Bioprocess designing for the synthesis of 2,5 Furandicarboxylic acid and its life cycle assessment

by

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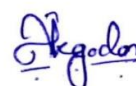Ghaziabad, U.P. – 201 002, India

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CERTIFICATE

This is to certify that the work incorporated in this Ph.D. thesis entitled, **"Bioprocess designing for the synthesis of 2,5 Furandicarboxylic acid and its life cycle assessment"**, submitted by **Mr. Godan T K** to the Academy of Scientific and Innovative Research (AcSIR) in fulfillment of the requirements for the award of the Degree of *Doctor of Philosophy in Science*, embodies original research work carried out by the student. We, further certify that this work has not been submitted to any other University or Institution in part or full for the award of any degree or diploma. Research materials obtained from other sources and used in this research work has been duly acknowledged in the thesis. Images, illustrations, figures, tables etc., used in the thesis from other sources, have also been duly cited and acknowledged.

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Dedicated to my family and friends

ACKNOWLEDGEMENTS

CONTENTS

LIST OF ABBREVIATIONS

%	Percent
°C	Degree Celsius
ANOVA	Analysis of Variance
BHMF	2,5-Bis(hydroxymethyl)furan/ HMF alcohol
bp	Base pair
cm	Centimeter
DFF	2,5-Diformylfuran
DNA	Deoxyribonucleic acid
FDCA	2,5-Furandicarboxylicacid
FFCA	5-Formyl-2-furancarboxylic acid
Fig.	Figure
FTIR	Fourier transform infrared spectroscopy
g/L	Gram per litre
GHG	Greenhouse gases
HMF	5-Hydroxymethylfurfural
HMFCA	5-Hydroxymethyl-2-furancarboxylic acid/ HMF acid
HPLC	High Performance Liquid Chromatography
L	Litre
M	Molar
meq	milliequivalent
mg	Milligram

mM	Millimolar
MSM	Mineral salt medium
NAD	Nicotinamide Adenine dinucleotide
NMR	Nuclear Magnetic Resonance
O.D	Optical density
PCR	Polymerase Chain Reaction
PDA	Photodiode Array Detector
rpm	Rotations per minute
RSM	Response Surface Methodology
Sp.	Species
UV	Ultraviolet
v/v	Volume per volume
vvm	Volume per volume per minute
w/v	Weight per volume
QMSM	Qingshengii mineral salt medium
CMPS	Chloromethylated polystyrene
DMF	N, N-dimethyformamide
DEA	Diethanolamine
PS-DEA	Polystyrene Diethanolamine
LCIA	Life cycle impact assessment
TRACI	Tool for the reduction and assessment of chemical and other environmental Impacts

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Chapter 1

Introduction and Review of Literature

1.1 Introduction

The excessive exploitation of the EARTH for improving the standard of living and industrialization has increased over the past decades which has created the increase in utilization of resources and energy. Now it's time to start thinking about the possibilities for resource utilization and efficient waste management. Sequentially, the term 'RECYCLING' was popular in every aspect of individual's life style. At the same time, people/scientists have started global level research, particularly on SUSTAINABLE technology/process, to reduce environmental burden. Sustainable technologies have always focused on the production of biofuels and platform chemicals from renewable biomass. In this context, industries are shifting from non-renewable energy to sustainable biomass based energy to bring down the emission of greenhouse gases (GHGs). The integrated biorefinery are looking for strategies to switch from non-renewable to renewable sources, especially from petroleum products. The global interest has increased on the development of an integrated and sustainable approaches for the conversion of surplus lignocellulosic biomass derived sugars to various platform chemicals.

In the energy context, according to the recent report by the International Energy Agency (IEA), India emitted 2,299 million tons of carbon dioxide in 2018, a 4.8% rise from last year. As per its commitments to the United Nations Framework Convention on Climate Change (UNFCCC), India has promised to reduce the emissions intensity of its economy by 2030, compared to 2005 levels. Furthermore, as per norms of CoP26, India has to achieve in the target of Net zero emission by 2070. Connecting to that, most of the chemical process which depends on coal or petroleum needs to be replaced in order to

achieve this aim. The dependence of coal and non-renewable resources are the major reasons for this high emission rate in India. Most of the Indian chemical industries are still using conventional technologies which affects the environmental GHG emission. In order to combat environmental emission issues, India has to adopt environmentally sound technologies and, in this scenario, use of renewable resources play an important role. There has been an increasing focus on biobased plastics in the last decade, thanks to its potential to reduce anthropogenic GHG emissions and ensuring the availability of raw materials. As India is an agrarian country, agricultural biomass is one of the easily available renewable resources. These lignocellulosic biomass consists of cellulose, hemicelluloses, and lignin, with cellulose being the most abundant carbohydrate on earth (Chen 2014). The cellulose and hemicellulose polymers can be converted to simple sugars which can be utilized for the production of various fuels and chemicals.

According to the US Department of Energy, the biomass-based compound 2,5-furandicarboxylic acid (FDCA) is one of the 12 uppermost chemical building blocks. FDCA ($C_6H_4O_5$) can be used for the production of polymers and resins and is often referred to as "the sleeping giant" as it can replace the fossil-based terephthalic acid for the creation of novel products (Wang et al., 2018). Polyethylene furanoate (PEF), a polymer of FDCA and ethylene glycol (EG), is considered to be the "biopolymer of the future", which could substitute the polyethylene-terephthalate (PET) (Papageorgiou et al., 2016). PEF could reduce the non-renewable energy (NREU) need from 51% to 43%, and the greenhouse gas emissions from 54% to 46% (Eerhart et al., 2012). First generation feedstock such as starch and cereal crops are the main source for HMF and FDCA production (Parshetti

et al., 2015), but lignocellulosic biomass (second-generation feedstock), which does not compete with food and feed markets, can also be a source of HMF/FDCA.

1.2 Hydroxymethylfurfural (HMF)

HMF ($C_6H_6O_3$) is an organic compound formed by the dehydration of reducing sugars and widely used in the chemical applications due to its functional groups (alcohol and aldehyde moiety) and furan aromatic ring. It can be oxidized to synthesize FDCA. The general oxidation pathway of HMF to FDCA is shown in Scheme 1.1. Through HMF oxidation 5-hydroxymethyl-2-furan carboxylic acid (HMFCA), 2,5-diformylfuran (DFF), 2-formyl5-furancarboxylic acid (FFCA), adipic acid, including 2,5-furandicarboxylic acid (FDCA) can be synthesized. Also, HMF can be transformed to 2,5-bis (hydroxymethyl)furan/ HMF alcohol (BHMF) via dehydrogenation. In route A, the aldehyde group is oxidized to carboxyl group and form HMFCA following which the alcohol group of HMFCA is oxidized to an aldehyde group, yielding FFCA, which is further oxidized to FDCA. In B route, the alcohol group of HMF is significantly oxidized to an aldehyde group to form DFF, following which the aldehyde groups of DFF are sequentially oxidized to carboxyl groups to yield FDCA via FFCA.

HMF oxidation and catalytic conversion produce FDCA (Hart et al., 2015). Oxidation can proceed through autoxidation or aerobic oxidation (Lilga et al., 2010). Two pathways are viable: (1) heterogeneous catalysis with precious catalysts (Ståhlberg et al., 2012) or non-precious metals catalysts (Li et al., 2017), and (2) biotransformation with enzymes (Karich et al., 2018) or whole cells (Koopman et al., 2010b). At present, the chemical method of the production of FDCA is costly due to the high energy demand and the process use of metal salts instead of environmentally friendly solvents (Koopman et

al., 2010a). In terms of cost and efficiency, the processing of C6 sugars into HMF as an intermediary product is more difficult than the route from HMF to FDCA (Bello et al., 2019).
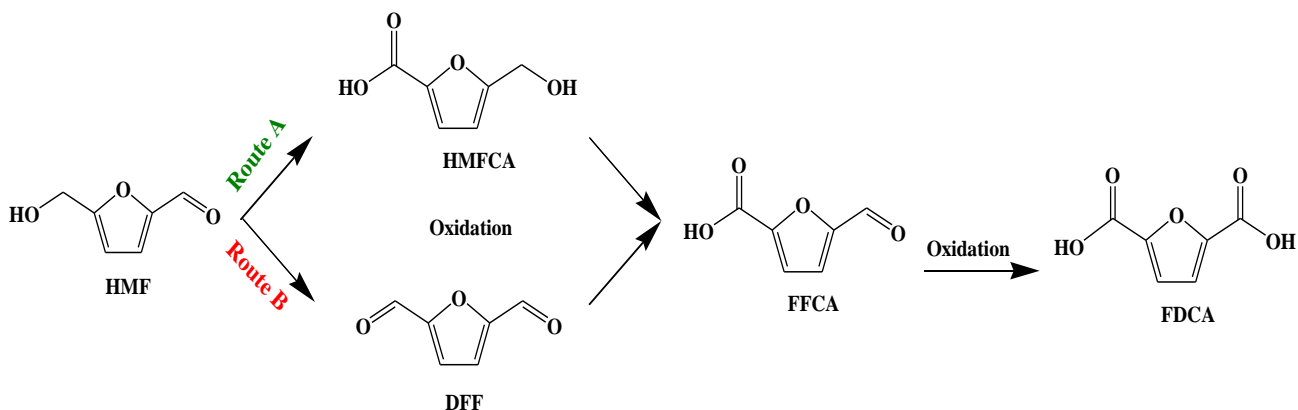
Scheme 1.1: HMF to FDCA general oxidation pathway

1.3 Chemocatalysis: Current scenario and problems

Most of the industries are following chemocatalysis due to the higher activity and availability of wide range of catalysts. Moreover, due to its robustness, larger volume of product can be synthesized. Apart from these advantages, industries are facing high amount of energy usages and environmental issues. Other drawbacks are low selectivity, high cost equipment and reagents using for the reactions and toxic chemicals make process unfavorable. These factors will lead to high waste generation and low recyclability issues (Troiano et al., 2020).

1.4 Biocatalytic production of FDCA

The biocatalytic production of FDCA has been stimulated by relatively recent discoveries pertaining to enzymes and organisms with oxidative activity on HMF. The origin of new biocatalytic processes often begin with an identification of new capable organism and their whole-cell/enzyme activities. To simplify the process of synthesizing cofactors whole metabolically active cells are used so that reduced cofactors can be reoxidized via the respiratory chain, as opposed to stoichiometric cofactor(s) and reoxidants. In addition to this, many enzymes have a broad substrate scope and high product specificity.

1.4.1 Whole cell biocatalysis

Whole cell biocatalytic production of FDCA has been investigated from several years with new organisms and also metabolic engineering of already known potential isolates for the improved production of FDCA. This section aims to provide an overview of whole cell biocatalytic production of FDCA using various isolates. In cell free biocatalysis, the aldehyde group (functional group) of HMF will react with enzyme and reactive oxygen would generate which inhibits the respected enzyme stability and finally led to denature the protein. In whole cell biocatalysis, the cell membrane can act as a barrier from bulk of reactive aldehyde substrate and using endogenous catalases hydrogen peroxide can be converted (eg; *Pseudomonas putida*). To provide the cellular environment the whole-cell biocatalytic production was first initiated by Koopman et al for the FDCA production using *Pseudomonas putida* S12. Initially they have identified and characterized the molecular pathway of *Cupriavidus basilensis* HMF14 which metabolizes HMF and furfural. Later the gene hmfH which is encoding for oxidoreductase was introduced in *Pseudomonas putida*

S12 (Koopman et al., 2010a; Koopman et al., 2010b). Employing fed-batch experiments, 30g/L of FDCA was produced with final yield of 97%.

Subsequently, a series of research on genetically engineered *Raoultella ornithinolytica* BF60 was carried out by altering HMF biotransformation pathway resulted in improved HMF conversion efficiency and FDCA yield (Hossain et al., 2017; Yuan et al., 2018a; Yuan et al., 2018b; Yuan et al., 2018c). Initial FDCA titer without any molecular alteration was 7.9 g/L by mutating dcaD gene and aldR gene to prevent FDCA degradation to furoic acid and catabolism of HMF to HMF alcohol respectively. Also, by overexpressing aldehyde dehydrogenase the FDCA yield was increased to 13.9 g/L. Later HMFO and HmfH were introduced into the same *Raoultella ornithinolytica* BF60 and whole-cell biocatalysis was performed and FDCA yield was further improved from 51.0 to 93.6 mM. Introducing combinatorial synthetic pathway fine-tuning and comparative transcriptomics approach could improve the FDCA yield up to 221.5 mM with the help of pulse-feeding strategy. AldH gene which is responsible for FFCA to FDCA, which was identified and characterized. Over expressing the mentioned gene in *Raoultella ornithinolytica* BF60 could produce the final yield of 264.7 mM of FDCA. These research evidences clearly opened the way that molecular identification and alteration strategy not only to improve the FDCA production but understanding the FDCA synthesis pathway.

