Jokić, S. 2015. Green solvents for green technologies. *Journal of Chemical Technology and Biotechnology,* 90(9)**:**1631-1639.

Dai, J.-Y., Cheng, L., He, Q.-F. & Xiu, Z.-L. 2015a. High acetoin production by a newly isolated marine bacillus subtilis strain with low requirement of oxygen supply. *Process Biochemistry,* 50(11)**:**1730-1734.

Dai, J.-Y., Liu, C.-J. & Xiu, Z.-L. 2015b. Sugaring-out extraction of 2, 3-butanediol from fermentation broths. *Process Biochemistry,* 50(11)**:**1951-1957.

Dai, J.-Y., Ma, L.-H., Wang, Z.-F., Guan, W.-T. & Xiu, Z.-L. 2017a. Sugaring-out extraction of acetoin from fermentation broth by coupling with fermentation. *Bioprocess and biosystems engineering,* 40(3)**:**423-429.

Dai, J., Guan, W., Ma, L. & Xiu, Z. 2017b. Salting-out extraction of acetoin from fermentation broth using ethyl acetate and k2hpo4. *Separation and Purification Technology,* 184**:**275-279.

Daneshi, A., Younesi, H., Ghasempouri, S. M. & Sharifzadeh, M. 2010. Production of poly-3-hydroxybutyrate by cupriavidus necator from corn syrup: Statistical modeling and optimization of biomass yield and volumetric productivity. *Journal of Chemical Technology and Biotechnology,* 85(11)**:**1528-1539.

Danielski, L. 2007. *Extraction and fractionation of natural organic compounds from plant materials with supercritical carbon dioxide.* PhD thesis, Technische Universität Hamburg.

Danielski, L., Ferreira, S. R., Hense, H., Martínez, J. & Brunner, G. 2008. Deterpenation of citrus peel oils with supercritical carbon dioxide–a review. *In:* BENKEBLIA, N. T. (ed.) *Citrus i. Tree and forestry science and biotechnology* (Special Issue 1) ed.: Global Books.

Dar, A. & Anuradha, N. 2018. Use of orthogonal arrays and design of experiment via taguchi l9 method in probability of default. *Accounting,* 4(3)**:**113-122.

Darani, K. K. & Mozafari, M. R. 2010. Supercritical fluids technology in bioprocess industries: A review. *Journal of Biochemical Technology,* 2(1)**:**144-152.

Das, S. P., Das, D. & Goyal, A. 2014. Statistical optimization of fermentation process parameters by taguchi orthogonal array design for improved bioethanol production. *Journal of Fuels,* 2014**:**11.

Davis, L., Rogers, P., Pearce, J. & Peiris, P. 2006. Evaluation of zymomonas-based ethanol production from a hydrolysed waste starch stream. *Biomass Bioenergy,* 30(8)**:**809-814.

Dawiec-Liśniewska, A., Szumny, A., Podstawczyk, D. & Witek-Krowiak, A. 2018. Concentration of natural aroma compounds from fruit juice hydrolates by pervaporation in laboratory and semi-technical scale. Part 1. Base study. *Food Chemistry,* 258**:**63-70.

De Castro, A. M., López, J. A., Dos Reis Castilho, L. & Freire, D. M. G. 2014. Techno-economic analysis of a bioprocess for the production of multienzyme solutions from the cake of babassu industrial processing: Evaluation of five different inoculum propagation strategies. *Biomass Conversion and Biorefinery,* 4(3)**:**237-247.

De La Vega, G. R., Cervantes, M. S., Alvarado, M. G., Romero-Martínez, A. & Hegel, P. 2016. Fractionation of vanilla oleoresin by supercritical co 2 technology. *The Journal of supercritical fluids,* 108**:**79-88.

Derringer, G. & Suich, R. 1980. Simultaneous optimization of several response variables. *Journal of quality technology,* 12(4)**:**214-219.

Dhillon, G. S., Kaur, S., Brar, S. K. & Verma, M. 2012. Potential of apple pomace as a solid substrate for fungal cellulase and hemicellulase bioproduction through solid-state fermentation. *Industrial Crops and Products,* 38**:**6-13.

Di Gioia, D., Luziatelli, F., Negroni, A., Ficca, A. G., Fava, F. & Ruzzi, M. 2011. Metabolic engineering of pseudomonas fluorescens for the production of vanillin from ferulic acid. *Journal of Biotechnology,* 156(4)**:**309-316.

Di Gioia, D., Sciubba, L., Setti, L., Luziatelli, F., Ruzzi, M., Zanichelli, D. & Fava, F. 2007. Production of biovanillin from wheat bran. *Enzyme and microbial technology,* 41(4)**:**498-505.

Díaz-Montaño, D. M. 2013. Continuous agave juice fermentation for producing bioethanol. *Biomass now-sustainable growth and use.* IntechOpen.

Díaz-Reinoso, B., Moure, A., Domínguez, H. & Parajó, J. C. 2006. Supercritical co$_2$ extraction and purification of compounds with antioxidant activity. *Journal of Agricultural and Food Chemistry,* 54(7)**:**2441-2469.

Doran, P. M. 2012. *Bioprocess engineering principles,* London, Academic press.

Dumancas, G. G., Adrianto, I., Bello, G. & Dozmorov, M. 2017. Current developments in machine learning techniques in biological data mining. *Bioinformatics and Biology Insights,* 11**:**1-4.

Ebrahimpour, A., Rahman, R. N. Z. R. A., Ch'ng, D. H. E., Basri, M. & Salleh, A. B. 2008. A modeling study by response surface methodology and artificial neural network on culture parameters optimization for thermostable lipase production from a newly isolated thermophilic geobacillus sp. Strain arm. *BMC Biotechnology,* 8(1)**:**96.

Edwinoliver, N., Thirunavukarasu, K., Purushothaman, S., Rose, C., Gowthaman, M. & Kamini, N. 2009. Corn steep liquor as a nutrition adjunct for the production of aspergillus niger lipase and hydrolysis of oils thereof. *Journal of agricultural and food chemistry,* 57(22)**:**10658-10663.

Effendi, C., Shanty, M., Ju, Y.-H., Kurniawan, A., Wang, M.-J., Indraswati, N. & Ismadji, S. 2013. Measurement and mathematical modeling of solubility of buttery-odor substance (acetoin) in supercritical co_2 at several pressures and temperatures. *Fluid Phase Equilibria,* 356**:**102-108.

Eller, F., Taylor, S., Compton, D., Laszlo, J. & Palmquist, D. 2008. Counter-current liquid carbon dioxide purification of a model reaction mixture. *The Journal of supercritical fluids,* 43(3)**:**510-514.

Eren, H., Avinc, O. & Eren, S. Supercritical carbon dioxide for textile applications and recent developments. IOP Conference Series: Materials Science and Engineering, 2017. IOP Publishing, 082011.

Evolva. 2017. *Vanillin : A sustainable production route* [Online]. Evolva. Available: http://www.evolva.com/vanillin/ [Accessed May 2017].

Fabre, C. E., Condoret, J. S. & Marty, A. 1999. Extractive fermentation of aroma with supercritical co2. *Biotechnology and bioengineering,* 64(4)**:**392-400.

Feng, Y. & Meier, D. 2015. Extraction of value-added chemicals from pyrolysis liquids with supercritical carbon dioxide. *Journal of analytical and applied pyrolysis,* 113**:**174-185.

Fornari, T., Hernández, E. J., Ruiz-Rodriguez, A., Señorans, F. J. & Reglero, G. 2009. Phase equilibria for the removal of ethanol from alcoholic beverages using supercritical carbon dioxide. *The Journal of supercritical fluids,* 50(2)**:**91-96.

Gao, J., Atiyeh, H. K., Phillips, J. R., Wilkins, M. R. & Huhnke, R. L. 2013. Development of low cost medium for ethanol production from syngas by clostridium ragsdalei. *Bioresource Technology,* 147**:**508-515.

Ghaffari, A., Abdollahi, H., Khoshayand, M., Bozchalooi, S., Dadgar, A. & Rafiee-Tehrani, M. 2006. Performance comparison of neural network training algorithms in modeling of bimodal drug delivery. *International Journal of Pharmaceutics,* 327(1)**:**126-138.

Ghosh, R. 2006. *Principles of bioseparations engineering*, World Scientific Publishing Co Inc.

Global Market Insight. 2016. *Bio vanillin market size by application ,price trend, competitive market share and forecast, 2016 – 2023* [Online]. Available: https://www.gminsights.com/industry-analysis/bio-vanillin-market [Accessed May 2017].

Groot, W. J., Soedjak, H. S., Donck, P. B., Van Der Lans, R. G. J. M., Luyben, K. C. a. M. & Timmer, J. M. K. 1990. Butanol recovery from fermentations by liquid-liquid extraction and membrane solvent extraction. *Bioprocess Engineering,* 5(5)**:**203-216.

Grosse, Y., Baan, R., Secretan-Lauby, B., El Ghissassi, F., Bouvard, V., Benbrahim-Tallaa, L., Guha, N., Islami, F., Galichet, L. & Straif, K. 2011. Carcinogenicity of chemicals in industrial and consumer products, food contaminants and flavourings, and water chlorination byproducts. *The lancet oncology,* 12(4)**:**328-329.

Gupta, A. & Verma, J. P. 2015. Sustainable bio-ethanol production from agro-residues: A review. *Renewable and Sustainable Energy Reviews,* 41**:**550-567.

Güvenç, A., Mehmetoglu, Ü. & Çalimli, A. 1998. Supercritical co_2 extraction of ethanol from fermentation broth in a semicontinuous system. *The Journal of supercritical fluids,* 13(1–3)**:**325-329.

Güvenç, A., Mehmetoğlu, Ü. & Çalimli, A. 1999. Supercritical co_2 extraction of ethanol. *Turkish Journal of Chemistry,* 23(3)**:**285-292.

Hahn, R. & Cecot, C. 2009. The benefits and costs of ethanol: An evaluation of the government's analysis. *Journal of Regulatory Economics,* 35(3)**:**275-295.

Harris, C. M. & Kell, D. B. 1985. The estimation of microbial biomass. *Biosensors,* 1(1)**:**17-84.