Thermally treated HCl macro algae containing HMF to FDCA biotransformation using *Burkholderia cepacia* H-2 strain 989.5 mg/L of FDCA was produced (Yang & Huang, 2016) and novel isolate *Methylobacterium radiotolerans* G-2 was reported with 50% of FDCA yield while 1 g/L of HMF was given (Yang & Huang, 2018). *Enterobacter* species showed low yield of FDCA (0.07 g/L) (Rajesh et al., 2018). In 2019, biotransformation of

HMF to FDCA by novel isolate *Acinetobacter oleivorans* S27 could produce FDCA yield of 65% (Godan et al., 2019) and from the same lab 6.6 mM of FDCA was produced when 8 mM of HMF was given using a novel fungal isolate *Aspergillus flavus* APLS-1 (Rajesh et al., 2019). Xin Wang (Wang et al., 2020) and co-workers constructed a biocatalyst by incorporating VDH1 and HmfH genes in *Escherichia coli* for the HMF to FDCA biotransformation with the yield of 96%. Hydroxymethylfurfural oxidase (HMFO) is the key gene involved in the HMF to FDCA synthesis pathway. Several reports on overexpression and heterogenous expression of HMFO. *Pseudomonas putida* S12 is the best example. HMFO gene expressed whole cell biocatalyst produced 35.7 mM FDCA from 50 mM HMF within 24 hours. Substrate inhibition was overcome by fed-batch strategy and increasing cell density. After 72 hours they could produce 542 mM FDCA, which is the highest production rate reported till today (Hsu et al., 2020). There was an interesting report on FDCA production from HMF by syntrophic consortium of engineered *Synechococcus elongatus* and *Pseudomonas putida.* In this study sucrose was produced by *S. elongatus* via photosynthesis. *Pseudomonas* could utilize the sucrose and catalyze the HMF to FDCA conversion. The two strains were surface engineered to improve the FDCA yield from 70% to 100% (Lin et al., 2020). Recent report on improved FDCA synthesis by introducing CRISPR technology for stable engineered strain. They could express both HMF oxidoreductase (HMFH) and HMF oxidase (HMFO) in *Pseudomonas putida S12* strain. Additionally, HMF transporter (HMFT1) was integrated which evidently increased the HMF (250 mM) biotransformation rate and final yield of FDCA was 196 mM (Pham et al., 2020). HMF (5 g/L) conversion using *Acinetobacter calcoaceticus* NL14 aerobic bacterium was reported with very low yield of FDCA (0.54 g/L) production (Sheng et al.,

2020). Apart from molecular level modification, cell immobilization was implemented for the improved FDCA production. In the latest study reported that immobilized *Burkholderia cepacia* H-2 cells could utilize the 2 g/L of HMF in presence of other lignocellulosic inhibitors and FDCA was produced with other valuable chemicals (Tsai et al., 2021). All the organisms with their substrate (HMF) concentrations, genes responsible for the FDCA production and FDCA yield are listed in the table 1.1.

Organism	HMF concentration (mM)	Genes responsible for FDCA production	FDCA Yield (% mol)
Pseudomonas putida S12	25	hmfH	97
Raoultella ornithinolytica BF60	100	HMFO, HmfH, AldH	94
Burkholderia cepacia H-2	16	Not identified	51
Methylobacterium radiotolerans G-2	8	Not identified	50
Acinetobacter oleivorans S27	8	Not identified	65
Aspergillus flavus APLS-1	8	Not identified	82.5
Escherichia coli	150	VDH1 and HmfH	96
Pseudomonas putida S12	50	HMFO	70
Pseudomonas putida	Not given	HmfH	100
Pseudomonas putida S12	250	HMFH,HMFO, HMFT1	78.4
Acinetobacter calcoaceticus NL14	40	Not identified	27

Table 1.1: Whole-cell biocatalysis (wild type and engineered strains) with their substrate concentration and FDCA yield (%)**.**

1.4.2 Isolated enzyme catalyzed production

Apart from whole-cell conversion, the enzymatic production of FDCA has more product recovery and also avoids undesirable side products. Compared to whole-cells, greater productivity and higher substrate scope may be achieved with enzyme catalysis. There are plenty of enzymes used for FDCA synthesis including single and multiple enzymes cascade systems. In this context, initial studies reported on equal concentration of HMFO enzyme along with FAD cofactor which catalyzes the 4 mM of HMF to FDCA with 96% of yield within 24 hours (Dijkman et al., 2014). However, HMFO activity is less towards DFF and FFCA without FAD cofactor also FAD is expensive and may not preferable for industrial process. Enzyme catalysis using lipase with immobilized TEMPO (2,2,6,6-tetramethylpiperidine-1-oxyl) was reported with 93% (Krystof et al., 2013). HMF oxidation by fungal enzyme was reported for the first time in 2015. Fungal aryl-alcohol oxidase (AAO) could oxidize alcohol moiety of HMF to FFCA via DFF. FDCA production (91% yield) was completed by introducing another fungal enzyme, peroxygenase within 120 hours. The fact, HMF to FDCA oxidation could not perform by single enzyme led the research focus on enzyme cascade systems. There were several reports on FDCA production by galactose oxidase M3–5 and aldehyde oxidase PaoABC with yield of 74% (McKenna et al., 2015). In other work, galactose oxidase and lipase were used to oxidize the HMF to DFF and DFF to FDCA respectively with the yield of 88% (Qin et al., 2015). Also, immobilization of periplasmic aldehyde oxidase (PaoABC) with galactose oxidase M3–5 was improved the FDCA yield as well as Diformylfuran (DFF) tolerance (McKenna et al., 2017). In the category of flavoprotein oxidase, HMFO has played major role in HMF oxidation. Another study reveals the improved HMFO stability and efficiency by

creating thermostable mutant HMFO and yield got increased compared to the original HMFO (Martin et al., 2018). Another group focused on three combined fungal enzymes; wild-type aryl alcohol oxidase (AAO), wild-type peroxygenase (AaeUPO), and recombinant galactose oxidase (GAO). This particular enzyme cascade system yielded FDCA of 80% within 24 hours (Karich et al., 2018). Another interesting work is on immobilized enzyme, the magnetic laccase catalyst with TEMPO mediated catalysis. After 96 hours 90.2% of FDCA yield was resulted which also increased the reusability (Wang et al., 2018). Hydrogen peroxide can be formed during HMF reactions, which inhibits the microbial cell system specifically. Interestingly, H_2O_2 was removed by catalases, which increased the HMF to FDCA conversion by AAO (Serrano et al., 2019). The FFCA and FDCA were synthesized through dual enzyme cascade systems that is galactose oxidase and alcohol dehydrogenases while FDCA yield was 95% after 60 hours (Jia et al., 2019). Recent studies on FDCA production using laccase enzyme with TEMPO with 87% of yield but very low yield (0.4 g/L) (Yang et al., 2020). To this end, FDCA was produced by consecutive enzyme reactions. HMFO and lipase Novozym 435 were used for HMF (20 mM) to FDCA conversion. The final yield was 94% after 20 hours (Wu et al., 2020). A recent study on engineered HMFO isolated from *Methylovorus sp.* Stabilization of the particular enzyme was improved through B-factor analysis which yielded 98% under 35^0 C temperature (Wu et al., 2021).

Enzyme (s)	HMF (mM)	FDCA yield (% mol)
laccase (with TEMPO)	30	10
HMFO (with FAD)	4.0	95
HMFO	5.0	90
Immobilized lipase (with immobilized TEMPO)	10.0	100
GO and lipase	30.0	88
HMFO and lipase	6.0	94
Immobilized laccase and lipase (Novozyme 435)	20	94

Table 1.2: Enzyme catalysis: FDCA production yield with substrate concentration.

1.5 Critical analysis on biocatalysis and future perspectives

Currently, HMF conversion to value-added chemicals is the hot topic in the bioprocess. Several biocatalytic approaches with a number of well-known advantages have gradually been reported in various years as a typical type of substitutes to chemocatalytic processes for the synthesis of FDCA. Even if a number of exciting and encouraging results were obtained on biocatalytic approaches, there are still many difficulties and obstacles in their useful applications, particularly in situations that are industrially sound. The major hurdle on whole-cells and enzymes are high toxicity and inhibitory effect of HMF on biocatalysts. The purification/downstream process is the critical factor for the catalytically (bio and chemo) produced FDCA. Both whole-cells and enzyme cascade systems are facing the issue on several intermediates along with desired product. On the other hand, the economic viability of the biocatalysis has to be further investigated. In case of whole-cells the equipment cost is high. Also in case of enzymes, cost of isolated proteins is high. Moreover, the enzyme cascade systems involved complicated reaction

kinetics. Advantages and disadvantages of both whole-cell and enzyme catalyzed FDCA synthesis has summarized in the table 1.3.

Whole-cell biocatalysis	Enzyme catalysis
Advantages ❖ Crude substrate can be used ❖ Mild reaction conditions ❖ Biodegradable ❖ Cofactors are present within the cell system ❖ Specific isolation of proteins is not required. ❖ Ideal method for large amount of chemicals **Disadvantages** ❖ High toxicity of HMF ❖ Intermediates along with desired product ❖ Equipment cost is high	**Advantages** ❖ Product specificity and high selectivity ❖ Cofactors can be recycled (enzyme cascade) ❖ Chances of contamination is very low ❖ Products can be recovered easily ❖ Multistep reaction at a time **Disadvantages** ❖ High toxicity of HMF ❖ High cost of isolated proteins ❖ Complicated reaction kinetics

Table 1.3. Summary of advantages and disadvantages of whole-cell and enzyme catalysis.

It is urgently necessary to produce more durable biocatalysts with high tolerance and selectivity in this study area. The following aspects should also be emphasized in future studies in order to further promote the biocatalytic conversion of HMF into FDCA on an industrial scale. (A) Investigating the metabolic processes and rate-limiting phases of HMF biotransformation in detail to provide some valuable references for the investigation and modification of novel transformation routes in the related microorganisms. (B) There should be a novel and inexpensive immobilization techniques of whole-cells and enzymes to improve the FDCA production. Also it should be reusable in a certain extend. (C) systematically developing physiochemical properties, high-efficiency, and energy-efficient separation and purification of desired products to develop

them into extensive application processes. Today, the complete process of HMF to FDCA in a large scale is still in its infancy stage. A deep investigation on economically sound and robust biocatalysis needs to be studied.

1.6 FDCA based Polymers

With its green routes, modern polymer technology is demonstrating its versatility and advantages as multifunctional, flexible, and highly versatile macromolecules in both natural and manmade technologies. Polyethylene terephthalate (PET), polybutylene terephthalate (PBT) and polytrimethylene terephthalate (PTT) are high quality polyesters commercially achieved more importance with various applications, but the dicarboxylic acid terephthalic acid or its ester dimethyl terephthalate is fossil based. In the current scientific community, the value of biobased products has much interest. (Hu et al., 2009). Studied are focuse on PEF (polyethylene furandicarboxylate) by catalytical conversion of biobased feedstocks to FDCA monomer later combined to polymers. There is an evident transition from fossil based feedstocks to sustainable biobased feedstocks. FDCA can be polymerized with ethylene glycol (EG) to form PEF (Nakajima et al., 2017). The thermal properties of PEF are superior to those of PET because it exhibits higher thermal stability (higher glass transition temperature) and a lower processing temperature (lower melting point). A detailed comparison of the Physical properties of PET and PEF are given in Table 1.3.

Property	Units	PET	PEF
Tensile strength	Mpa	72.5	77
Density	g/cm^3	1.36	1.43
O_2 permeability		0.114	0.0107
CO_2 permeability		0.46	0.026
Glass transition	^{o}C	76	88
Melting temperature	^{o}C	250-270	210-230
E-modulus	GPa	2.1–2.2	3.1–3.3
Yield stress	MPa	50–60	90–100
Quiescent crystallization time	Min	2–3	20–30

Table 1.4: Comparison of the physical properties of PEF and PET.

1.7 Applications of FDCA

Recent attention has been paid to FDCA because of its wide application in many fields, especially as a replacement for petrochemical-derived terephthalic acid in the synthesis of useful polymers. There are numerous applications of FDCA, including green chemicals and biopolymers. Although it is chemically stable, it undergoes the reaction typical of carboxylic acids, generating carboxylic dihalides, esters, and amides (Zhang & Deng, 2015), (Van Haveren et al., 2016), (Harmsen & Hackmann, 2013), (de Jong et al., 2012), (Teong et al., 2014). There are multiple billions of euros in materials business, comprising plastics, plasticizers, thermosets, and coatings. FDCA applications are summarized in table 1.4.

Applications	Role of FDCA
Polyesters, Polyamides, Polyurethanes	Monomers of FDCA has involved in the polymerization with wide range of polymers; bottles, containers, films and nylons.
Fire foams	Presence polycarboxylic acids (ingredient of fire foams).
Plasticizers	Esters of FDCA can substitute phthalate plasticizers for PVC
Precursor of levulinic and succinic acids	FDCA as a platform chemical
Pharmacology	Anti-bacterial properties of FDCA derivatives. Presence of FDCA in tetrahydrofuran is used for artificial veins preparation in transplantation.

Table 1.5: FDCA applications.

1.8 Objectives of the study

Currently, chemical routes are followed in industries for the production of FDCA via oxidation of HMF where the process requires high temperature, high pressure, metal salts, organic solvents and toxic chemicals. Also, the purification of FDCA after the chemical reactions make the processes expensive and it generates lots of chemical pollutants which are not eco-friendly. So, the industries are looking for an alternative environmentally friendly method for the production of this chemical via microbial conversion process where the starting raw materials include 5-hydroxymethyl furfural (HMF), fructose or renewable biomass (Scheme 1.2). The biological process is still in infancy and there exists a wide gap in developing an economically efficient process for the production of FDCA.
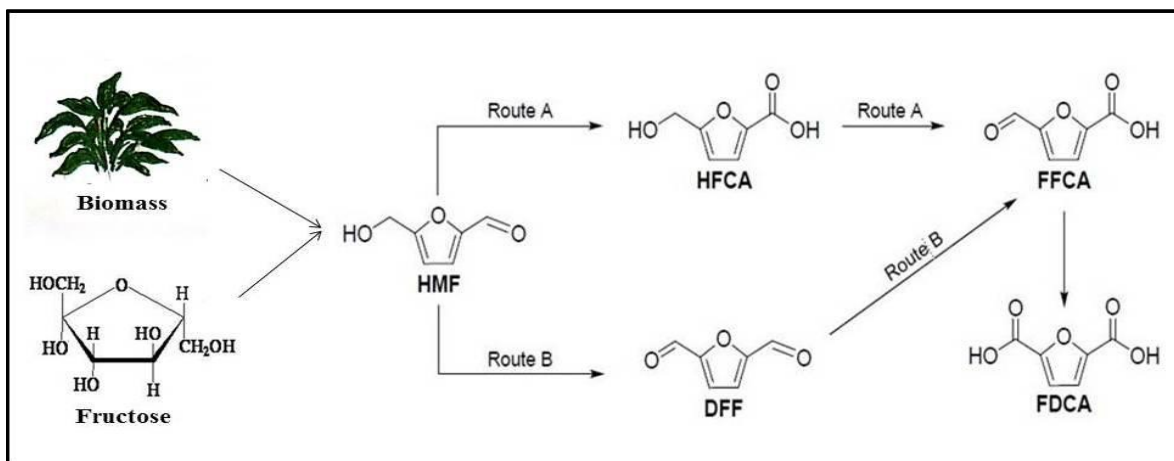
Scheme 1.2: Schematic representation of the biological conversion of biomass derived HMF into FDCA.

With the above background the objectives of the study are

1. Conversion and optimization of biomass derived carbohydrate to 5-hydroxymethylfurfural (HMF).

2. Exploration of non-pathogenic microorganism(s) for 2,5-furandicarboxylic acid production.

3. To develop an optimized bio process for the FDCA production by the selected strain.

4. Evaluation and designing of different biotransformation strategies; batch, fed-batch and immobilization for the FDCA production.

5. Downstream process of FDCA.

6. Comparative Life Cycle Assessment (LCA) of the complete process from biomass to FDCA and to understand the environmental impacts.
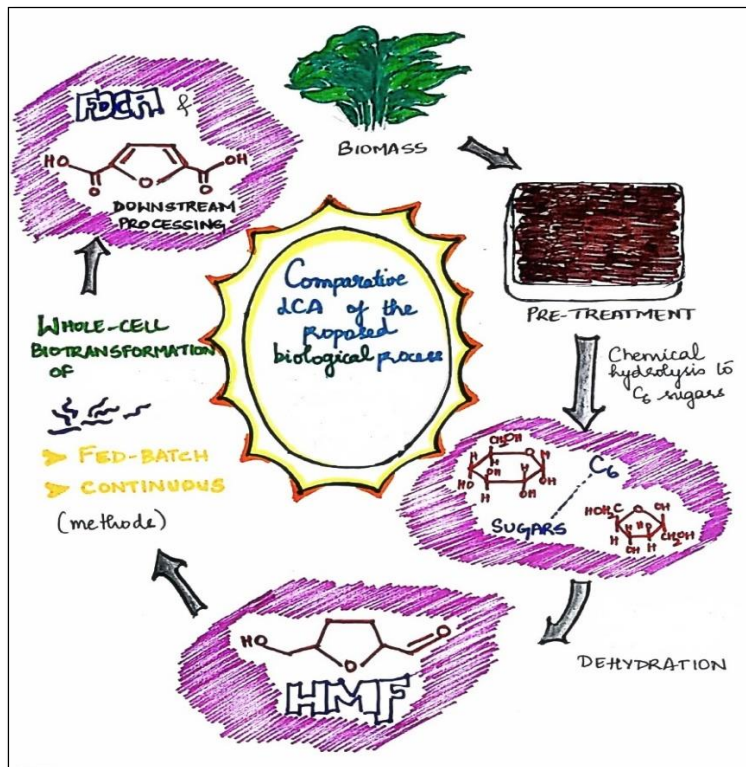
Scheme 1.3: Hand drawn illustration of objectives.

Organization of the thesis

The chapter comprises of eight chapters. Chapter 1 is introduction and review of literature. Chapter 2 discusses the HMF synthesis from sorghum syrup derived fructose dehydration using solid acid catalyst. In chapter 3, isolation, screening and identification of potential microorganisms for the FDCA are discussed. This chapter also deals with the physical parameter optimization of selected FDCA producers. Chapter 4 deals with the different biotransformation strategies and immobilization for the improved FDCA production by the best strain. Chapter 5 discusses the complete process of sorghum syrup derived HMF to FDCA through biotransformation in flask and fermenter level (1 L). In chapter 6, the downstream process for the separation and purification of synthesized FDCA. Chapter 7 discusses the environmental impacts of the complete process by

comparative life cycle assessment (LCA) studies. Finally, the chapter 8 summarizes the whole work and conclusions are described based on the present study.

Chapter 2

Chemo-catalytic dehydration of sorghum syrup derived fructose for the synthesis of HMF

2.1. Introduction

In the platform chemical synthesis, the biomass derived processes is one of the most preferred and viable alternative. Utilization of agriculture waste as the starting material for the industrially important chemicals is an art of process. However, the main challenge lies in finding suitable carbon rich biomass and availability. After finding a suitable biomass, the next challenge is to develop a process with minimum cost and energy. Besides this, reduction in environment burden by reducing usage of toxic chemicals and solvents is yet another challenge. Among biobased chemical, Hydroxymethylfurfural (HMF) is well known platform chemical widely used as building block for fuels and chemicals. It can be derived from hexose by dehydration (described detail in chapter 1).

With all these priorities in mind, we continued our research interest with an aim to develop an integrated and sustainable process for the conversion of lignocellulosic biomass derived sugars to FDCA. Therefore, the studies were initiated on HMF synthesis from biomass derived sugars. Sorghum syrup is the juice derived from the stalks of sweet sorghum plants. It is a concentrated juice chemically comprised of sugars such as sucrose, glucose and fructose with minor constitution of starch, protein, and amino acid (Kaur et al., 2013). Sucrose is the predominant sugar present in the sorghum syrup that can be catalytically hydrolyzed to yield glucose and fructose. Catalytic dehydration of fructose to HMF has been widely reported using homogenous and heterogeneous catalyst (Wang et al., 2014; Qi et al., 2011; Chheda et al., 2007; Hu et al., 2012; Hu et al., 2009; Wei et al., 2011; Zhang et al., 2014). The main objective of this study was to utilize the sugars from the selected biomass. Secondly, efforts were made to reduce the usage

of toxic solvents because most of these dehydration reactions are not environment friendly and they use high energy too.

In the current study, we synthesized HMF from sorghum plant extract (syrup) through strong acidic cation resin mediated dehydration and the HMF was purified to synthesis FDCA through biotransformation.

2.2. Materials and methods

2.2.1. Chemicals and reagents

Chloroform was bought from Merck (India). Seralite SRC 120 and Amberlyst-15 was purchased from SRL Pvt Ltd (India). Sorghum syrup was obtained upon request from Indian Institute of Millets Research (Hyderabad, India).

2.2.2. HPLC analysis

The sorghum syrup derived HMF was analysed using reversed phase HPLC (Shimadzu, Japan) with ultraviolet detector on an organic acid column (Phenomenex; 300 mm × 7.8 mm). The mobile phase was 0.01N H_2SO_4 in deionized water with flow rate of 0.6 ml/min with column temperature 55 °C. The HMF was detected at 283 nm.

2.2.3. Screening of catalysts

In the current study, solid acid catalyst namely cationic resins were considered selected for the conversion of fructose to HMF. Two different cationic resin catalyst, Amberlyst 15 and seralite SRC120 were screened for the dehydration reaction. Screening of catalyst was performed on standard fructose. The reaction was conducted in water using fructose and catalyst in 1:0.5 ratio respectively for 1 h under reflux condition. After the reaction,

HMF yield was calculated after the quantitative estimation of HMF by HPLC analysis. The residual fructose was estimated by HPLC for the determination of percentage conversion. The reaction was extended till 3 h under the same reaction conditions.

2.2.4. Optimization of reaction parameters by response surface methodology

The resin which gave high HMF yield was used for the hydrolysis of sucrose in the syrup and further dehydration of fructose to HMF. Response surface methodology was adapted for the optimization of reaction parameters. A Box- Beheken design was employed for the optimization of time, catalyst and substrate concentration in the dehydration of fructose to HMF. The design and analysis of result was performed using minitab software (Minitab Inc. Ver. 17.1.0). The experiments were conducted in a round bottom flask equipped with reflux condenser and magnetic stirrer. The temperature was set at 100º C for all the reactions. All the experiments were performed at 20 ml reaction volume with three different parameters; reaction time (1 to 6 h), catalyst concentration (2.5% to 10% w/v) and sorghum syrup volume (2.5% to 10% w/v). The design's adequacy was validated experimentally by randomly selecting 6 solutions out of 40 solutions predicted by the software. The results obtained experimentally were compared to the predicted ones. After the reaction, HMF present in the reaction mixture was analyzed by HPLC. The experimental run yielding high HMF was further subjected to dehydration reaction with fresh resin at optimized concentration and time for the conversion of residual unreacted fructose to HMF.

2.2.5. Recovery and characterization of HMF

HMF present in the reaction mixture was extracted using chloroform. NaCl was added to the reaction mixture at the concentration of 5% w/v. Extraction was done thrice with equal volume of reaction mixture and chloroform. The chloroform layers were separated (organic layer and aqueous layer), combined and concentrated in rotavac to recover HMF. After the evaporation of chloroform, the left out HMF was dried in an oven set at $50^{O}C$ for 6h. Dried HMF was characterized by HPLC and NMR. Glucose and unreacted fructose was used as carbon source for inoculum preparations of *Rhodococcus qingshengii* C27 in the next stage of biotransformation experiments.

2.3. Results and discussion

2.3.1. Screening of catalysts

From the comparative study on the effect of reaction by the two catalysts tested, seralite SRC120 yielded high fructose conversion with high HMF yield (Figure 2.1). After 1 h of reaction, Seralite SRC yielded 261 mg of HMF and Amberlyst-15 yielded 115 mg of HMF. Control experiments without resin rendered low HMF yield indicating the catalytic action of resin on the dehydration of fructose. Nearly 99% conversion of standard fructose was obtained using Seralite SRC120 with 99% selectivity for HMF. Hence Seralite SRC120 was selected for the study on dehydration of fructose in sorghum syrup. Amberlyst-15 has been widely reported for the dehydration of fructose yielding 50-99% HMF yield (Caratzoulas & Vlachos, 2011; Sampath & Kannan, 2013; Shimizu et al., 2009; Takagaki et al., 2009; Upare et al., 2018; Zhu et al., 2011). Literature reports indicated incorporation of organic solvents (Butanol, Methyl isobutyl ketone), high boiling solvents (DMF and

DMSO) or ionic liquids for improving the conversion of fructose to HMF. In the current study, the reaction was conducted in the water as sucrose is the major sugar component in the sorghum syrup which needs to be catalytically hydrolyzed to fructose for subsequent dehydration to HMF. Though Amberlyst-15 had high catalytic sites (4.7 meq/g resin) than seralite SRC120 (4.5 meq/g resin) as provided by the manufacturer, seralite SRC120 rendered high HMF yield. This may be due to high catalytic site in aqueous phase for seralite SRC120 (2.5 meq/ml) in comparison to Amberlyst-15 (1.7 meq/ml). The next reaction was conducted with fructose and catalyst in 1:1 ratio till 3h, 99% conversion of fructose was achieved with 84 % selectivity to HMF (Figure 2.2).
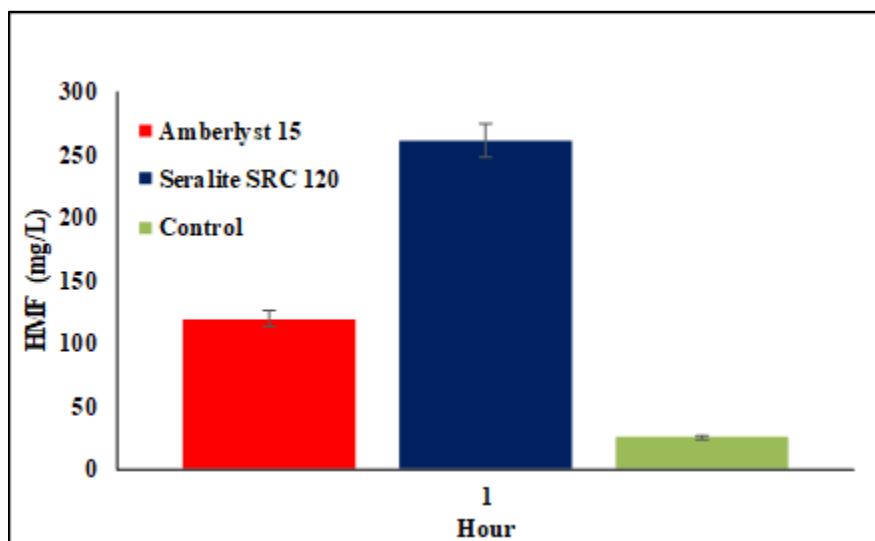
Figure 2.1. Screening of resin for the dehydration of fructose; Reaction conditions: 1g fructose, 0.5g of Seralite SRC 120 and Amberlyst 15, reaction periods of 1 h, temperature 100 °C, 20 ml reaction volume.
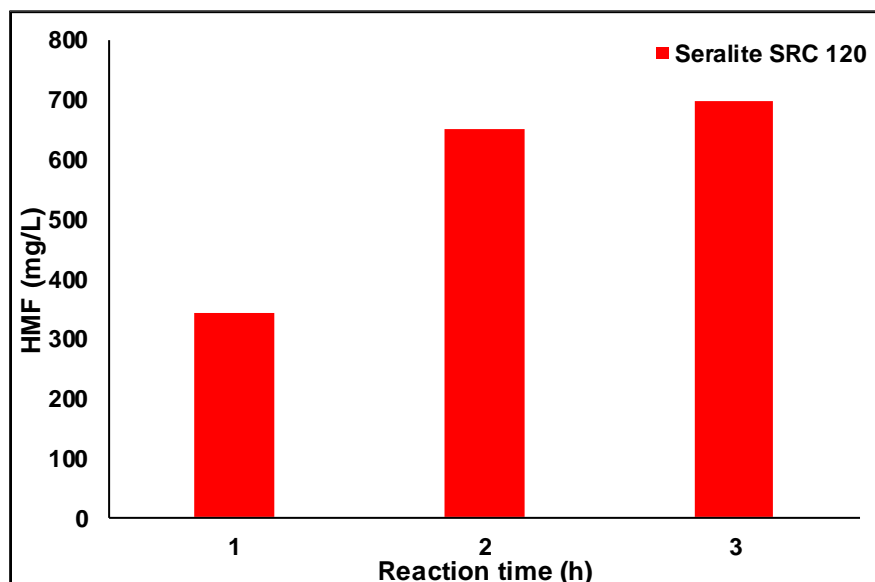
Figure 2.2. Seralite SRC 120 mediated fructose dehydration for HMF synthesis; Reaction conditions: 1g fructose, 1g of Seralite SRC 120, reaction periods of 3 h, temperature 100°C, 20 ml reaction volume.