Harrison, R. G. 2014. Bioseparation basics. *Chemical Engineering Progress,* 110(10)**:**36-42.

Harrison, R. G., Todd, P. W., Rudge, S. R. & Petrides, D. P. 2015. *Bioseparations science and engineering,* Oxford University Press.

Hattori, T. & Morita, S. 2010. Energy crops for sustainable bioethanol production; which, where and how? *Plant Production Science,* 13(3)**:**221-234.

Heinzle, E., Biwer, A. P. & Cooney, C. L. 2007. *Development of sustainable bioprocesses: Modeling and assessment,* John Wiley & Sons.

Herrero, M. & Ibáñez, E. 2015. Green processes and sustainability: An overview on the extraction of high added-value products from seaweeds and microalgae. *The Journal of supercritical fluids,* 96**:**211-216.

Hrnčič, M. K., Cör, D., Verboten, M. T. & Knez, Ž. 2018. Application of supercritical and subcritical fluids in food processing. *Food Quality and Safety,* 2(2)**:**59-67.

Hua, D., Ma, C., Song, L., Lin, S., Zhang, Z., Deng, Z. & Xu, P. 2007. Enhanced vanillin production from ferulic acid using adsorbent resin. *Applied Microbiology and Biotechnology,* 74(4)**:**783-790.

Hull, S. R., Yang, B. Y., Venzke, D., Kulhavy, K. & Montgomery, R. 1996. Composition of corn steep water during steeping. *Journal of agricultural and food chemistry,* 44(7)**:**1857-1863.

Ighose, B. O., Adeleke, I. A., Damos, M., Junaid, H. A., Okpalaeke, K. E. & Betiku, E. 2017. Optimization of biodiesel production from thevetia peruviana seed oil by adaptive neuro-fuzzy inference system coupled with genetic algorithm and response surface methodology. *Energy conversion and management,* 132**:**231-240.

Ikawa, N., Nagase, Y., Tada, T., Furuta, S. & Fukuzato, R. 1993. Separation process of ethanol from aqueous solutions usihg supercritical carbon dioxide. *Fluid Phase Equilibria,* 83**:**167-174.

Jadhav, A., Kareparamban, J., Nikam, P. & Kadam, V. 2012. Spectrophotometric estimation of ferulic acid from ferula asafoetida by folin-ciocalteu's reagent. *Der Pharmacia Sinica,* 3(6)**:**680-684.

Jeon, S., Kim, D.-K., Song, H., Lee, H. J., Park, S., Seung, D. & Chang, Y. K. 2014. 2, 3-butanediol recovery from fermentation broth by alcohol precipitation and vacuum distillation. *Journal of bioscience and bioengineering,* 117(4)**:**464-470.

Ji, X.-J., Huang, H. & Ouyang, P.-K. 2011. Microbial 2, 3-butanediol production: A state-of-the-art review. *Biotechnology advances,* 29(3)**:**351-364.

Jin, H., Liu, R. & He, Y. 2012. Kinetics of batch fermentations for ethanol production with immobilized saccharomyces cerevisiae growing on sweet sorghum stalk juice. *Procedia Environmental Sciences,* 12**:**137-145.

Joana Gil-Chávez, G., Villa, J. A., Fernando Ayala-Zavala, J., Basilio Heredia, J., Sepulveda, D., Yahia, E. M. & González-Aguilar, G. A. 2013. Technologies for extraction and production of bioactive compounds to be used as nutraceuticals and food ingredients: An overview. *Comprehensive Reviews in Food Science and Food Safety,* 12(1)**:**5-23.

Joglekar, A. & May, A. 1987. Product excellence through design of experiments. *Cereal foods world,* 32(12)**:**857-868.

Kaneko, T., Watanabe, Y. & Suzuki, H. 1990. Enhancement of diacetyl production by a diacetyl-resistant mutant of citrate-positive lactococcus lactis ssp. Lactis 3022 and by aerobic conditions of growth. *Journal of Dairy Science,* 73(2)**:**291-298.

Karmakar, B., Vohra, R., Nandanwar, H., Sharma, P., Gupta, K. & Sobti, R. 2000. Rapid degradation of ferulic acid via 4-vinylguaiacol and vanillin by a newly isolated strain of bacillus coagulans. *Journal of Biotechnology,* 80(3)**:**195-202.

Katoh, S., Yoshida, F. & Horiuchi, J.-I. 2015. *Biochemical engineering,* Wiley Online Library.

Kaur, B. & Chakraborty, D. 2013. Statistical media and process optimization for biotransformation of rice bran to vanillin using pediococcus acidilactici. *Indian Journal of Experimental Biology,* 51(11)**:**935-943.

Kennedy, C. R. 2015. The flavor rundown: Natural vs. Artificial flavors. *Harvard University: Science in the News,* 21.

Keskinen, K. I., Kinnunen, A., Nyström, L. & Aittamaa, J. Efficient approximate method for packed column separation performance simulation. Proceedings of the International Conference on Distillation & Absorption (on CD), 2002.

Khaw, K.-Y., Parat, M.-O., Shaw, P. N. & Falconer, J. R. 2017. Solvent supercritical fluid technologies to extract bioactive compounds from natural sources: A review. *Molecules,* 22(7)**:**1186.

Khosravi-Darani, K. & Vasheghani-Farahani, E. 2005. Application of supercritical fluid extraction in biotechnology. *Critical Reviews in Biotechnology,* 25(4)**:**231-242.

Kim, J. K., Iannotti, E. L. & Bajpai, R. 1999. Extractive recovery of products from fermentation broths. *Biotechnology and Bioprocess Engineering,* 4(1)**:**1-11.

Knez, Ž. 2009. Enzymatic reactions in dense gases. *The Journal of supercritical fluids,* 47(3)**:**357-372.

Knez, Ž., Škerget, M. & Uzunalić, A. P. 2007. Phase equilibria of vanillins in compressed gases. *The Journal of supercritical fluids,* 43(2)**:**237-248.

Knutson, B., Strobel, H., Nokes, S., Dawson, K., Berberich, J. & Jones, C. 1999. Effect of pressurized solvents on ethanol production by the thermophilic bacterium clostridium thermocellum. *The Journal of supercritical fluids,* 16(2)**:**149-156.

Kohli, R. 2019. Applications of supercritical carbon dioxide for removal of surface contaminants. *Developments in surface contamination and cleaning: Applications of cleaning techniques.* Elsevier.

Kollerup, F. & Daugulis, A. J. 1985. Screening and identification of extractive fermentation solvents using a database. *The Canadian Journal of Chemical Engineering,* 63(6)**:**919-927.

Koutinas, A. A., Yepez, B., Kopsahelis, N., Freire, D. M., De Castro, A. M., Papanikolaou, S. & Kookos, I. K. 2016. Techno-economic evaluation of a complete bioprocess for 2, 3-butanediol production from renewable resources. *Bioresource Technology,* 204**:**55-64.

Kumoro, A. & Hasan, M. 2007. Supercritical carbon dioxide extraction of andrographolide from andrographis paniculata: Effect of the solvent flow rate, pressure, and temperature. *Chinese Journal of Chemical Engineering,* 15(6)**:**877-883.

Kundiyana, D. K., Huhnke, R. L. & Wilkins, M. R. 2010. Syngas fermentation in a 100-l pilot scale fermentor: Design and process considerations. *Journal of Bioscience and Bioengineering,* 109(5)**:**492-498.

Laitinen, A. 2000. *Supercritical fluid extraction of organic compounds from solids and aqueous solutions.* Doctor of Technology dissertation, Helsinki University of Technology.

Lang, Q. & Wai, C. M. 2001. Supercritical fluid extraction in herbal and natural product studies—a practical review. *Talanta,* 53(4)**:**771-782.

Lawford, H. G. & Rousseau, J. D. 1997. Corn steep liquor as a cost-effective nutrition adjunct in high-performancezymomonas ethanol fermentations. *Applied biochemistry and biotechnology,* 63(1)**:**287.

Li, L., Wei, X., Yu, W., Wen, Z. & Chen, S. 2017a. Enhancement of acetoin production from bacillus licheniformis by 2, 3-butanediol conversion strategy: Metabolic engineering and fermentation control. *Process Biochemistry,* 57**:**35-42.

Li, Z., Wang, D. & Shi, Y.-C. 2017b. Effects of nitrogen source on ethanol production in very high gravity fermentation of corn starch. *Journal of the Taiwan Institute of Chemical Engineers,* 70**:**229-235.

Liggett, R. W. & Koffler, H. 1948. Corn steep liquor in microbiology. *Bacteriological reviews,* 12(4)**:**297-311.

Lim, J. S., Lee, Y.-W., Kim, J.-D., Lee, Y. Y. & Chun, H.-S. 1995. Mass-transfer and hydraulic characteristics in spray and packed extraction columns for supercritical carbon dioxide-ethanol-water system. *The Journal of supercritical fluids,* 8(2)**:**127-137.

Lim, J. S., Lee, Y. Y. & Chun, H. S. 1994. Phase equilibria for carbon dioxide-ethanol-water system at elevated pressures. *The Journal of supercritical fluids,* 7(4)**:**219-230.

Liu, D., Chen, Y., Ding, F., Guo, T., Xie, J., Zhuang, W., Niu, H., Shi, X., Zhu, C. & Ying, H. 2015. Simultaneous production of butanol and acetoin by metabolically engineered clostridium acetobutylicum. *Metabolic Engineering,* 27**:**107-114.

Liu, J., Kim, Y. & Mchugh, M. A. 2006. Phase behavior of the vanillin–co$_2$ system at high pressures. *The Journal of supercritical fluids,* 39(2)**:**201-205.

Liu, W., Fu, Y.-J., Zu, Y.-G., Tong, M.-H., Wu, N., Liu, X.-L. & Zhang, S. 2009. Supercritical carbon dioxide extraction of seed oil from opuntia dillenii haw. And its antioxidant activity. *Food Chemistry,* 114(1)**:**334-339.

Liu, Y., Zhang, S., Yong, Y.-C., Ji, Z., Ma, X., Xu, Z. & Chen, S. 2011. Efficient production of acetoin by the newly isolated bacillus licheniformis strain mel09. *Process Biochemistry,* 46(1)**:**390-394.

Long, B., Ryan, K. M. & Padrela, L. 2019. From batch to continuous—new opportunities for supercritical co2 technology in pharmaceutical manufacturing. *European Journal of Pharmaceutical Sciences***:**104971.

Lu, Z. M., Xu, W., Yu, N. H., Zhou, T., Li, G. Q., Shi, J. S. & Xu, Z. H. 2011. Recovery of aroma compounds from zhenjiang aromatic vinegar by supercritical fluid extraction. *International journal of food science & technology,* 46(7)**:**1508-1514.

Lukin, I., Merz, J. & Schembecker, G. 2018. Techniques for the recovery of volatile aroma compounds from biochemical broth: A review. *Flavour and fragrance journal,* 33(3)**:**203-216.

Luo, Q., Wu, J. & Wu, M. 2014. Enhanced acetoin production by bacillus amyloliquefaciens through improved acetoin tolerance. *Process Biochemistry,* 49(8)**:**1223-1230.

Macedo, S., Fernandes, S., Lopes, J. A., De Sousa, H. C., Pereira, P. J., Carmelo, P. J., Menduiña, C., Simões, P. C. & Da Ponte, M. N. 2008. Recovery of wine-must aroma compounds by supercritical co 2. *Food and Bioprocess Technology,* 1(1)**:**74-81.

Maddipati, P., Atiyeh, H. K., Bellmer, D. D. & Huhnke, R. L. 2011. Ethanol production from syngas by clostridium strain p11 using corn steep liquor as a nutrient replacement to yeast extract. *Bioresource Technology,* 102(11)**:**6494-6501.

Maiti, S., Gallastegui, G., Kaur Brar, S., Lebihan, Y., Buelna, G., Drogui, P. & Verma, M. 2015. Quest for sustainable bio-production and recovery of butanol as a promising solution to fossil fuel. *International Journal of Energy Research,* 40(4)**:**411-438.

Malherbe, S., Bauer, F. & Du Toit, M. 2007. Understanding problem fermentations: A review. *South African Journal for Enology and Viticulture,* 28(2)**:**169-186.

Manjare, S. D. & Dhingra, K. 2019. Supercritical fluids in separation and purification: A review. *Materials Science for Energy Technologies*.

Martens, D. E., Van Den End, E. J. & Streefland, M. 2014. Configuration of bioreactors. *In:* PÖRTNER, R. (ed.) *Animal cell biotechnology. Methods in molecular biology(methods and protocols).* Humana Press,Totowa, NJ.

Mccabe, W. L., Smith, J. C. & Harriott, P. 1993. *Unit operations of chemical engineering,* McGraw-hill New York.

Menet, J.-M. & Thiebaut, D. 1999. Chromatography science series. *In:* CAZE, S. (ed.) *Countercurrent chromatography.* Cherry Hill, New Jersey: CRC Press.

Mezule, L. & Dalecka, B. 2017. Adjustment of yeast growth media for the fermentation of lignocellulosic sugars. *Chemical Engineering Transactions,* 57**:**25-30.

Miller, G. L. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry,* 31(3)**:**426-428.

Moghaddam, M. G., Ahmad, F. B. H., Basri, M. & Rahman, M. B. A. 2010. Artificial neural network modeling studies to predict the yield of enzymatic synthesis of betulinic acid ester. *Electronic Journal of Biotechnology,* 13(3)**:**3-4.

Mota, M. I. F., Rodrigues Pinto, P. C., Loureiro, J. M. & Rodrigues, A. E. 2016. Recovery of vanillin and syringaldehyde from lignin oxidation: A review of separation and purification processes. *Separation & Purification Reviews,* 45(3)**:**227-259.

Mourabet, M., El Rhilassi, A., El Boujaady, H., Bennani-Ziatni, M., El Hamri, R. & Taitai, A. 2012. Removal of fluoride from aqueous solution by adsorption on apatitic tricalcium

phosphate using box–behnken design and desirability function. *Applied Surface Science,* 258(10)**:**4402-4410.

Nguyen, K., Barton, P. & Spencer, J. S. 1991. Supercritical carbon dioxide extraction of vanilla. *The Journal of supercritical fluids,* 4(1)**:**40-46.

Nielsen, D. R., Yoon, S. H., Yuan, C. J. & Prather, K. L. 2010. Metabolic engineering of acetoin and meso-2, 3-butanediol biosynthesis in e. Coli. *Biotechnology Journal,* 5(3)**:**274-284.

Nielsen, J. 2008. Fermentation monitoring, design and optimization. *Encyclopedia of Bioprocess Technology.*

Obom, K. M., Magno, A. & Cummings, P. J. 2013. Operation of a benchtop bioreactor. *Journal of Visualized Experiments: JoVE,* (79).

Oliveira, A. L., Kamimura, E. S. & Rabi, J. A. 2009. Response surface analysis of extract yield and flavour intensity of brazilian cherry (eugenia uniflora l.) obtained by supercritical carbon dioxide extraction. *Innovative Food Science and Emerging Technologies,* 10(2)**:**189-194.

Osunkanmibi, O. B., Owolabi, T. O. & Betiku, E. 2015. Comparison of artificial neural network and response surface methodology performance on fermentation parameters optimization of bioconversion of cashew apple juice to gluconic acid. *International Journal of Food Engineering,* 11(3)**:**393-403.

Pansuriya, R. C. & Singhal, R. S. 2009. Supercritical fluid extraction of lovastatin from the wheat bran obtained after solid-state fermentation. *Food Technology and Biotechnology,* 47(2)**:**159-165.

Patnaik, P. 2017. *Handbook of environmental analysis: Chemical pollutants in air, water, soil, and solid wastes,* Boca Raton, CRC Press.

Paz, A., Outeiriño, D., Pinheiro De Souza Oliveira, R. & Domínguez, J. M. 2018. Fed-batch production of vanillin by bacillus aryabhattai ba03. *New Biotechnology,* 40**:**186-191.

Pereira, C., Rocha, R., Flp, P. & Mendes, M. Phorbol esters extraction from jatropha curcas seed cake using supercritical carbon dioxide. III Iberoamerican Conference on Supercritical Fluids Cartagena de Indias 2013. Colômbia.

Pereira, F. B., Guimarães, P. M., Teixeira, J. A. & Domingues, L. 2010. Optimization of low-cost medium for very high gravity ethanol fermentations by saccharomyces cerevisiae using statistical experimental designs. *Bioresource Technology,* 101(20)**:**7856-7863.

Perrut, M. 2000. Supercritical fluid applications: Industrial developments and economic issues. *Industrial and Engineering Chemistry Research,* 39(12)**:**4531-4535.

Perrut, M. 2003. Supercritical fluids applications in the pharmaceutical industry. *STP pharma sciences,* 13(2)**:**83-91.

Pfeiffer, T. & Morley, A. 2014. An evolutionary perspective on the crabtree effect. *Frontiers in Molecular Biosciences,* 1**:**17.

Pieck, C. A., Crampon, C., Charton, F. & Badens, E. 2015. Multi-scale experimental study and modeling of the supercritical fractionation process. *The Journal of supercritical fluids,* 105**:**158-169.

Prado, J. M., Prado, G. H. & Meireles, M. a. A. 2011. Scale-up study of supercritical fluid extraction process for clove and sugarcane residue. *The Journal of supercritical fluids,* 56(3)**:**231-237.

Priefert, H., Rabenhorst, J. & Steinbüchel, A. 2001. Biotechnological production of vanillin. *Applied Microbiology and Biotechnology,* 56(3-4)**:**296-314.

Prokofeva, M., Miroshnichenko, M., Kostrikina, N., Chernyh, N., Kuznetsov, B., Tourova, T. & Bonch-Osmolovskaya, E. 2000. Acidilobus aceticus gen. Nov., sp. Nov., a novel anaerobic thermoacidophilic archaeon from continental hot vents in kamchatka. *International journal of systematic and evolutionary microbiology,* 50(6)**:**2001-2008.

Qureshi, N. & Eller, F. 2018. Recovery of butanol from clostridium beijerinckii p260 fermentation broth by supercritical CO_2 extraction. *Journal of Chemical Technology and Biotechnology,* 93(4)**:**1206-1212.

Qureshi, N. & Maddox, I. S. 1995. Continuous production of acetone-butanol-ethanol using immobilized cells of clostridium acetobutylicum and integration with product removal by liquid-liquid extraction. *Journal of fermentation and bioengineering,* 80(2)**:**185-189.

Rana, R., Mathur, A., Jain, C., Sharma, S. & Mathur, G. 2013. Microbial production of vanillin. *International Journal of Biotechnology and Bioengineering Research,* 4(3)**:**227-234.

Rebnegger, C., Vos, T., Graf, A. B., Valli, M., Pronk, J. T., Daran-Lapujade, P. & Mattanovich, D. 2016. Pichia pastoris exhibits high viability and a low maintenance energy requirement at near-zero specific growth rates. *Applied and environmental microbiology,* 82(15)**:**4570-4583.

Rodríguez-López, L., Vecino, X., Barbosa-Pereira, L., Moldes, A. & Cruz, J. 2016. A multifunctional extract from corn steep liquor: Antioxidant and surfactant activities. *Food and Function,* 7(9)**:**3724-3732.

Rojas, L. B., Quideau, S., Pardon, P. & Charrouf, Z. 2005. Colorimetric evaluation of phenolic content and gc-ms characterization of phenolic composition of alimentary and cosmetic argan oil and press cake. *Journal of agricultural and food chemistry,* 53(23)**:**9122-9127.

Roncal, T., Caballero, S., De Guereñu, M. D. M. D., Rincón, I., Prieto-Fernández, S. & Ochoa-Gómez, J. R. 2017. Efficient production of acetoin by fermentation using the newly isolated mutant strain lactococcus lactis subsp. Lactis cml b4. *Process Biochemistry,* 58**:**35-41.

Ruphuy, G., Souto-Lopes, M., Paiva, D., Costa, P., Rodrigues, A., Monteiro, F., Salgado, C., Fernandes, M., Lopes, J. & Dias, M. 2018. Supercritical co₂ assisted process for the production of high-purity and sterile nano-hydroxyapatite/chitosan hybrid scaffolds. *Journal of Biomedical Materials Research Part B: Applied Biomaterials,* 106(3)**:**965-975.