2.3.2. Optimization of reaction parameters

Box Behnken design was adapted for the optimization of the dehydration process using Seralite SRC120 (Table 2.1). Optimization was studied with respect to time, catalyst concentration and substrate concentration (sorghum syrup) to be used for the dehydration reaction. Water was added and total reaction volume was maintained at 20 mL for all the experiments. Sorghum syrup has good nutritional value equivalent to honey and has 75% of carbohydrates (Moreau et al., 2004). HPLC profile of the sorghum syrup on the carbohydrate column indicated 77% sucrose, 13.2% glucose and 3.52% fructose. An additional peak was observed near to sucrose which may be partially hydrolyzed oligomers (Figure 2.3). Sucrose is the major sugar component present in the syrup and hence it needs to be catalytically hydrolyzed to yield fructose for further dehydration to HMF. The set up for the conversion is shown in Figure 2.4.
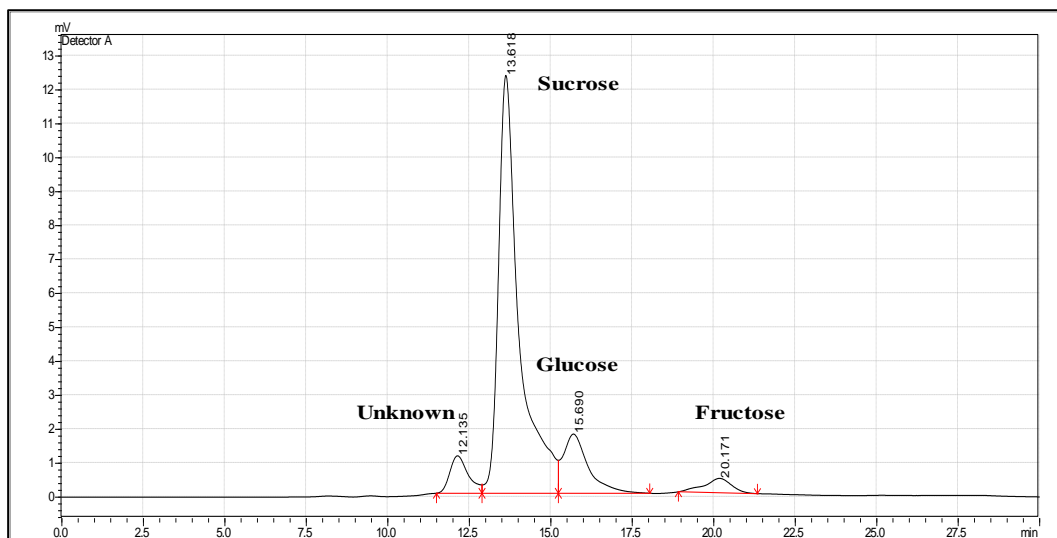
Figure 2.3. HPLC profile of sorghum syrup.

Experimental findings suggested that sucrose was completely hydrolyzed to glucose and fructose in an hour. However, it was observed that only fructose could further dehydrate to yield HMF. Glucose obtained after the hydrolysis remained unaffected throughout the reaction. Theoretically, glucose can directly dehydrate to HMF or glucose can isomerize to fructose and then dehydrate to yield HMF. The latter one is most studied and investigated. Literature reports use of strong Lewis acid for the isomerization of glucose to fructose. In the current study, the catalyst used was strong cation exchange resin bearing sulphonic group. Hence isomerization of glucose to HMF was least expected. Figure 2.5 depicts the contour plots illustrating the effect of time, catalyst concentration and substrate concentration (syrup volume) on the HMF yield. Contour plot between catalyst concentration and time (Figure 2.5A) indicated high HMF yield at middle level of catalyst loading and time. At lower catalyst loading and short reaction time, there was not enough catalytic site available for the dehydration of fructose to HMF. HPLC analysis indicated high unreacted fructose concentration for reaction conducted at lower

catalyst loading with shorter reaction time. This was attributed to the fact that the dehydration of fructose to HMF is incomplete due to lack of catalytic sites or insufficient reaction time. As the time proceeds, HMF was formed with catalyst equivalent to substrate loading. However, with further increase in reaction time HMF can further undergo rehydration to form Levunilic acid and with it increase in catalyst concentration, more catalytic sites are available to rehydrate or polymerize the HMF to Levunilic acid and humins respectively. Similar findings were observed for time versus substrate concentration (Syrup volume) as well (Figure 2.5B). When the substrate concentration is increased, most of the catalytic sites are utilized for the hydrolysis of sucrose to fructose and glucose. In addition to that, increased incorporation of sorghum syrup leads to enhanced concentration of other constituents of syrup namely starch and protein which can irreversible block the catalytic site. In case of substrate concentration versus catalyst concentration (Figure 2.5C), the contour plot for high HMF yield were found to be broader at intermediate concentrations indicating the significant interaction between catalyst and substrate concentration.

Run Order	Time (h)	Catalyst Concentration in (%)	Substrate Concentration (%)	Yield (%)
1	3.5	6.25	6.25	56.4
2	3.5	6.25	6.25	56.4
3	1	6.25	2.5	39.24
4	3.5	2.5	2.5	5.17
5	3.5	6.25	6.25	56.4
6	6	6.25	10	45.35
7	1	6.25	10	10.105
8	3.5	10	10	35.015
9	1	10	6.25	11.515
10	1	2.5	6.25	1.15
11	3.5	2.5	10	4.7
12	6	6.25	2.5	27.73
13	6	10	6.25	48.88
14	6	2.5	6.25	11.55
15	3.5	10	2.5	48.66

Table 2.1. Box-Behnken design for optimization of reaction parameters for optimum

HMF yield from sorghum syrup.
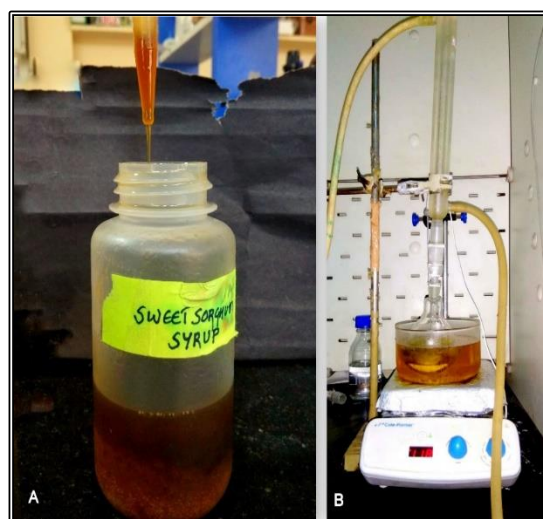
Figure 2.4. Sorghum syrup (A) and Reaction setup for sorghum syrup to HMF

synthesis (B).
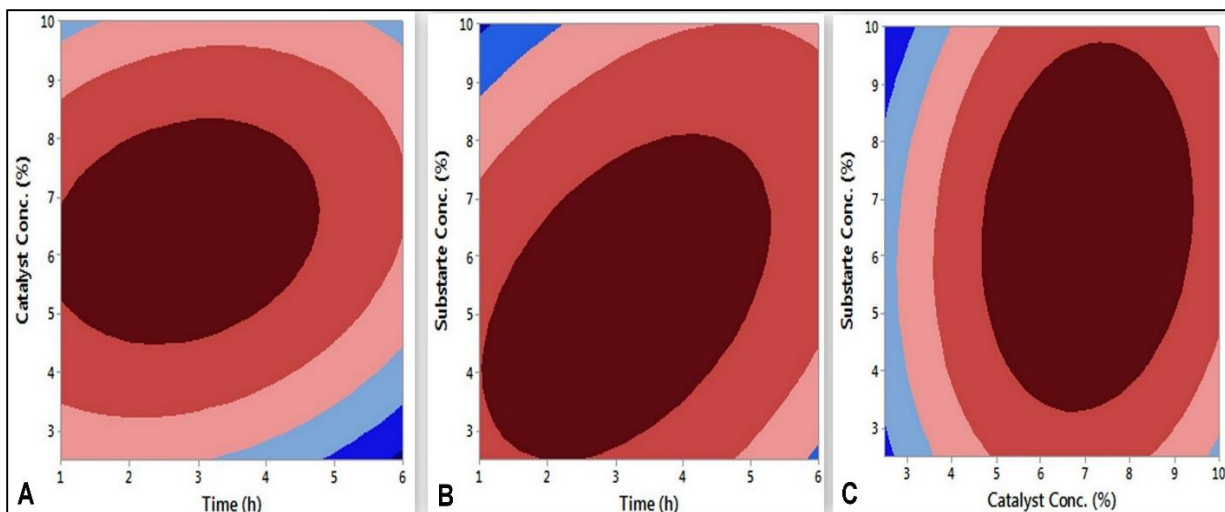
Figure 2.5. Contour plots of the parameters which include, Interaction of catalyst concentration versus time (A), interaction of substrate concentration versus time (B), Interaction of catalyst concentration versus substrate concentration (C) on the HMF yield. All the reactions were at 100º C and water as reaction medium.

The model's suitableness was determined by Analysis of Variance (ANOVA). Time and catalyst concentration were identified as the most significant factor with p values less than 0.01. Regression coefficient (R^2) was found to 96% indicating that the model can explain 96% variability in HMF yield. Validation experiments selected from the various solutions provided by the model indicated that HMF yield obtained experimentally was nearly equivalent to the predicted yield as shown in table 2.2. Maximum fructose conversion of 65% was achieved when the substrate and the catalyst concentration were 6.25% & 6.25% respectively with reaction time of 3.5 h. Experimental findings suggest that dehydration reaction time is the most crucial factor to maximize the fructose conversion with high HMF yields. Hydrolysis of sucrose was the primary reaction taking place by the treatment of aqueous sorghum syrup solution with seralite SRC120 resin. Sucrose was completely hydrolyzed within 1 h of reaction. From the HPLC analysis, it was clear that the glucose did not participate in the HMF synthesis because there was no change in the

glucose concentration after the reaction. The analysis of the result gave optimum condition as catalyst concentration 7.5%, substrate concentration 7.5% and reaction time 5 h with HMF yield of 65.8% (RSM validation run number 2). Literature reports suggested the reason for low HMF yield in water was due to side reactions (rehydration and polymerization) (Hansen et al., 2009). Previous studies on the reaction mechanism of fructose dehydration indicated the closed ring dehydration and the furanoid (five membered ring) form of fructose as the most stable tautomer leading to the synthesis of HMF (Upare et al., 2018). Caratzoulas et al. reported the reaction mechanism of fructose to HMF by molecular and quantum mechanics. They studied the effect of solvent dynamics on the energetic of the reaction and indicated that hydride transfer from C4 to C5 carbon was the rate limiting step with activation energy of 31.8 kcal/mol (Caratzoulas & Vlachos, 2011). The reaction mixture after separating the used resin was further treated with fresh resin catalyst in concentration equivalent to the initial reaction. The reaction was further carried out under optimized condition as per RSM model. From the experimental observation, the fructose conversion rate was increased but there was no significant increase in the HMF yield. Literature results suggest high conversion of fructose to HMF using solvents like butanol, DMF and DMSO with acidic resin as catalyst (Shimizu et al., 2009; Zhu et al., 2011). However, since the reaction was conducted in water, there is high probability of generation of side products namely levunilic acid, water soluble polymers and humins. The rationale of the study was to effectively utilize the sorghum syrup components for the production of FDCA. HMF after extraction with chloroform was used for the biotransformation to FDCA and the aqueous solution containing glucose and minor fructose was used for the cell enrichment of the organism.