Saffarionpour, S. & Ottens, M. 2018. Recent advances in techniques for flavor recovery in liquid food processing. *Food Engineering Reviews,* 10(2)**:**81-94.

Sarangi, P. K., Nanda, S. & Sahoo, H. 2010. Maximization of vanillin production by standardizing different cultural conditions for ferulic acid degradation. *New York Science Journal,* 3(7)**:**77-79.

Sarks, C., Jin, M., Sato, T. K., Balan, V. & Dale, B. E. 2014. Studying the rapid bioconversion of lignocellulosic sugars into ethanol using high cell density fermentations with cell recycle. *Biotechnology for Biofuels,* 7(1)**:**73.

Sarma, S. J., Dhillon, G. S., Hegde, K., Brar, S. K. & Verma, M. 2014. Utilization of agro-industrial waste for the production of aroma compounds and fragrances. *Biotransformation of waste biomass into high value biochemicals.* Springer.

Sarrade, S., Guizard, C. & Rios, G. M. 2003. New applications of supercritical fluids and supercritical fluids processes in separation. *Separation and Purification Technology,* 32(1–3)**:**57-63.

Sarteshnizi, R. A., Hosseini, H., Bondarianzadeh, D. & Colmenero, F. J. 2015. Optimization of prebiotic sausage formulation: Effect of using β-glucan and resistant starch by d-optimal mixture design approach. *LWT - Food Science and Technology,* 62(1)**:**704-710.

Saxena, J. & Tanner, R. S. 2012. Optimization of a corn steep medium for production of ethanol from synthesis gas fermentation by clostridium ragsdalei. *World Journal of Microbiology and Biotechnology,* 28(4)**:**1553-1561.

Schaechter, M. 2009. *Encyclopedia of microbiology*, Academic Press.

Schmidhuber, J. 2015. Deep learning in neural networks: An overview. *Neural networks,* 61**:**85-117.

Seifan, M., Samani, A. K. & Berenjian, A. 2017. New insights into the role of ph and aeration in the bacterial production of calcium carbonate (caco₃). *Applied Microbiology and Biotechnology,* 101(8)**:**3131-3142.

Senanayake, S. N. & Shahidi, F. 2002. Lipase-catalyzed incorporation of docosahexaenoic acid (dha) into borage oil: Optimization using response surface methodology. *Food Chemistry,* 77(1)**:**115-123.

Seo, H.-B., Kim, S. S., Lee, H.-Y. & Jung, K.-H. 2009. High-level production of ethanol during fed-batch ethanol fermentation with a controlled aeration rate and non-sterile glucose

powder feeding of saccharomyces cerevisiae. *Biotechnology and Bioprocess Engineering,* 14(5)**:**591.

Shah, A. & Darr, M. 2016. A techno-economic analysis of the corn stover feedstock supply system for cellulosic biorefineries. *Biofuels, Bioproducts and Biorefining,* 10(5)**:**542-559.

Shahavi, M. H., Hosseini, M., Jahanshahi, M., Meyer, R. L. & Darzi, G. N. 2016. Clove oil nanoemulsion as an effective antibacterial agent: Taguchi optimization method. *Desalination and Water Treatment,* 57(39)**:**18379-18390.

Shao, P. & Kumar, A. 2009. Recovery of 2, 3-butanediol from water by a solvent extraction and pervaporation separation scheme. *Journal of membrane science,* 329(1-2)**:**160-168.

Sharma, P. & Noronha, S. 2014. Comparative assessment of factors involved in acetoin synthesis by bacillus subtilis 168. *ISRN microbiology,* 2014.

Shukla, A., Naik, S., Goud, V. V. & Das, C. 2019. Supercritical co$_2$ extraction and online fractionation of dry ginger for production of high-quality volatile oil and gingerols enriched oleoresin. *Industrial Crops and Products,* 130**:**352-362.

Sindhwani, G., Uk, I. & Aeri, V. 2012. Microbial transformation of eugenol to vanillin. *Journal of Microbiology and Biotechnology Research,* 2**:**313-318.

Singh, S. P., Ekanem, E., Wakefield Jr, T. & Comer, S. 2003. Emerging importance of bio-based products and bio-energy in the us economy: Information dissemination and training of students. *International Food and Agribusiness Management Review,* 5(3)**:**14.

Solana, M., Qureshi, N., Bertucco, A. & Eller, F. 2016. Recovery of butanol by counter-current carbon dioxide fractionation with its potential application to butanol fermentation. *Materials,* 9(7)**:**530.

Souza, A. F., Rodriguez, D. M., Ribeaux, D. R., Luna, M. A., Lima E Silva, T. A., Andrade, R. F. S., Gusmão, N. B. & Campos-Takaki, G. M. 2016. Waste soybean oil and corn steep liquor as economic substrates for bioemulsifier and biodiesel production by candida lipolytica ucp 0998. *International Journal of Molecular Sciences,* 17(10)**:**1608.

Sovová, H. 2012. Modeling the supercritical fluid extraction of essential oils from plant materials. *Journal of Chromatography A,* 1250**:**27-33.

Speckman, R. & Collins, E. 1982. Specificity of the westerfeld adaptation of the voges-proskauer test. *Applied and environmental microbiology,* 44(1)**:**40-43.

Sreenath, H. K. & Jeffries, T. W. 1996. Effect of corn steep liquor on fermentation of mixed sugars by candida shehatae fpl-702. *Appl. Biochem. Biotechnol.,* 57(1)**:**551-561.

Streng, W. H. 2012. *Characterization of compounds in solution: Theory and practice*, Springer Science & Business Media.

Sumbhate, S., Nayak, S., Goupale, D., Tiwari, A. & Jadon, R. S. 2012. Colorimetric method for the estimation of ethanol in alcoholic-drinks. *Journal of Analytical Techniques,* 1**:**1-6.

Sun, J., Zhang, L., Rao, B., Han, Y., Chu, J., Zhu, J., Shen, Y. & Wei, D. 2012. Enhanced acetoin production by serratia marcescens h32 using statistical optimization and a two-stage agitation speed control strategy. *Biotechnology and Bioprocess Engineering,* 17(3)**:**598-605.

Taiwo, A., Madzimbamuto, T. & Ojumu, T. 2018a. Optimization of corn steep liquor dosage and other fermentation parameters for ethanol production by saccharomyces cerevisiae type 1 and anchor instant yeast. *Energies,* 11(7)**:**1740.

Taiwo, A. E., Ojumu, T. V. & Madzimbamuto, T. N. 2018b. Statistical optimization of acetoin production using corn steep liquor as a low-cost nitrogen source by bacillus subtilis cicc 10025. *In:* JACOB-LOPES, E. & QUEIROZ ZEPKA, L. (eds.) *Renewable resources and biorefineries.* IntechOpen.

Tan, J., Jahim, J., Wu, T., Harun, S. & Mumtaz, T. 2016. Use of corn steep liquor as an economical nitrogen source for biosuccinic acid production by actinobacillus succinogenes. *IOP Conference Series: Earth and Environmental Science,* 36(1)**:**012058.

Tan, M., Liew, S., Maskat, M. Y., Aida, W. W. & Osman, H. 2015. Optimization of vanillin production using isoeugenol as substrate by aspergillus niger i-1472. *International Food Research Journal,* 22(4)**:**1651.

Tang, Y., An, M., Liu, K., Nagai, S., Shigematsu, T., Morimura, S. & Kida, K. 2006. Ethanol production from acid hydrolysate of wood biomass using the flocculating yeast saccharomyces cerevisiae strain kf-7. *Process Biochemistry,* 41(4)**:**909-914.

Teixeira, R. M., Cavalheiro, D., Ninow, J. & Furigo Jr, A. 2002. Optimization of acetoin production by hanseniaspora guilliermondii using experimental design. *Brazilian Journal of Chemical Engineering,* 19(2)**:**181-186.

Tesfaw, A. & Assefa, F. 2014. Current trends in bioethanol production by saccharomyces cerevisiae: Substrate, inhibitor reduction, growth variables, coculture, and immobilization. *International Scholarly Research Notices,* 2014.

Tian, Y., Fan, Y., Liu, J., Zhao, X. & Chen, W. 2016. Effect of nitrogen, carbon sources and agitation speed on acetoin production of bacillus subtilis sf4-3. *Electronic Journal of Biotechnology,* 19**:**41-49.

Tian, Y., Fan, Y., Zhao, X., Zhang, J., Yang, L. & Liu, J. 2014. Optimization of fermentation medium for acetoin production by bacillus subtilis sf4-3 using statistical methods. *Preparative Biochemistry and Biotechnology,* 44(5)**:**529-543.

Tilay, A., Bule, M. & Annapure, U. 2010. Production of biovanillin by one-step biotransformation using fungus pycnoporous cinnabarinus. *Journal of agricultural and food chemistry,* 58(7)**:**4401-4405.

Tu, J. V. 1996. Advantages and disadvantages of using artificial neural networks versus logistic regression for predicting medical outcomes. *Journal of Clinical Epidemiology,* 49(11)**:**1225-1231.

Turner, C., Whitehand, L. C., Nguyen, T. & Mckeon, T. 2004. Optimization of a supercritical fluid extraction/reaction methodology for the analysis of castor oil using experimental design. *Journal of Agricultural and Food Chemistry,* 52(1)**:**26-32.

Tyśkiewicz, K., Konkol, M. & Rój, E. 2018. The application of supercritical fluid extraction in phenolic compounds isolation from natural plant materials. *Molecules,* 23(10)**:**2625.

Van Hee, P., Hoeben, M., Van Der Lans, R. & Van Der Wielen, L. 2006. Strategy for selection of methods for separation of bioparticles from particle mixtures. *Biotechnology and Bioengineering,* 94(4)**:**689-709.

Van Urk, H., Postma, E., Scheffers, W. A. & Van Dijken, J. P. 1989. Glucose transport in crabtree-positive and crabtree-negative yeasts. *Journal of General Microbiology,* 135(9)**:**2399-2406.

Vijayan, S., Byskal, D. & Buckley, L. 1994. Separation of oil from fried chips by a supercritical extraction process; an overview of bench-scale test experience and process economics. *Supercritical Fluids Processing of Food and Biomaterials,* 751401846.

Wang, Z., Zhou, Q., Guo, H., Yang, P. & Lu, W. 2018. Determination of water solubility in supercritical co2 from 313.15 to 473.15 k and from 10 to 50 mpa by in-situ quantitative raman spectroscopy. *Fluid Phase Equilibria,* 476**:**170-178.

Webb, C. & Atkinson, B. 1992. The role of chemical engineering in biotechnology. *The Chemical Engineering Journal,* 50(1)**:**B9-B16.

Weibel, G. L. & Ober, C. K. 2003. An overview of supercritical co_2 applications in microelectronics processing. *Microelectronic Engineering,* 65(1-2)**:**145-152.

Welti-Chanes, J. & Velez-Ruiz, J. F. 2016. *Transport phenomena in food processing*, CRC press.

Wesolowski, M. & Suchacz, B. 2012. Artificial neural networks: Theoretical background and pharmaceutical applications: A review. *Journal of AOAC International,* 95(3)**:**652-668.

Williams, D. F. 1981. *Fundamental aspects of biocompatibility*, CRC Pressl Llc.

Wu, W., Yenkie, K. & Maravelias, C. T. 2017. A superstructure-based framework for bio-separation network synthesis. *Computers & Chemical Engineering,* 96**:**1-17.

Wu, Y.-T., Feng, M., Ding, W.-W., Tang, X.-Y., Zhong, Y.-H. & Xiao, Z.-Y. 2008. Preparation of vanillin by bioconversion in a silicon rubber membrane bioreactor. *Biochemical Engineering Journal,* 41(2)**:**193-197.

Xiao, X., Hou, Y., Liu, Y., Liu, Y., Zhao, H., Dong, L., Du, J., Wang, Y., Bai, G. & Luo, G. 2013. Classification and analysis of corn steep liquor by uplc/q-tof ms and hplc. *Talanta,* 107**:**344-348.

Xiao, Z., Liu, P., Qin, J. Y. & Xu, P. 2007. Statistical optimization of medium components for enhanced acetoin production from molasses and soybean meal hydrolysate. *Applied Microbiology and Biotechnology,* 74(1)**:**61-68.

Xiao, Z. & Lu, J. R. 2014. Strategies for enhancing fermentative production of acetoin: A review. *Biotechnology advances,* 32(2)**:**492-503.

Xiao, Z., Wang, X., Huang, Y., Huo, F., Zhu, X., Xi, L. & Lu, J. R. 2012. Thermophilic fermentation of acetoin and 2, 3-butanediol by a novel geobacillus strain. *Biotechnology for Biofuels,* 5**:**88.

Xiu, Z.-L. & Zeng, A.-P. 2008. Present state and perspective of downstream processing of biologically produced 1, 3-propanediol and 2, 3-butanediol. *Applied Microbiology and Biotechnology,* 78(6)**:**917-926.

Xu, H., Jia, S. & Liu, J. Production of acetoin by bacillus subtilis th-49. Consumer Electronics, Communications and Networks (CECNet), 2011 International Conference on, 16-18 April 2011. IEEE, 1524-1527.

Xu, Q., Xie, L., Li, Y., Lin, H., Sun, S., Guan, X., Hu, K., Shen, Y. & Zhang, L. 2015. Metabolic engineering of escherichia coli for efficient production of (3r)-acetoin. *Journal of Chemical Technology and Biotechnology,* 90(1)**:**93-100.

Yan, L., Chen, P., Zhang, S., Li, S., Yan, X., Wang, N., Liang, N. & Li, H. 2016. Biotransformation of ferulic acid to vanillin in the packed bed-stirred fermentors. *Scientific reports,* 6**:**34644.

Yang, S.-T. 2011. *Bioprocessing for value-added products from renewable resources: New technologies and applications,* Elsevier.

Yang, T.-W., Rao, Z.-M., Zhang, X., Xu, M.-J., Xu, Z.-H. & Yang, S.-T. 2013. Effects of corn steep liquor on production of 2, 3-butanediol and acetoin by bacillus subtilis. *Process Biochemistry,* 48(11)**:**1610-1617.

Yiyong, D. & Hong, C. 2011. A new bioprocess to produce natural vanillin by microbial fermentation. *Flavour Fragrance Cosmetics,* 3**:**003.

Yusoff, M., Akita, H., Hassan, M., Fujimoto, S., Yoshida, M., Nakashima, N. & Hoshino, T. 2017. Production of acetoin from hydrothermally pretreated oil mesocarp fiber using metabolically engineered escherichia coli in a bioreactor system. *Bioresource Technology,* 245**:**1040-1048.

Zamzuri, N., Abd-Aziz, S., Rahim, R., Phang, L., Alitheen, N. & Maeda, T. 2014. A rapid colorimetric screening method for vanillic acid and vanillin-producing bacterial strains. *Journal of applied microbiology,* 116(4)**:**903-910.

Zhang, L., Chen, S., Xie, H., Tian, Y. & Hu, K. 2012. Efficient acetoin production by optimization of medium components and oxygen supply control using a newly isolated paenibacillus polymyxa cs107. *Journal of Chemical Technology and Biotechnology,* 87(11)**:**1551-1557.

Zhang, Q.-W., Lin, L.-G. & Ye, W.-C. 2018. Techniques for extraction and isolation of natural products: A comprehensive review. *Chinese Medicine,* 13(1)**:**20.

Zhang, S., Huang, X., Qu, C., Suo, Y., Liao, Z. & Wang, J. 2017. Extractive fermentation for enhanced isopropanol and n-butanol production with mixtures of water insoluble aliphatic acids and oleyl alcohol. *Biochemical Engineering Journal,* 117**:**112-120.

Zhang, X., Heinonen, S. & Levänen, E. 2014. Applications of supercritical carbon dioxide in materials processing and synthesis. *RSC Advances,* 4(105)**:**61137-61152.

Zhang, X., Yang, T.-W., Lin, Q., Xu, M.-J., Xia, H.-F., Xu, Z.-H., Li, H.-Z. & Rao, Z.-M. 2011. Isolation and identification of an acetoin high production bacterium that can reverse transform 2, 3-butanediol to acetoin at the decline phase of fermentation. *World Journal of Microbiology and Biotechnology,* 27(12)**:**2785-2790.

Zhang, Y., Li, S., Liu, L. & Wu, J. 2013. Acetoin production enhanced by manipulating carbon flux in a newly isolated bacillus amyloliquefaciens. *Bioresource Technology,* 130**:**256-260.

Zhao, J., Hao, Y., Cao, L., Wang, J., Wang, W. & Wu, X. 2017. Preparation of phema/nhap nanocomposites via in situ polymerization in supercritical carbon dioxide for biomedical applications. *Fibers and Polymers,* 18(5)**:**868-874.

Zheng, L., Zheng, P., Sun, Z., Bai, Y., Wang, J. & Guo, X. 2007. Production of vanillin from waste residue of rice bran oil by aspergillus niger and pycnoporus cinnabarinus. *Bioresource Technology,* 98(5)**:**1115-1119.

Appendices

Appendix A: Standard graphs and tables

Table A.1: Reducing sugar standard table

Concentration (g/L)	Absorbance (nm)
0	0
0.1	0.195504
0.2	0.407788
0.3	0.741793
0.4	0.8345
0.5	0.9543
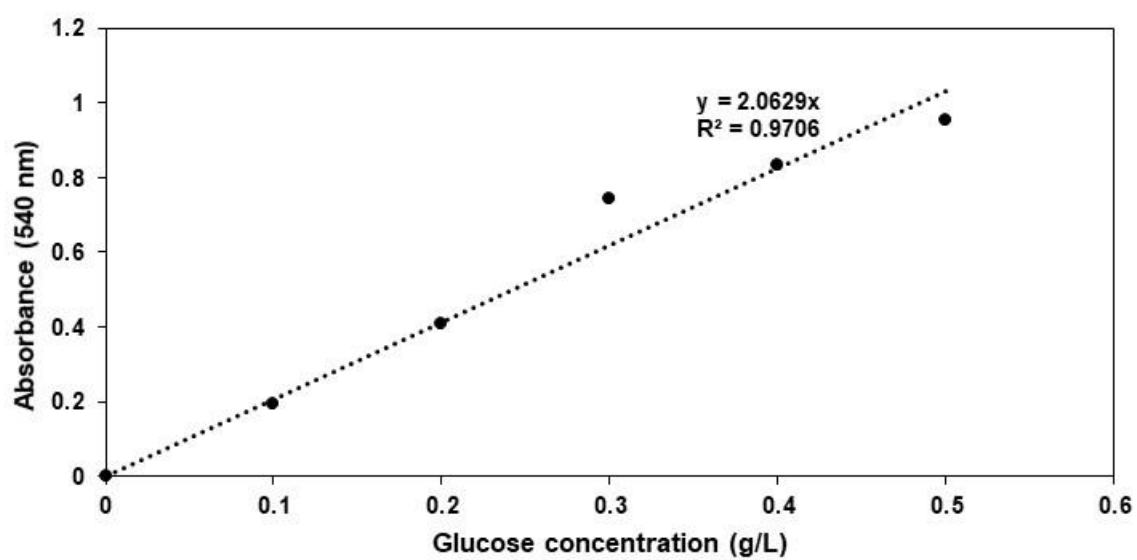
Figure A.1: Graph of absorbance at 540 nm against reducing sugar concentration (g/L)