RUN	Biomass Loading (%)	Catalyst Concentration (%)	Reaction time (h)	Predicted yield (%)	Experimental Yield (%)
1	7.5	7.5	5	61.33	56.4±
2	7.5	7.5	6	60.16	65.8±
3	10.0	10.0	5	46.76	58.75±
4	7.5	10.0	5	61.10	64.65±
5	10.0	7.5	5	54.45	44.65±
6	10.0	10.0	6	47.00	56.40±

Table 2.2. Predicted and experimental HMF yield (%) production from sorghum syrup from Box-Behnken validation results.

2.3.3. Recovery and Characterization of HMF

Chloroform extraction was performed for the recovery of HMF (Figure 2.6). HMF recovered from the reaction mixture using chloroform was analyzed by HPLC (Figure 2.7). HPLC analysis indicated 99% purity. The residual unextracted HMF left behind in the reaction mixture after extracting with chloroform was also determined by HPLC. It was observed that 95% of HMF was recovered. HMF obtained was further characterized by NMR. The NMR spectral pattern of the recovered HMF was similar to that of standard HMF (Figure 2.8). Strong signals were obtained for aldehydic (9.3 ppm), ring methylene (7.4, 6.5 ppm) and aliphatic CH_2 protons (4.5 ppm). Unlike the standard HMF, few additional peaks were also obtained for the recovered HMF indicating the presence of some impurities in the sample.

Figure 2.6. Chloroform extraction to purified HMF (left to right)
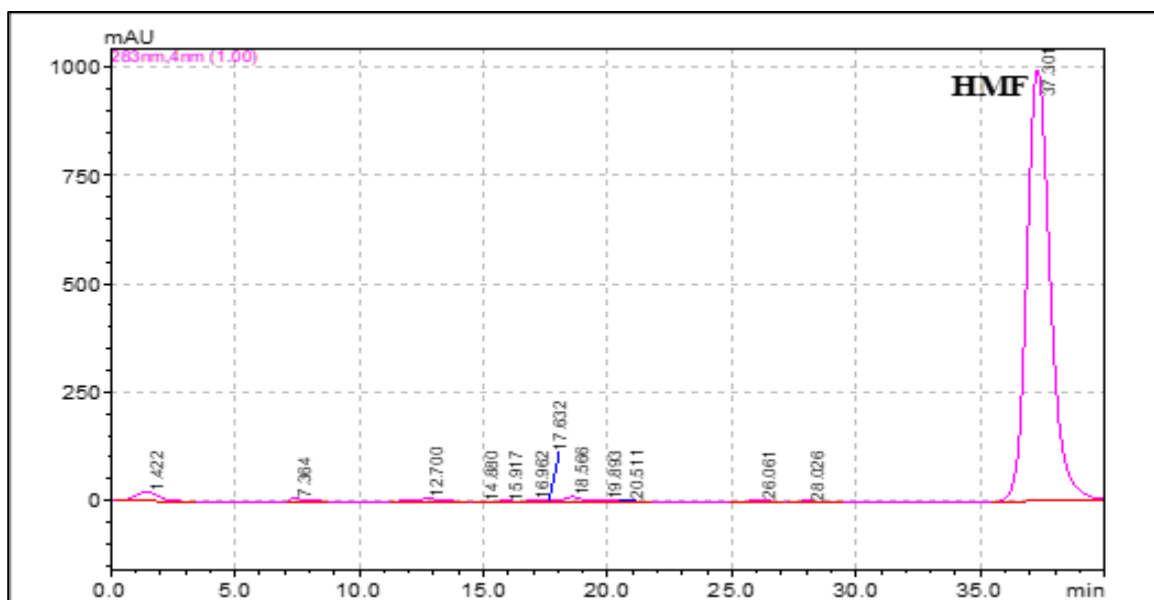
Figure 2.7. HPLC chromatogram of sorghum syrup derived HMF.
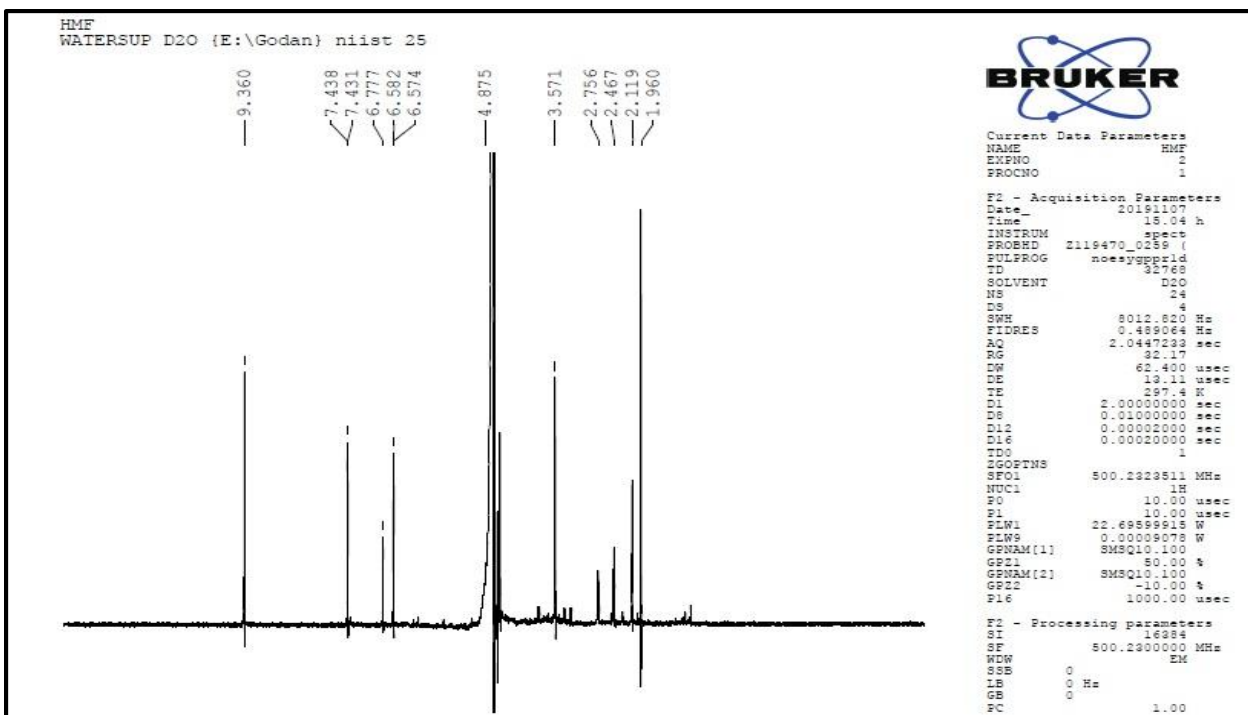
Figure 2.8. NMR spectra of sorghum syrup derived HMF.

2.4. Conclusion

An integrated approach with sustainable resources is an important driving force for the synthesis of valuable platform chemicals. Sucrose component of sorghum syrup was chemo-catalytically converted to HMF in water albeit with low conversion and selectivity. 72 mg of HMF was obtained from 1 ml of sorghum syrup. Seralite SRC 120 catalytically hydrolysed the sucrose component of the syrup to glucose and fructose. However, Seralite SRC 120 could dehydrate only fructose to HMF and the glucose remained unchanged. The synthesized HMF was recovered from the reaction mixture and purified using chloroform extraction. The hydrolysed and unreacted sugar component of the syrup needs to be effectively utilized or redirected to other processes.

Chapter 3

Isolation, screening & identification of potential microorganisms and physical parameters optimization for the production of 2,5-Furandicarboxylic acid

3.1. Introduction

Biological production of platform chemicals has gained attention for decades because of its environment friendly nature compared to chemical process. In case of FDCA, biological process still in its infancy. The very first challenge was to obtain a potential microbial strain for producing FDCA. Since HMF is toxic and it inhibits the microbial growth due to its aldehyde moiety and also involves three consecutive oxidations (pathway and other details are provided in chapter 1). Very few report are available on the production of FDCA by whole-cell biocatalytic. So the challenges and work flow involved in the production of FDCA using microbial strains are given below.

1. Selection and identification of potential microbial strain.
2. Physical parameters optimization and media engineering.
3. Optimization of bioprocess strategies (upstream) to improve the product yield.
4. Downstream process to separate and purify the desired product.
5. Complete process evaluation and scale-up (fermenter scale).

In the current study, isolations were carried out using onsite enrichment technique and later isolates were screened on HMF containing media. The North-East region of India is not much explored in terms microflora. So the isolation aims to explore the microorganisms from the North-east soil and Marcha which is an amylolytic starter used for the preparation of various alcoholic beverages in north-east states of India. A total of 324 microorganisms were isolated from different soils and marcha samples which were able to grow in media supplemented with different concentration of HMF. Then it was screened and identified. In this chapter, isolation, screening, identification of organism

and the important physical parameter optimization was described. Finally, the obtained results were compared and concluded for the further investigation.

3.2. Materials and methods

3.2.1. Chemicals

Analytical grades of HMF and its derivatives HMFCA, HMF Alcohol, DFF, FDCA, FFCA and furoic acid standards were purchased from Sigma-Aldrich, India. H_2SO_4 (analytical grade) was purchased from Fluka Pvt Ltd, and HPLC grade methanol and acetonitrile were purchased from Merck (India).

3.2.2. Analytical methods

HMF and its derivatives (HMFCA, HMF alcohol, DFF, FFCA, FDCA and furoic acid) were analyzed using gradient HPLC (Shimadzu, Japan).

HPLC Conditions

Column: C-18 column (250mm × 4.4mm× 5µm)

Column temperature: 40 °C

Mobile phase solvent A: Acidic water (0.02N H_2SO_4 in deionized water)

Mobile phase solvent B: Acetonitrile

Flow rate: 1 mL/min

Detector: PDA ultraviolet

Loading volume: 10 µl

Initially started with mobile phase A (100%) for 1min, then 94% of A and 6% of B for next one min. From 2nd min to 15th min, 98% of A and 2% of B and final 5 min running with 94% of A and 6% of B. The flow rate was 1.0 mL/min and total running time was 20 min. The UV wavelength for the detection of HMF and FFCA was 280 nm, HMFCA and furoic acid was fixed at 254nm and FDCA, HMF Alcohol and DFF at 263 nm, 225 nm and 290 nm respectively. The OD_{600} was measured using UV/Vis spectrophotometer (UV-1601 Shimadzu, Japan) set at 600nm.

3.2.3 Isolation and enrichment of 5-HMF to 2,5-FDCA biotransforming microbes

Isolation of microorganisms was carried out from marcha and soil samples collected from 25 different locations of Sikkim state in India with altitude ranging from 1120-4272 metre above sea level. To isolate the potential microorganisms for the production of FDCA from 5-Hydroxymethyl furfural, the collected soil samples were pre-cultured on minimal salt media (MSM composition: g/L: $MgSO_4.7H_2O$, 0.2g; $CaCl_2.2H_2O$, 0.002g; KH_2PO_4, 0.5g; K_2HPO_4, 0.5g; NH_4Cl, 0.5g; Trace elements solution 10mL, Trace element composition mg/l: $FeSO_4.7H_2O$, 300mg; $MnSO_4.H_2O$, 50mg; $CoCl_2.6H_2O$, 34mg; $Na_2MoO_4.2H_2O$, 34mg; $ZnSO_4.7H_2O$, 40mg; $CuSO_4.5H_2O$, 50mg;) with varying concentrations of HMF (500 mg/L to 2500 mg/L) for 24 h. This was followed by spread plating of pre-cultured samples following standard serial dilution procedure on four different media (LB agar, nutrient agar, potato dextrose agar and yeast extract peptone dextrose agar) supplemented with different concentration of HMF (500mg/l to 2500mg/l). The plates were incubated for a maximum of 72 h at 30°C and 37°C. Morphologically distinct isolates were streaked on fresh plates for obtaining single colony. Individual pure cultures were

screened further for the production of FDCA. The isolated cultures were further screened

for FDCA production and glycerol stocks were maintained at -80°C for future use.

NO	Given Name	SOURCE	ORGANISM
1	S6	BULBULAY ZOO-1	YEAST
2	S7	BULBULAY ZOO-1	BACTERIA
3	S13	MARCHA- ASSAM L.	BACTERIA
4	S21	BULBULAY ZOO-2	BACTERIA
5	**S27**	**HANUMAN TOK-1**	**BACTERIA**
6	S30	HANUMAN TOK-1	BACTERIA
7	**C27**	**EAST SIKKIM**	**BACTERIA**
8	C44	EAST SIKKIM	BACTERIA
9	C47	EAST SIKKIM	BACTERIA
10	C54	EAST SIKKIM	BACTERIA

Table 3.1: Details of screened Isolates for the FDCA production.

Initial screening was completed based on growth of strains in the presence of different

concentration of HMF. From the initial results the best two strains were selected for the

further study (Table 3.1). The selected strains were; **Code number S27 & C27**. Screened

isolates were identified by molecular level. Then the process parameters including

incubation time, inoculum size, pH, temperature were optimized. In this context, following

procedures and experiments were performed.