Table A.2: Ethanol standard table

Concentration (g/L)	Absorbance (nm)
0	0
4	0.064
8	0.121
12	0,153333
16	0.218
20	0.295333
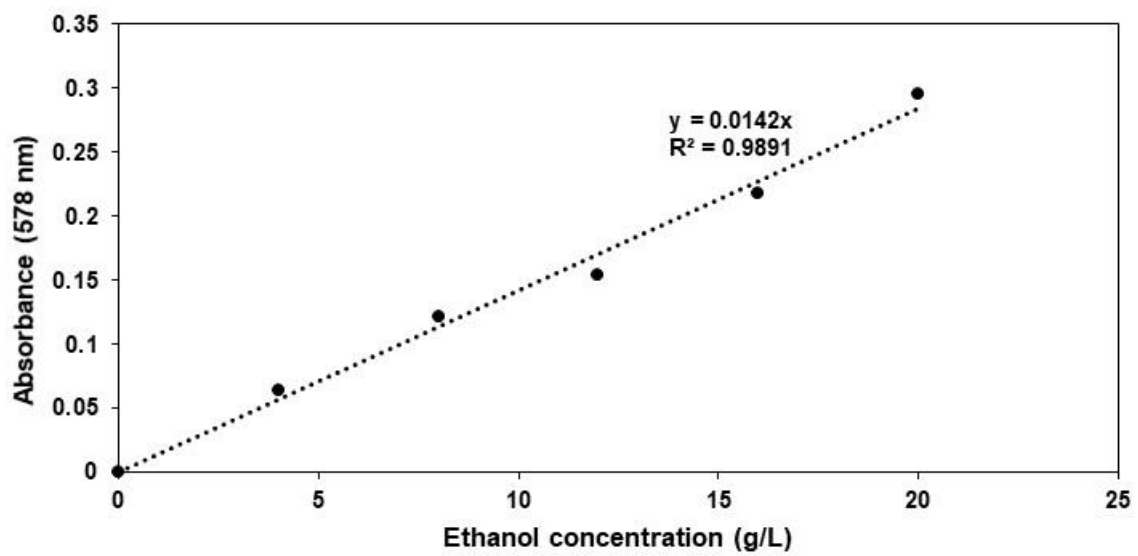
Figure A.2: Graph of absorbance at 578 nm against ethanol concentration (g/L)

Table A.3: Acetoin standard table

Concentration (g/L)	Absorbance (nm)
0	0
0.1	0.083
0.2	0.358
0.3	0.477
0.4	0.7197
0.5	0.802
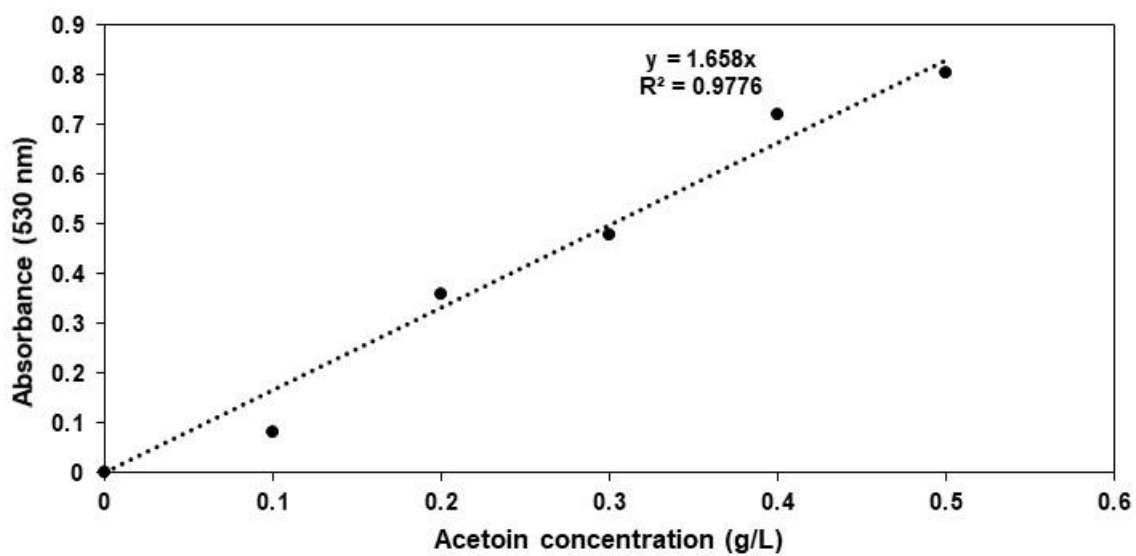
Figure A.3: Graph of absorbance at 530 nm against acetoin concentration (g/L)

Table A.4: Ferulic acid standard table

Concentration (μg/mL)	Absorbance (nm)
1	0.156086
2	0.289335
3	0.400877
4	0.502998
5	0.615873
6	0.756066
7	0.849739
8	0.933239
calibration coefficient	0.997227
Maximum error	0.2164
Concentration = 8.891926*reading-0.506412	
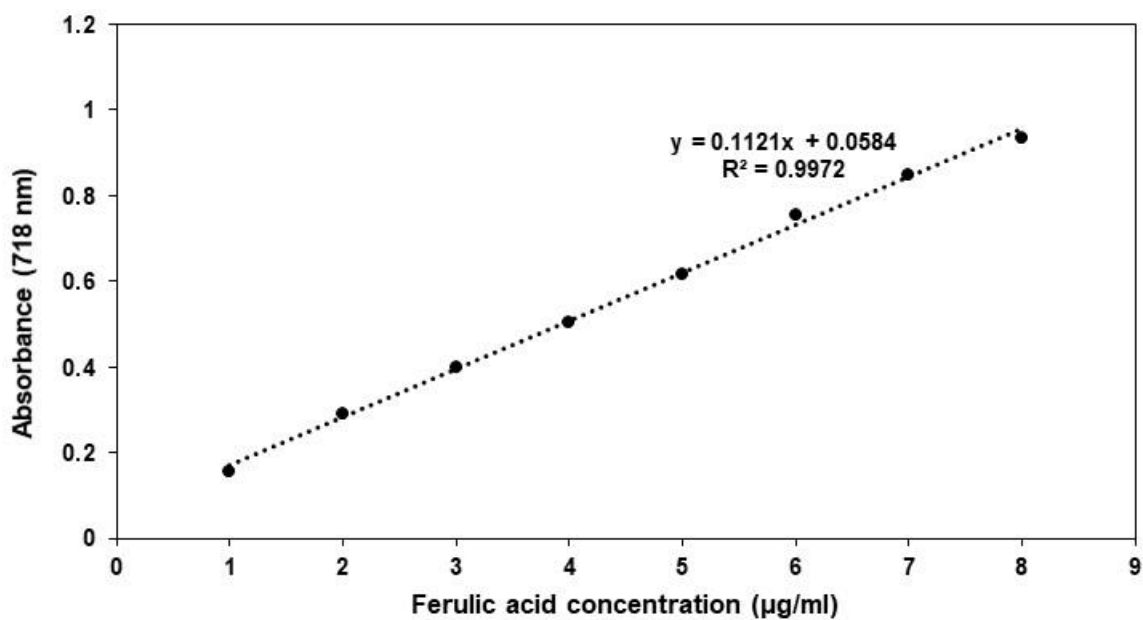
Figure A.4: Graph of absorbance at 718 nm against ferulic acid concentration (μg/mL)

Table A.5: Vanillin standard table

Concentration (mg/mL)	Absorbance (nm)
0	0
0.2	0.125498
0.4	0.24562
0.6	0.395734
0.8	0.586303
1	0.70108
Calibration coefficient	0.993697
Maximum error	0.0337

Concentration =+1.33217*reading+0.0526822
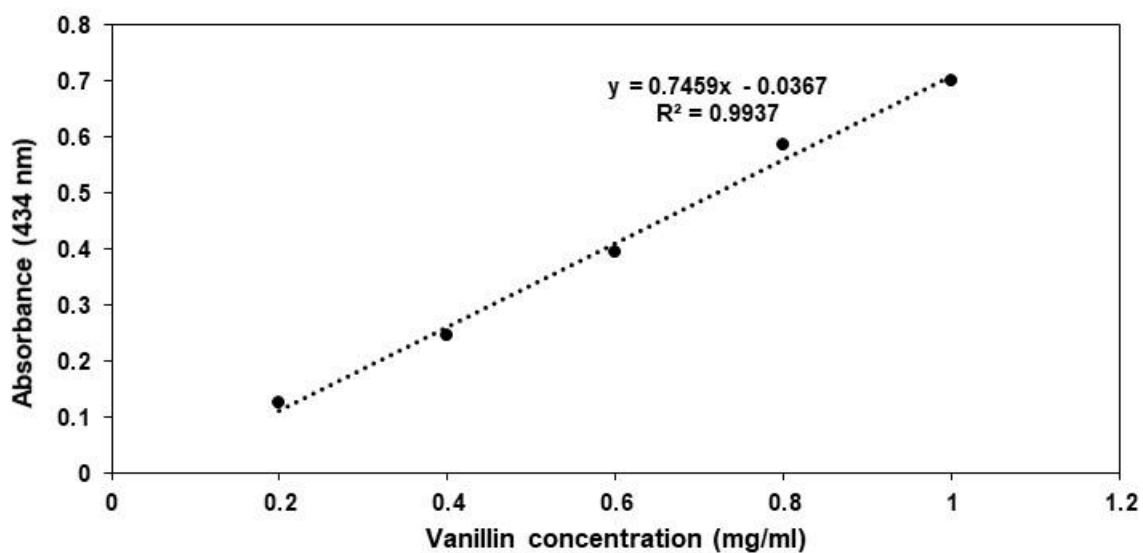
Figure A.5: Graph of absorbance at 434 nm against vanillin concentration (mg/mL)