3.2.4 Molecular identification of bacterial strain

Genomic DNA was isolated using known traditional method for both gram negative and

positive bacteria. 16S rRNA fragment amplification was done by PCR using universal

primers 27F and 1492R (5′-AGAGTTTGATCMTGGCTCAG-3′ and 5′-

GGTTACCTTGTTACGACTT-3′ respectively). The amplified 16SrRNA gene was purified

by QIA quick gel extraction kit (Qiagen). Later purified PCR products were sequenced

(ABI 3130 automated DNA sequencer). Sequences have been deposited in GenBank.

<u>FDCA production using *Acinetobacter oleivorans* S27 whole-cell biocatalyst</u>

3.2.5A. Preparation of whole-cell biocatalyst

The pre-inoculum was prepared by growing *Acinetobacter oleivorans* S27 cells at 30 °C for 12 h on a rotary shaker (200rpm) in a Luria-Bertani (LB) medium (100mL). From the pre-inoculum 1% (v/v) were transferred to freshly prepared LB medium (100mL) and kept for 27 h for incubation with same conditions. After 27 hours of incubation, cells were harvested by centrifugation at 12000 RPM for 15 min at 4°C. Cells were washed twice with sterilized water and phosphate buffer (pH 8) to remove complete media components. The cell pellet was then transferred to mineral salt media (50mL) supplemented with HMF (500 mg/L) as a sole carbon source. The production media is kept for further analysis at 30°C on rotary shaker (200rpm). The supernatant from the reaction mixture was withdrawn in every 24h and analysed using high performance liquid chromatography (HPLC).

3.2.5B. Process development by optimization of parameters

All the parameters were optimised using 50mL reaction mixture supplemented with HMF concentration of 0.5 g/L. For optimization studies, priority was given to FDCA production. To optimise the temperature, the reaction was run at variable temperature at 30°C and 37°C with pH 7 and 200rpm. For pH optimization, reaction mixtures were prepared with variable pH ranges (6,6.5,7,7.5,8 and 8.5) at 30°C and 200rpm. The substrate concentration is an important parameter to be optimized and various concentration of HMF (g/L) such as 0.5,1,1.5,2,2.5 & 3 were maintained at 30°C, pH at 6.5 and 200 rpm. Inoculum age is an important parameter especially in a bio-catalytic process and for the

inoculum age optimisation, reaction conditions were 30°C, pH 7.0 and 200rpm with variable hours. Initially experiment was started from 6th hour to 30th hour, and later it was observed that the production happens between 24 hours and 28 hours. Hence this variable was kept in ranges from 24h-28h. The supernatant was recovered by centrifugation at 12000 rpm for 15 min for HPLC analysis. All experiments were repeated for three times and standard deviation (SD) was calculated.

3.2.5C. Response Surface Methodology (RSM)

Further optimization of the whole-cell biotransformation process using *Acinetobacter oleivorans* S27 was done by statistical optimization methods.

Experiment Order	Inoculum age (H)	Pre-inoculum size(mL)	pH
1	30	5	7.5
2	24	2	7.5
3	30	2	5.5
4	27	3.5	6.5
5	27	6.02	6.5
6	27	3.5	6.5
7	27	3.5	8.18
8	27	0.98	6.5
9	27	3.5	6.5
10	27	3.5	6.5
11	24	2	5.5
12	27	3.5	6.5
13	27	3.5	6.5
14	24	5	7.5
15	24	5	5.5
16	30	2	7.5
17	30	5	5.5
18	27	3.5	4.82
19	21.95	3.5	6.5
20	32.05	3.5	6.5

Table 3.2: RSM experimental design

Response Surface Methodology (RSM) studies were conducted using Minitab software version 15.1.10. Three parameters such as inoculum age, pre-inoculum size and pH were selected with respective ranges for Central Composite Design (CCD). The experimental design contained 20 runs as given in Table 3.2.

Rhodococcus qingshengii C27 whole-cell biocatalyst for FDCA production

3.2.6A. Isolation and identification of _Rhodococcus qingshengii_ C27

R. qingshengii C27 was isolated from soil samples of East Sikkim, India (altitude 2862 m above sea level). The screening and isolation protocol for the potential strain for the HMF biotransformation has already described in section 3.2.3 Also the genetic identification was done by 16s ribosomal RNA analysis (3.2.4). Cells were maintained in -20°C in 15% (w/v) glycerol.

3.2.6B Pre-inoculum and inoculum preparation

R. qingshengii C27 cells were grown in Luria-Bertani (LB) broth at 30 °C for 12 h on a rotary shaker (200 rpm). After 12h, 4% seed culture was inoculated into freshly prepared LB broth for 24h. The cells were then harvested by centrifugation at 13000 rpm for 8 minutes at 4 °C. Media components were removed by washing twice with sterile water followed by phosphate buffer (pH 8.0). The final cell concentration was 25 mg/mL (cell wet weight).

3.2.6C. Whole-cell biocatalyst preparation

The mineral salt media (MSM) with pH 6.52 (without adjusting) was prepared and supplemented with 1 g/L of HMF as sole carbon source. The cultivated _R. qingshengii_

C27 cells (25 mg/mL) were incubated at 30 °C and 200 rpm. The clear supernatant was collected from the reaction mixture in every 12 h interval and analysed using HPLC. The percentage of HMF conversion and percentage of FDCA yield were calculated using standard protocol. The pH variation was routinely monitored. All the experiments were conducted at least in triplicates with standard deviation <0.05.

3.2.6D. Process parameters optimization

All parameters were optimized with general procedure including cell harvesting (detail in section 3.2.). The effects of inoculum age (23-28 h), temperature (20 °C, 30 °C and 37 °C), pH (5.5-8.0) and HMF concentrations (8 mM, 16 mM, 24 mM, 32 mM) were studied. Reaction mixtures were taken in every 24 hours. The maximum HMF conversion and FDCA yield were calculated.

3.2.6E. Optimization of production media components

After optimizing the inoculum age and temperature, production media components were modified and optimized. All the experimental conditions were mentioned in section 3.2. The inoculum age and temperature were 24 hours and 30°C respectively. Both mineral salt media and modified media were compared with respect to FDCA production.

3.2.6F. HMF derivatives as substrate for FDCA production

To understand the capability/potentiality of *Rhodococcus qingshengii* C27 strain for the utilization of HMF derivatives other than HMF, experiments were performed supplementation of these derivatives in the media. These experiments provide an idea of FDCA synthesis pathway by the strain. Also it will provide the range of substrate tolerance of the desired strain. In this experiment we used HMFCA, BHMF (HMF alcohol), DFF and

FFCA (1g/L). HMF was used as the control for the comparison study. Cells were harvested and inoculated in QMSM medium. Samples were taken in every 24 h for HPLC analysis.

3.3 Results and Discussion

FDCA production using *Acinetobacter oleivorans* S27 whole-cell biocatalyst

3.3.1A. Isolation and identification of *Acinetobacter oleivorans* S27

Initially 10 strains were selected based on their capability of growing in presence of HMF. From the selected list of strains, S27 could grow in HMF up to 3000 mg/L concentration. After 16s rRNA gene sequencing, the organism was identified as *Acinetobacter Oleivorans.* Sequence were deposited in GenBank with an accession number (**MK359024**). The *Acinetobacter oleivorans* S27 was found to be a gram-negative and oxidase negative. It shows good activity on FDCA production when HMF is given as a sole carbon source.

Acinetobacter Oleivorans **S27** in presence of HMF (1g/L)

Sequencing result of 16S rDNA region (1404 bp)

GCAGTCGAGCGGAGAGAGGTAGCTTGCTACTGATCTTAGCGGCGGACGGGTGAG
TAATGCTTAGGAATCTGCCTATTAGTGGGGGACAACATTTCGAAAGGAATGCTAAT
ACCGCATACGTCCTACGGGAGAAAGCAGGGGATCTTCGGACCTTGCGCTAATAGA
TGAGCCTAAGTCGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGAT
CTGTAGCGGGTCTGAGAGGATGATCCGCCACACTGGGACTGAGACACGGCCCAG
ACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGGAAGCCTGATC
CAGCCATGCCGCGTGTGTGAAGAAGGCCTTATGGTTGTAAAGCACTTTAAGCGAG
GAGGAGGCTACTTTAGTTAATACCTAGAGATAGTGGACGTTACTCGCAGAATAAGC
ACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATC
GGATTTACTGGGCGTAAAGCGCGCGTAGGCGGCTAATTAAGTCAAATGTGAAATC
CCCGAGCTTAACTTGGGAATTGCATTCGATACTGGTTAGCTAGAGTGTGGGAGAG
GATGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCG
ATGGCGAAGGCAGCCATCTGGCCTAACACTGACGCTGAGGTGCGAAAGCATGGG
GAGCAAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGATGTCTACTAGCC
GTTGGGGCCTTTGAGGCTTTAGTGGCGCAGCTAACGCGATAAGTAGACCGCCTGG
GGAGTACGGTCGCAAGACTAAAACTCAAATGAATTGACGGGGGGCCCGCACAAGC
GGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGCCTTGAC
ATAGTAAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTTACATACAGGTGC
TGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAG
CGCAACCCTTTTCCTTATTTGCCAGCGAGTAATGTCGGGAACTTTAAGGATACTGC
CAGTGACAAACTGGAGGAAGGCGGGGACGACGTCAAGTCATCATGGCCCTTACG
GCCAGGGCTACACACGTGCTACAATGGTCGGTACAAAGGGTTGCTACCTAGCGAT
AGGATGCTAATCTCAAAAAGCCGATCGTAGTCCGGATTGGAGTCTGCAACTCGACT
CCATGAAGTCGGAATCGCTAGTAATCGCGGATCAGAATGCCGCGGTGAATACGTT
CCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTTGCACCAGAAGT
AGCTAGCCTAACTGCAAAGAGGGCGGT

3.3.1B. Effect of inoculum age, temperature, pH and substrate concentration

The tuning of physical parameters of whole-cell biocatalyst has an important role in the improved production of HMF derivatives. An ideal whole-cell biocatalyst should have some good features such as genetically flexible, fast growing capability in a simple media, enzyme production in fair amount and downstream process should be compatible with current system of approaches (Klatte et al. 2014). Among all HMF derivatives, FDCA got a special interest because of its wide applications. Hence the focus of this study was majorly on FDCA production and biotransformation parameters were optimized. The

optimization was started with inoculum age because the time of cell harvesting phase has a strong influence in the production level. Most of the enzymes, which are involved in the biotransformation, will be produced in a specific phase. Moreover, studies suggested late exponential phase is the crucial time for the production (Hossain et al. 2017). It was found that maximum conversion happens between 24-28 hours (Fig 3.1.1) and 27th hours were found to be optimum. Single parameter optimization for incubation temperature resulted 30°C (Fig 3.1.2). When the temperature increases up to 37 °C, less activity was noted and most probably this is happening because of the enzyme denaturation. Increase in temperature will not make much difference and also in the case of gram negative *Acinetobacter* species 30 °C was reported as optimum temperature for diesel oil degradation (Yoon-Suk Kang et al. 2011). For pH optimization studies, wide range of pH (6 to 8.5) were selected and results showed that pH 7.5 was optimum (Fig 3.1.3). Tuning of pH had an important role in the production level. Because of the other derivatives which include alcohol and acids, pH of the reaction mixture becomes acidic. Additionally, pH was maintained throughout the incubation period. The study shows that the optimum substrate (HMF) concentration ranges from 500 mg to 3000 mg (Fig 3.1.4). The strain could utilize 90% the HMF up to 2000 mg/L concentration. It was found FDCA yield was almost 51% when the initial HMF concentration of 500 mg/L. Higher than 2000 mg/L of HMF concentration, the reaction got inhibited because of the toxicity to the organism.
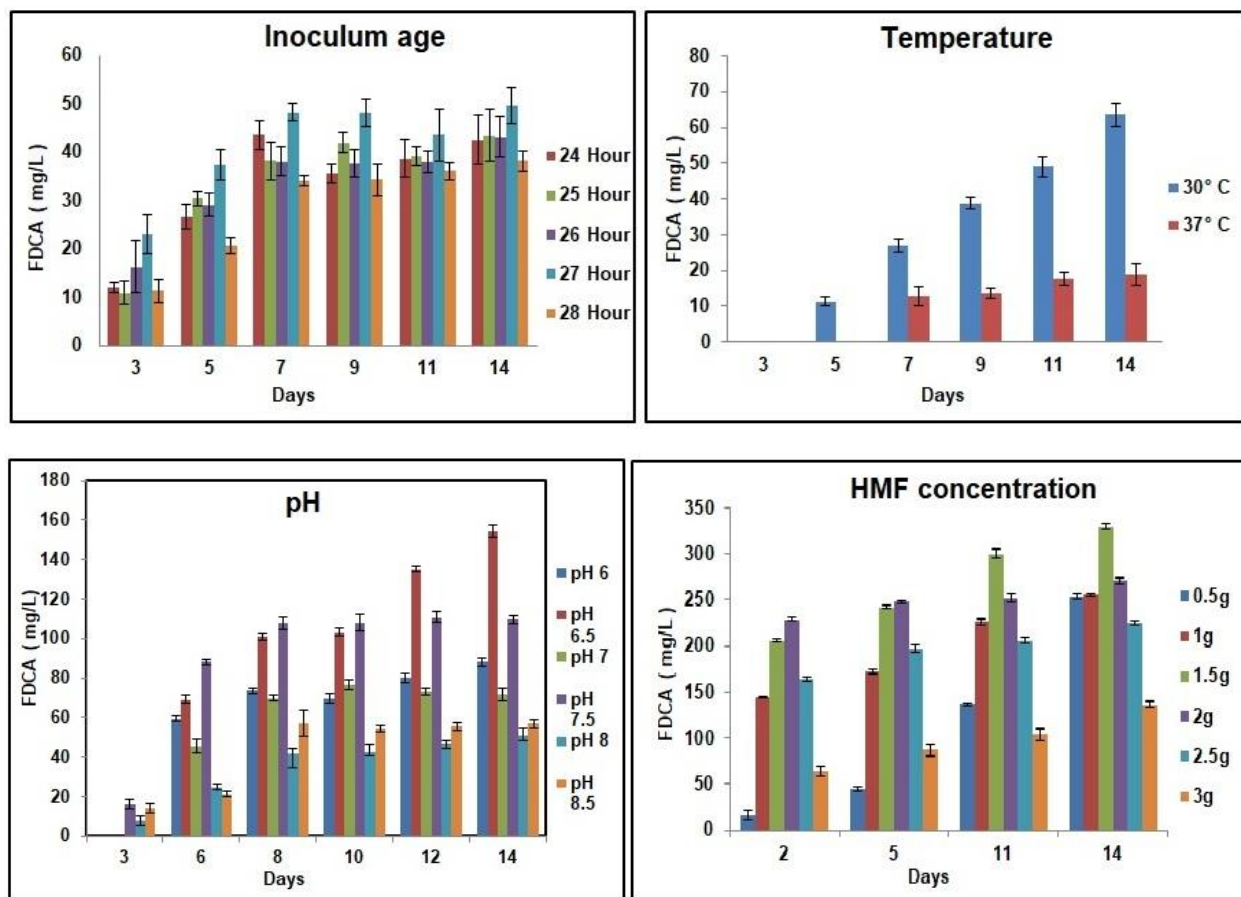
Figure 3.1.1: Optimization of inoculum age**, Figure 3.1.2**: Optimization of incubation temperature**, Figure 3.1.3**: Optimization of pH, **Figure 3.1.4**: Optimization of HMF concentration.