Table A.6: Vanillic acid standard table

Vanillic acid Concentration (µg/mL)	Absorbance (nm)
1	-0.26239
2	0.0523447
3	0.672589
4	1.21497
5	1.24761
6	1.94958
7	2.71477
8	2.91515
Calibration result	
calibration coefficient	0.98057
Maximum error	0.6368
Concentration = 2.08957*reading+1.75623	
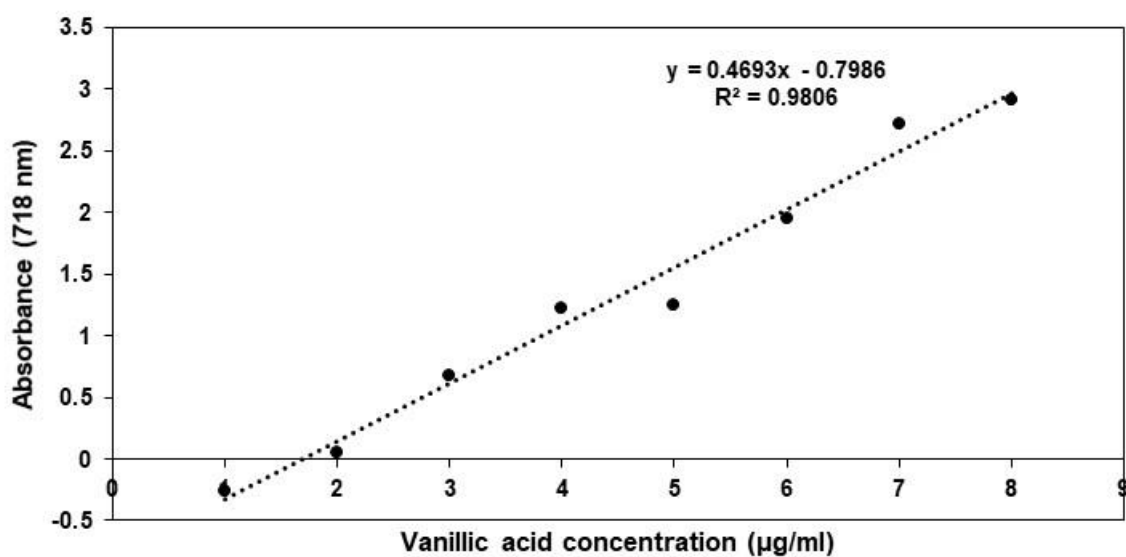
Figure A.6: Graph of absorbance at 718 nm against vanillic acid concentration (mg/mL)

Appendix B: Acetoin broth analysis using GC-MS and UV

Table B.1: Estimation of acetoin broth concentration using GC-MS

Broth	Acetoin (mg/L)	Acetoin (g/L)
Acetoin fresh broth	304.98	0.30
Acetoin stale broth	183.36	0.18

Concentration of AC (Acetoin) in shake flask study from UV analysis: 10.70 g/L

Concentration of AC (Acetoin) detected form GC-MS analysis after extraction: 0.30 g/L

$$\% \text{ Relative amount of AC present in the broth} = \frac{\text{Extracted broth quantified from GC/MS}}{\text{Total unpurified fermentation broth UV}}$$

$$\% \text{ Relative amount of AC present in the broth} = \frac{0.30}{10.70} = 2.80\%$$

Table B.2: Organic compounds in acetoin fermentation broth

| Compound	Area Ratio	Retention time
trans-3-penten-2-ol	0.134	11.54
Acetoin	14.814	16.82
2 ethyl butanoic acid	0.186	21.94
2-acetoxyl-3-butanone	0.220	22.86
Trimethyl pyrazine	0.450	23.20
Acetic acid	0.011	27.59
1-hydroxyl-2-propanone	0.018	27.94
2-ethyl-1-hexanol	0.095	28.80
2-hexanedione	0.210	29.06
Benzaldehyde	0.033	29.79
(2S,3S)-butane diol	3.154	30.98
Iso-butytric acid	0.489	32.02
Meso-2, 3-Butanediol	6.301	32.52
4-hydroxy-butanoic acid	1.927	33.93
Furfuryl alcohol	1.392	35.61
Isovaleric acid	0.812	35.75
3methylthio-1-propanol	0.470	37.13
5-methyl-2-furanmethanol	0.138	37.42
Methyl-guaiacol	0.080	37.49
Trim ethylene acetate	0.032	37.42
2(5H)-Furanone	0.181	37.91
Methyl phenethyl-alcohol	0.001	38.93
3-methyl-1,2-cyclopentanedione	0.109	39.04
Hexanoic acid	0.131	39.23

2-methoxyphenol (guaiacol)	0.133	39.44
Guaiacol	0.102	39.44
2,2.4. trimethyl-1,3, pentanedioldisobuytryrate	0.379	39.56
Benzyl alcohol	0.743	39.61
Phenylethyl alcohol	2.599	39.96
1,2,4, Trimethoxy benzene	6.038	40.23
Phenylethyl guaiacol	0.279	41.01
Dehydromevaloniclactone	1.354	41.02
2-methoxy-4-vinlyphenol	0.176	42.17
Octadecanoic acid	0.035	43.11
Diethylpthalate	0.856	43.23
Oleic acid	0.033	43.92
9,12, octadecadienoic acid, ethyl ester	0.042	44.11
N-phenethyl-acetamide	0.162	44.87

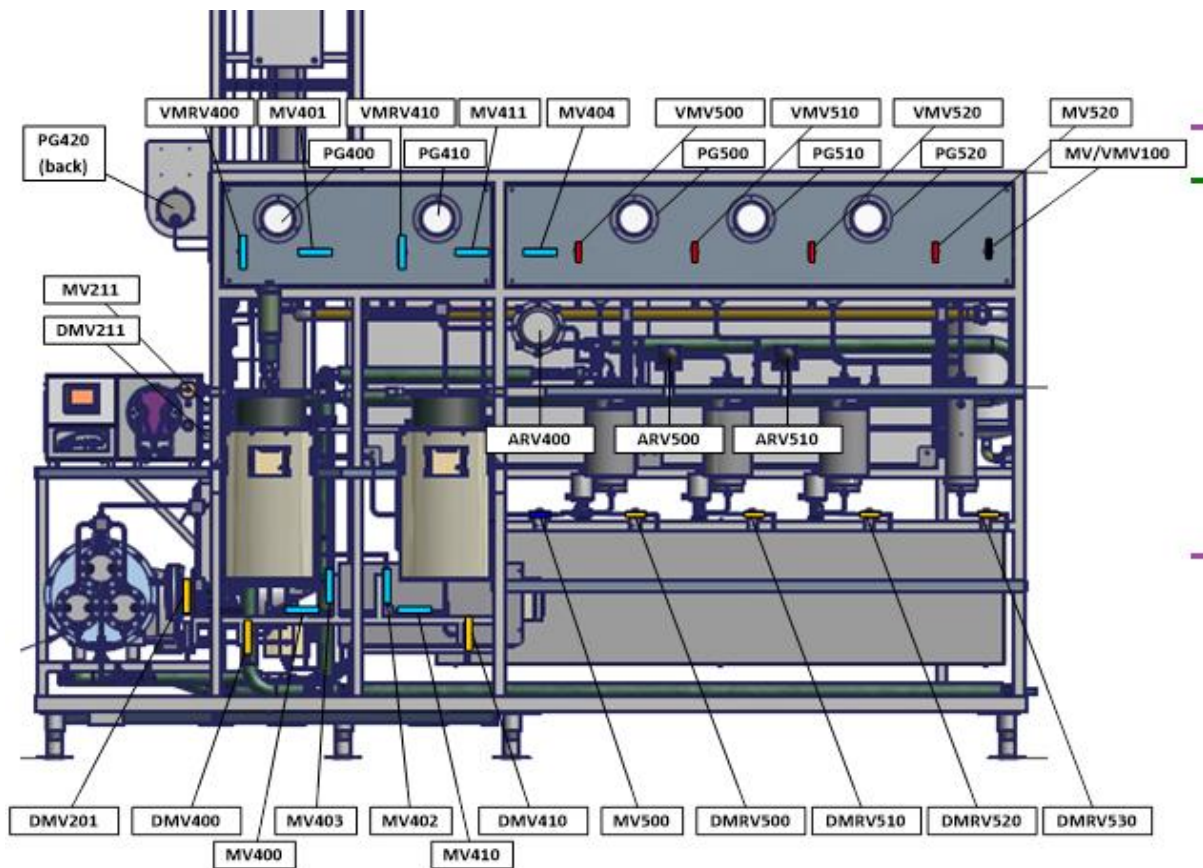## Appendix C: Supercritical fluid extraction equipment layouts

Figure C.1: Front side of the equipment with manual valves necessary to control extraction
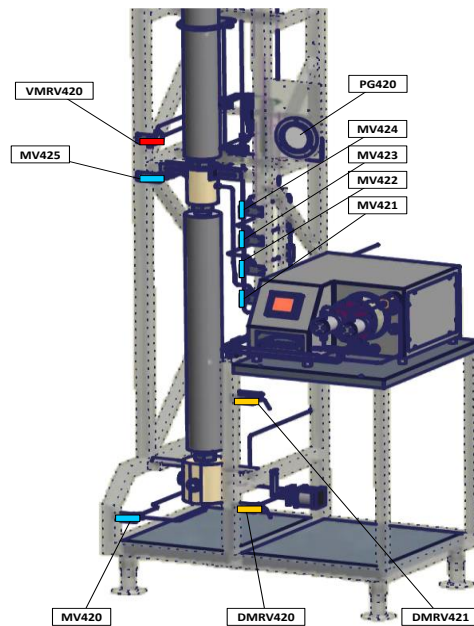

Figure C.2: Back side of the equipment with counter current extraction column, reflux pump, and manual valves to control the operation of the column
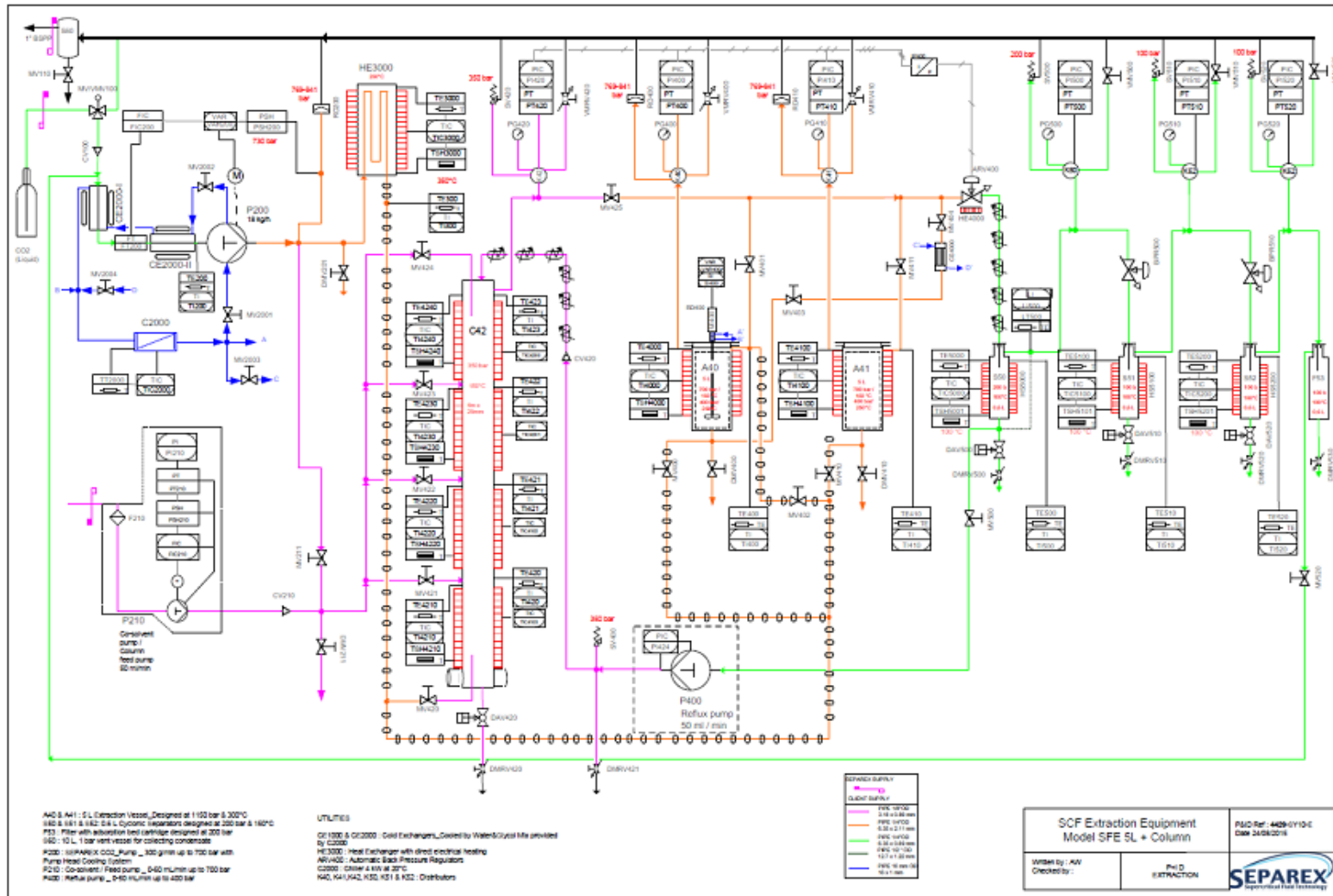
Figure C.3: P&ID of Supercritical CO₂ pilot plant

Appendix D: Checklist and operation of counter-current column

The checklist shown below must be used in preparation for the start-up:

- Ensure that the air (nitrogen), and CO_2 supply are available by inspecting the supply tanks outside the laboratory. Make sure that the bottle and shut-off valves are open.

- Switch on the HMI, and open the LabVIEW application for the column operation. Have a flash drive handy for data storage after the experiment.

- Turn on the High Voltage power supply to the machine, both on the wall and on the machine. The power light signal should switch on

- Ensure that the chiller is plugged into the mainframe of the machine and that it is set to "ON".

- Switch on the HMI, and open the application named "Column". Reset the alarms shown at the bottom of the screen, and switch the machine on by clicking on the green button at the top of the screen. The chiller should turn on. The over-temperature alarm will remain activated.

- Set all the thermal equipment to their required settings. Wait until the chiller and heaters reach the set temperature before filling the machine with CO_2.

- In the meantime, make sure that all the draining valves and venting valves are closed, vessels closed, and temperature balanced inside vessels before pressurizing the equipment.

- When both the chiller and the machine have reached a steady-state temperature, open CO_2 supply valve MV100 to fill P200 pump. Select the valves corresponding to your process conditions.

Table D.1: Check if all vent and drain valves are closed

Vent valve code	Checked	Drain valve code	Checked
VMRV400		DMV201	
VMRV410		DMV401	
VMRV420		DMV410	
VMRV500		DMRV500	
VMRV510		DMRV510	
VMRV520		DMRV520	
		DMRV530	

Table D.2: Check if MV400 – MV410 and MV500 are closed

Vent valve	Checked	Drain valve	Checked
MV400		MV420	
MV401		MV421	
MV402		MV422	
MV403		MV423	
MV404		MV424	
MV211		MV425	

Appendix E: Counter current column operation

❖ Pressurize the CO_2 pump by opening MV/VMV100

❖ After reaching a pressure > 50 bar in C42 check the setpoint pressure on ARV400, open MV425, and start the CO_2 pump P200

❖ Check the set points of the temperatures of the four sections of the column. Watch the development of the temperatures in the column.

❖ Wait for reaching pressure and temperature equilibria. Meanwhile, adjust the pressures and temperatures in the separators where necessary.

❖ Circulate until the system is in equilibrium

❖ In the meantime, prime the injection pump via DMV211

❖ When the column is ready, close DMV211, open the desired feed valve (MV421 – MV424) and start pump P210.

❖ Watch the pressure on Pump 210 to raise and reach the pressure in the column.

❖ If the pressure in Pump P210 remains lower than the column pressure, close the feed valve (MV421 – MV424), and prime the pump another time.

❖ Continue extraction and watch the development of the temperatures on the column

❖ Samples can be taken from the separators via the drain valves DMRV500 – DMRV520

❖ Samples can be taken from the bottom of the column via DMRV420.

❖ As extract is received in separator S50 it can be refluxed via Pump P400. In order to achieve this:

❖ The Pump P400 must be primed via DMRV421.

❖ The separation rate of the extract in separator S50 needs to be determined by sampling over time.

❖ The flow rate of the pump P400 must not exceed the rate of extract collected in the separator. It will not lead to a dangerous situation as the pump will continue to reflux CO_2 into the column, but such process conditions will not make much sense.

Appendix F: Concentration factor and percentage recovery data for calculations

Table F.1: Experimental Run 1 data collected at the raffinate and extract of SFE pilot plant

	Run 1		
Separators	Mass of the extract after 2hrs 30 min(g)	Acetoin concentration at extract (g/L)	Time (min)
1	59.79	0.96	90
2	3.72	3.99	90
Total mass l	63.51	4.95	
Separators	Mass of the extract after 4hrs 30 min (g)	Acetoin concentration at extract (g/L)	Time (min)
1	69.52	0.71	270
2	3.06	4.09	270
Total mass	72.58	4.8	
Separators	Mass of extract after operation (g)	Acetoin concentration at extract drained (g/L)	Time (min)
1&2	19.51	2.15	270
Starting feed mass(g)	mass of feed in column volume (g)	CO_2 flow rate(kg/h)	mass of raffinate after operation (g)
789.97	441.28	10	222.89

Table F.2: Experimental Run 2 data collected at the raffinate and extract of SFE pilot plant

	Run 2		
Mass of feed for the experiment (g)			
603.99			
Time (min)	Separators	Acetoin concentration at extract (g/L)	Average acetoin concentration at extract (g/L) (E)
60	1	1.78	1.71
60	2	1.63	
75	Separator 1&II	2.19	
Time (min)	Mass after draining (g)	Acetoin concentration at raffinate (g/L)	Average acetoin concentration at raffinate (g/L) (R)
0	495.76		0.44

20	0.48
25	0.42
30	0.42

Table F.3: Experimental Run 3 data collected at the raffinate and extract of SFE pilot plant

Run 3					
Mass of the feed (g)				Time (min)	CO$_2$ flow rate (kg/h)
623.65				30	23.052
				45	23.801
		Acetoin concentration at the raffinate			
Sample	Time (min)	(g/L)		55	27.04
				70	30.409
R5	5	0.60		85	32.313
R15	15	0.41		100	35.116
R20	20	0.40		115	36.97
				Extract	Acetoin
R25	25	0.38	Time (min)	Separators	(g/L)
Mass after draining (g)			50	Separator 1	2.176
445.06 g			50	Separator 2	3.894
			105	Separator 1&2	1.808

Table F.4**:** Experimental Run 4 data collected at the raffinate and extract of SFE pilot plant

Run 4				
Mass of the feed (g)				
	Time (min)	Acetoin concentration (g/L)	Time (min)	CO_2 flow rate (kg/h)
532.73				
Sample	0	1	15	43.88
Raffinate	5	0.371	30	50.792
R5	15	0.304	60	51.103
R15	20	0.275		
R20	25	0.189		
R25	30	0.186		
	Time (min)	Extract Separators	Acetoin concentration (g/L)	
R30	60	Separator 1	3.829980392	
mass of the drained raffinate (g)				
118.26	60	Separator 2	3.834009466	

Table F.5: Experimental Run 5 data collected at the raffinate and extract of SFE pilot plant

Run 5				
Mass of the feed (g)	Feed volume	Time (min)	Extract Separator	Acetoin concentration (g/L)
603.99	600 ml	30	Separator 1	1.410

Sampling time (min)	Acetoin concentration at the raffinate (g/L)
0	1.00
5	0.79
15	0.59
25	0.60
30	0.44

Table F.6: Experimental Run 6 data collected at the raffinate and extract of SFE pilot plant

Run6				
Mass of feed (g)			Time (min)	CO_2 flow rate (kg/h)
603.99	Time (min)	Acetoin concentration at the raffinate (g/L)	0	12.318
	0	1.0	5	13.238
	5	0.5	10	14.081
	10	0.3	15	14.907
	20	0.2	20	15.576
	25	0.2	25	16.645
Time (min)	Extract Separator	Acetoin (g/L)	30	17.566
30	Separator 1	0.910		

Table F.7: Experimental Run 7 data collected at the raffinate and extract of SFE pilot plant

Run 7				
Mass of feed (g)	Time (min)	Acetoin concentration at the raffinate (g/L)	Time (min)	CO_2 flow rate (kg/h)
617	0	1	10	21.809
	5	0.37	15	23.119
	10	0.1	25	24.276
	15	0.09	30	25.807
	20	0.15		
Time (min)	Extract Separator	Acetoin concentration at the extract (g/L)	Mass of extract (g) separator 1	Mass of extract (g) separator 2
30	Separator 1	0.410	236	239
30	Separator 2	0.650		
30	Separator 3	2.150		