3.3.1C. Experimental design and RSM analysis on biosynthesis of FDCA

The result of 20 experiments based on three important parameters (inoculum age, pre-inoculum size and pH) for the biotransformation of HMF into major value added products like HMFCA and FDCA were analysed using Minitab software version 15.1.10.The biocatalytic production of FDCA involves three subsequent oxidation steps (Wierckx et al., 2015). Results showed that the particular strain had no inhibition on given HMF concentration because it could degrade the toxic HMF to less toxic carboxylic acids and

alcohols. The conversion of HMF to HMFCA was a comparatively faster than HMFCA to FDCA. Availability of oxygen for the further oxidation may be the reason and also compared to HMF, the strain may more tolerant towards HMFCA because it is less toxic than aldehyde. A detailed contour plot was obtained and analyzed (Figure 3.2). The model equation is as follows.

$$Y = 7.03-0.36(x_1)-0.40(x_2) +0.60(x_3) +0.004(x_1)^2 +0.008 (x_2)^2 -0.067 (x_3)^2 +0.013(x_1* x_2) + 0.005(x_1* x_3) + 0.02(x_2* x_3)$$

Where Y is FDCA yield; Inoculum age- x_1, Pre-inoculum size- x_2, pH- x_3.

 Here FDCA was produced more while giving high pre-inoculum size (5mL). It should be noted that while pH is changing towards acidic (5.5-6.5) FDCA yield got increased. At the same time initial pH was decreased and showed slightly alkaline towards as incubation time is increasing. This result was quite similar with the previous work of *Methylobacterium radiotolerance* mediated FDCA production from HMF in algal acid hydrolysate (Yang and Huang, 2017). More precisely, in the present work, in run number 15 (Table no.3.2) complete HMF degradation occurred and approximately 65% yield of FDCA was obtained. Moreover, pre-inoculum size was high and pH was at 5.5, and from this, it can be concluded that HMF toxicity could be decreased by increasing the cell concentration. To connect with the result of optimum pH for biotransformation, several fungal enzymes like aryl alcohol oxidase and recombinant galacto oxidase can be oxidized the HMF into HMF derivatives with the pH range of 5.5 to 7. This indicates the action of enzymes prefer slightly alkaline conditions (Karich et al., 2018). The study revealed that *Acinetobacter oleivorans* S27 strain has comparatively wide range of pH

tolerance. Actual relation between pH tolerance and FDCA production is to be further investigated.
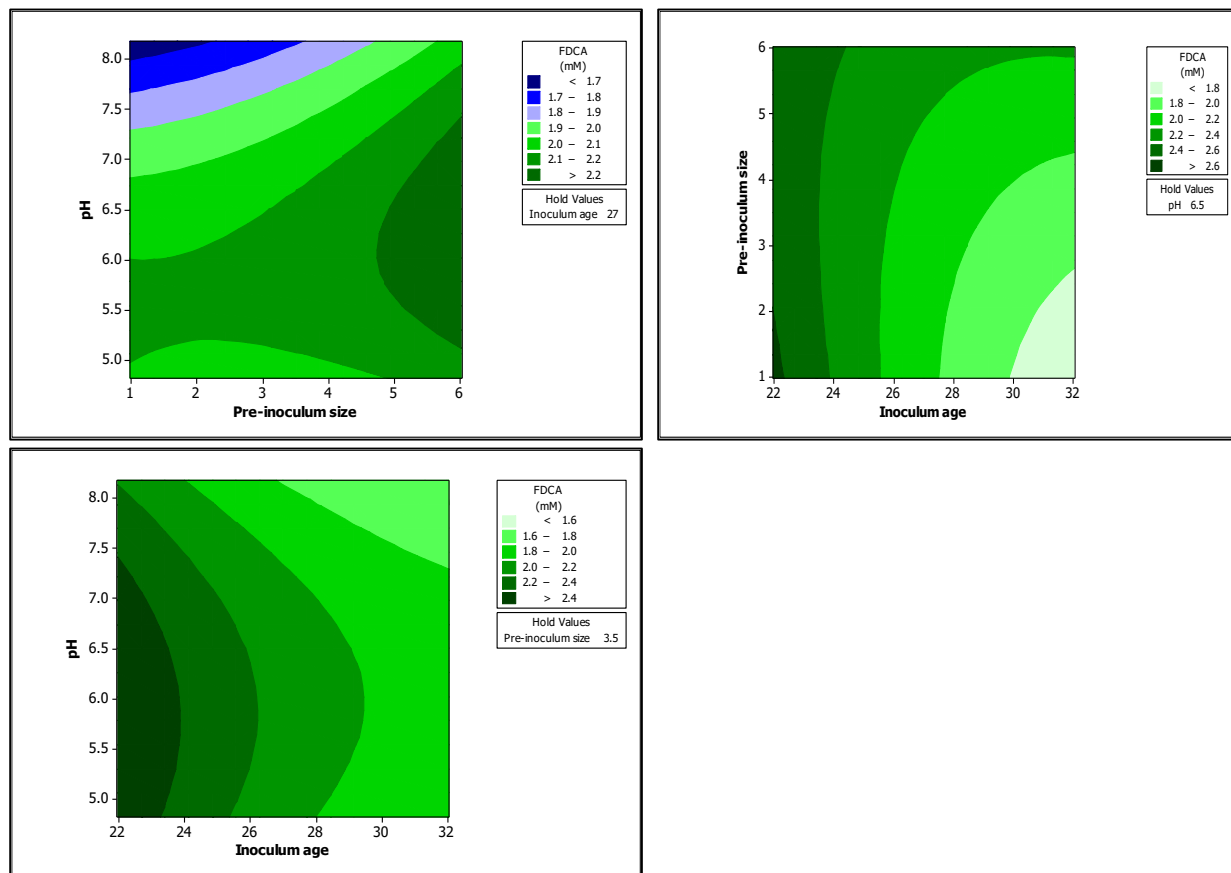
Figure 3.2; Contour plot of FDCA production: pH vs pre-inoculum size, pH vs inoculum age, pre-inoculum size vs inoculum age.

Rhodococcus qingshengii C27 whole-cell biocatalyst for FDCA production

3.3.2A. Isolation and identification of *Rhodococcus qingshengii* C27

Rhodococcus qingshengii C27 were isolated from east Sikkim. The genetic identification was done by 16S ribosomal RNA analysis and confirmed as *Rhodococcus qingshengii* using NCBI-BLAST and sequence alignment data obtained from EzTaxon server. The sequence is available in the GenBank with an accession number - **MW692850**). *R. qingshengii* C27 is a gram positive and aerobic bacteria It could grow in higher HMF concentrations compared to the isolated *Acinetobacter Oleivorans* S27. Figure 3.3 displays the phylogenetic tree based on 16s rDNA sequences of *Rhodococcus* strains which were found to be similar to the present isolated strain C27.

Culture Details

- ✓ *Rhodococcus qingshengii*
- ✓ Isolated from East Sikkim (near Changu Lake
- ✓ Altitude: 2862m

16S rRNA gene sequence of *Rhodococcus qingshengii* C27 1308 bp

CTTTCGGGGTACACGAGCGGCGAACGGGTGAGTAACACGTGGGTGATCTGCCCT
GCACTTCGGGATAAGCCTGGGAAACTGGGTCTAATACCGGATATGACCTCCTRTY
GCATGRYGKGKGGTGGAAAGATTTATCGGTGCAGGATGGGCCCGCGGCCTATCA
GCTTGTTGGTGGGGTAATGGCCTACCAAGGCGACGACGGGTAGCCGACCTGAGA
GGGTGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAG
CAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAG
GGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGACGAAGCGCAAGTGAC
GGTACCTGCAGAAGAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGT

AGGGTGCAAGCGTTGTCCGGAATTACTGGGCGTAAAGAGTTCGTAGGCGGTTTGT
CGCGTCGTTTGTGAAAACCAGCAGCTCAACTGCTGGCTTGCAGGCGATACGGGCA
GACTTGAGTACTGCAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAG
ATATCAGGAGGAACACCGGTGGCGAAGGCGGGTCTCTGGGCAGTAACTGACGCT
GAGGAACGAAAGCGTGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCACGCC
GTAAACGGTGGGCGCTAGGTGTGGGTTCCTTCCACGGAATCCGTGCCGTAGCTAA
CGCATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAAT
TGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGATTAATTCGATGCAACGCGA
AGAACCTTACCTGGGTTTGACATATACCGGAAAGCTGCAGAGATGTGGCCCCCCT
TGTGGTCGGTATACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTT
GGGTTAAGTCCCGCAACGAGCGCAACCCCTATCTTATGTTGCCAGCACGTTATGGT
GGGGACTCGTAAGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTC
AAGTCATCATGCCCCTTATGTCCAGGGCTTCACACATGCTACAATGGCCAGTACAG
AGGGCTGCGAGACCGTGAGGTGGAGCGAATCCCTTAAAGCTGGTCTCAGTTCGGA
TCGGGGTCTGCAACTCGACCCCGTGAAGTCGGAGTCGCTAGTAATCGCAGATCAG
CAACGCTGCGGKGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCAC

Phylogenetic tree constructed using 16S rRNA of C27 and different Type strains of Rhodococuss sp
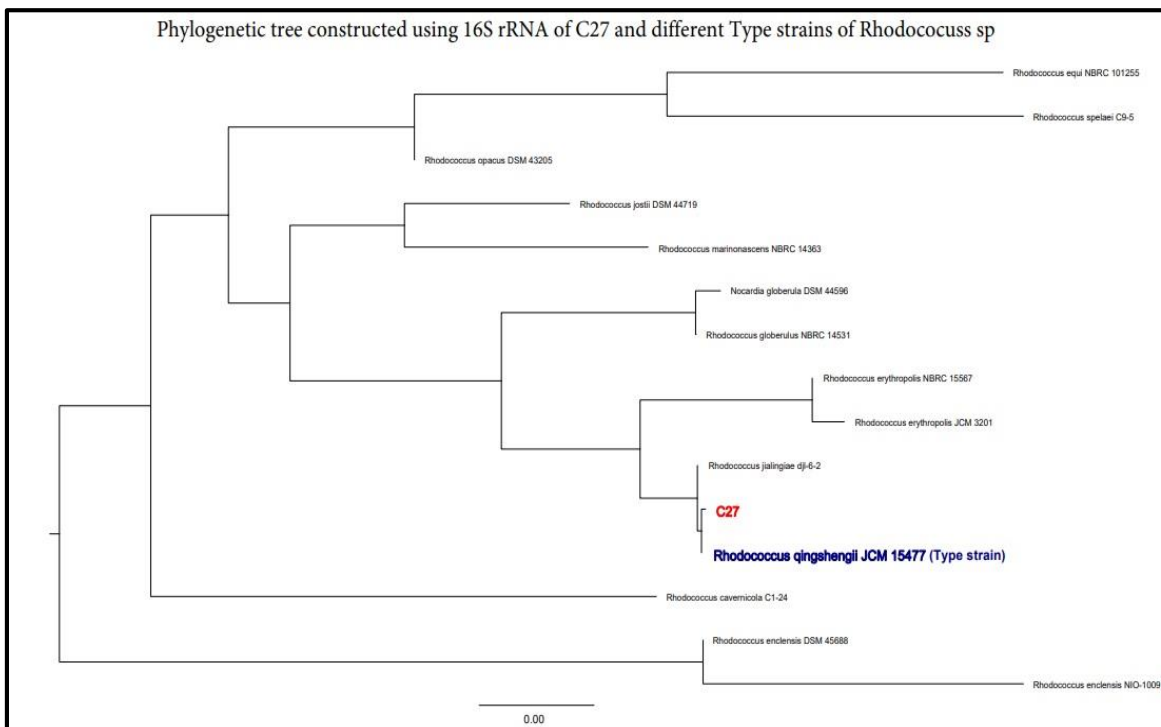
Figure 3.3 Maximum likelihood tree constructed using the 16s rDNA gene nucleotide sequences displaying the phylogenetic position of C27 strain in *Rhodococcus* genus.

3.3.2B. Effect of inoculum age and temperature

The cell harvesting phase is critical factor for the whole-cell biotransformation. The inoculum age for the FDCA biosynthesis were studied (Fig 3.4). The experiment shows the effect of FDCA production and HMF conversion on inoculum age (cell age). FDCA yield was maximum at 24th h and it was observed that complete HMF was consumed by the strain within 24 h. FDCA yield differs from 24h to 27h perhaps due to the change of growth phase. In whole cell biotransformation, most of the catalytic enzymes will be produced during particular growth phase. The FDCA yield was maximum (55%) at late exponential phase (OD_{600} value was 0.6).
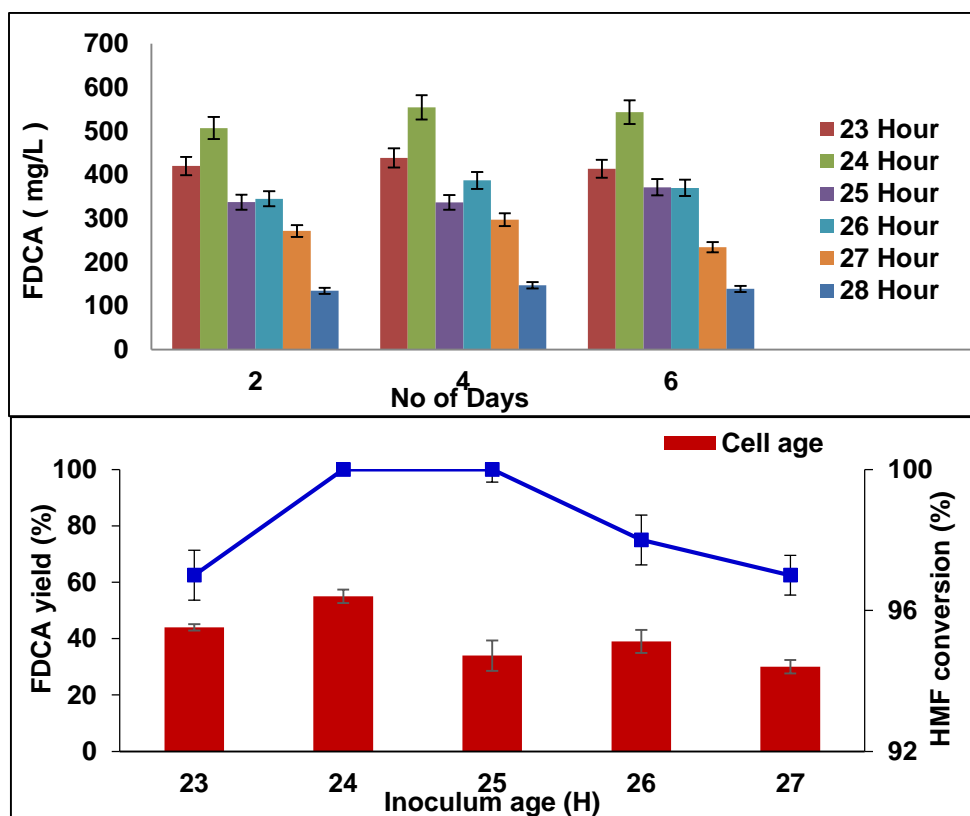
Figure 3.4: Effect of inoculum age; 1 g/L HMF, 25 mg mL−1 microbial cells, 50 mL MSM (pH 6.5), 200 rpm, 30 °C temperature.

Figure 3.5 shows the impact of reaction temperature from 20 °C to 37 °C on FDCA synthesis. Consumption of HMF was directly proportional to the increase in temperature, however, the FDCA yield was very low (14%) at 37 °C possibly due to the thermal inactivation of enzymes involved in the oxidation of HMF to FDCA. As the strain was isolated from the eastern Himalayan regions of India (east Sikkim) it is viable at 4 °C. As expected, FDCA yield (48%) was comparatively good at 20 °C. Nevertheless, the substrate conversion was decreased from 100% to 76%. The optimized temperature of 30 °C was found to be maximum FDCA yield (60%).
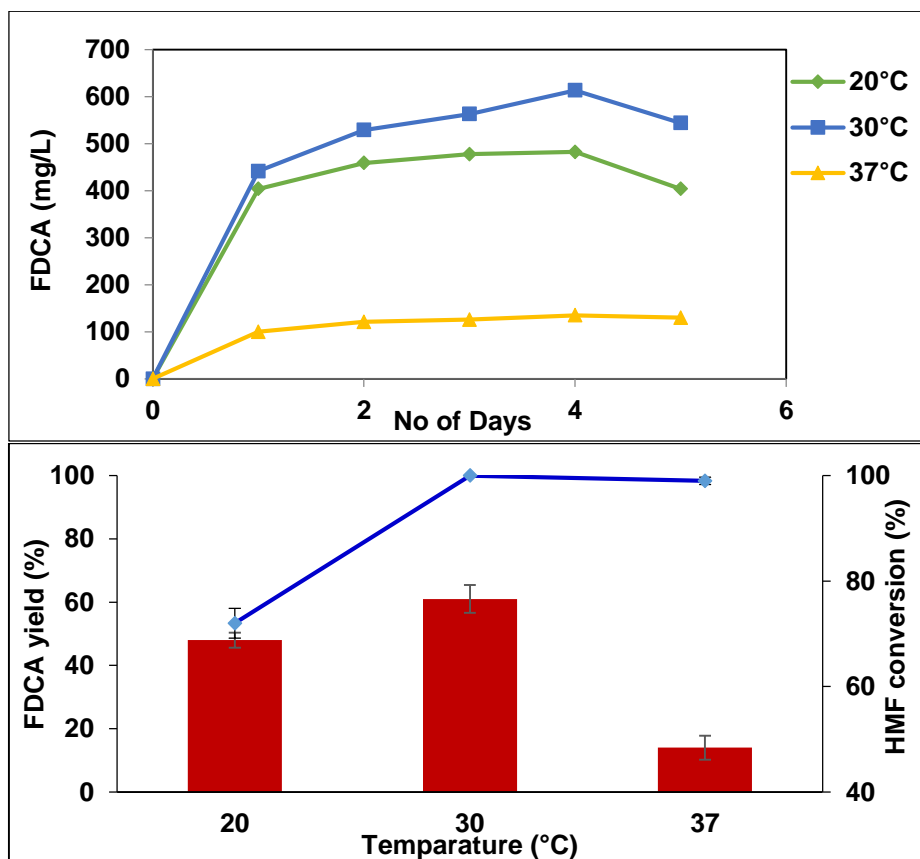
Figure 3.5: Effect of temperature; 1 g/L HMF, 24th h of inoculum age, 25 mg mL−1 microbial cells,50 mL MSM (pH 6.5), 200 rpm.

3.3.2C. Media optimization on FDCA production

HMF to FDCA biotransformation involves three consecutive oxidations as mentioned in chapter one. In general, biocatalytic reactions require specific genes/enzymes. The aldehyde moiety of HMF gets oxidized in the first step which is in fact a common occurrence. Most of the aldehydes inhibit and destroy the protein, nucleic acid and organelles by forming reactive oxygen species. In many microbial strains, HMF will convert into HMFCA/ acid/alcohol forms to reduce the toxicity using generic aldehyde dehydrogenases. Further oxidations should happen for the FDCA production. The role of cofactors was observed in previous studies of Wierckx et al., {2009}. Two reducing equivalents required for one mole of HMF to one mole of FDCA conversion. NADH/FADH cofactors are generally involved in metabolic and complex reactions. Therefore, availability of more oxygen, nitrogen etc. should be high for the HMF to FDCA biotransformation. The modified mineral salt media, termed as Qingshengii mineral salt medium (QMSM), consists of more oxygen content than MSM and also modified specifically for *R. qingshengii* C27 (Schedler et al., 2014).

MSM	QMSM
❖ KH_2PO_4- 0.5g/L ❖ K_2HPO_4- 0.5g/L ❖ NH_4Cl- 0.5g/L ❖ $MgSO_4.7H_2O$- 0.2g/L ❖ $CaCl_2.2H_2O$- 0.002g/L ❖ Trace elements	❖ Na_2HPO_4- 2.6 g/L ❖ KH_2PO_4- 1.3 g/L ❖ $(NH_4)_2SO_4$-1.0 g/L ❖ $MgSO_4.7H_2O$- 0.2 g/L ❖ Trace elements

Table 3.3: Media components of MSM and QMSM.

The media components of MSM and QMSM are given in Table 3.3. There was a remarkable change in the HMF utilization and increase in the FDCA production (Figure 3.9). In presence of QSM, *R. qingshengii* C27 cells could utilize the complete HMF (Q-HMF) within 15 hours. But in control MSM, unutilized HMF (C-HMF) was remaining even after 96 h ($\approx$ 400 mg/L). Hence, in both media HMFCA accumulation was high (Q-HMFCA & C-HMFCA). This result indicates the HMFCA to FFCA is the rate limiting step. Both BHMF and FFCA are produced less than 100 mg/L. There was 50% of increase (Figure 3.6) in the FDCA production (almost doubled). The proper modification of media components helps the *R. qingshengii* C27 cell to utilize the complete HMF (8 mM) and also might activated the gene/enzymes to oxidize the HMF to FDCA.
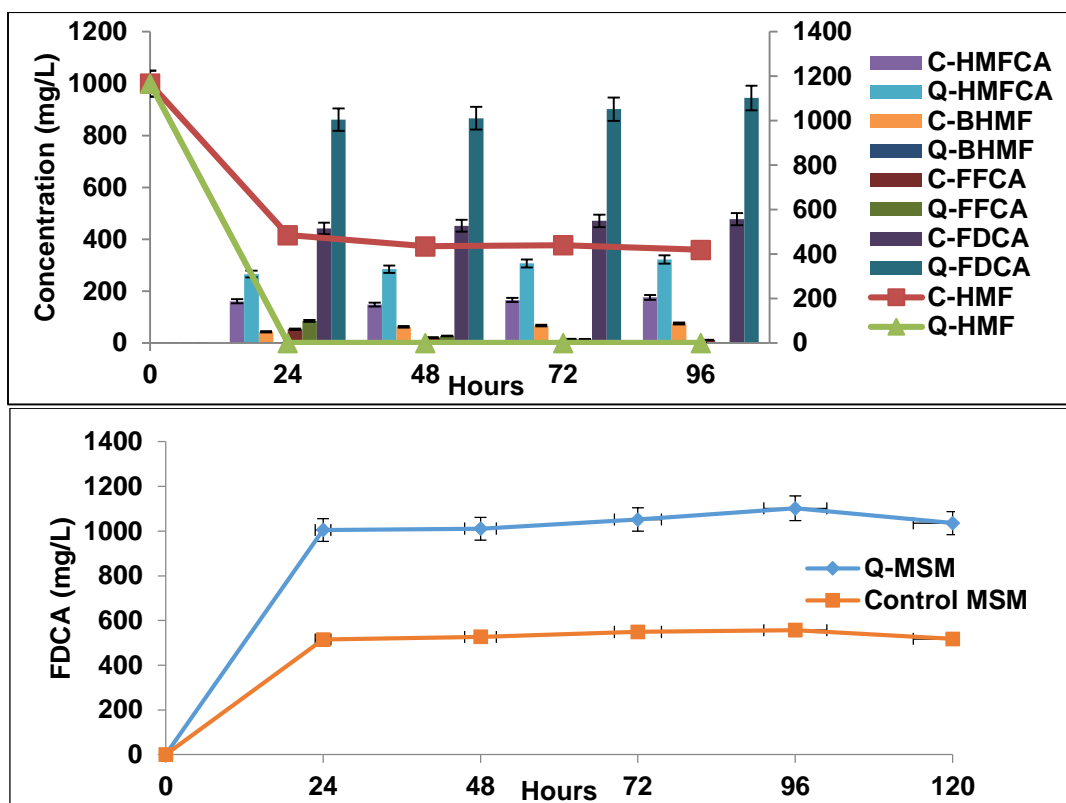
Figure 3.6: QMSM vs MSM; HMF utilization and FDCA production.

3.3.2D. Effect of pH

The QMSM was used for the optimization of pH. Effect of pH was very critical for the HMF to FDCA biotransformation. Figure 3.7 shows the various range of pH (5.5 to 8.0) on FDCA synthesis. The complete substrate conversion was achieved when pH range increased from 5.5 to 8.0 (85% to 100%). A remarkable 33% increment in the FDCA yield was observed when pH was tuned to 7.0.
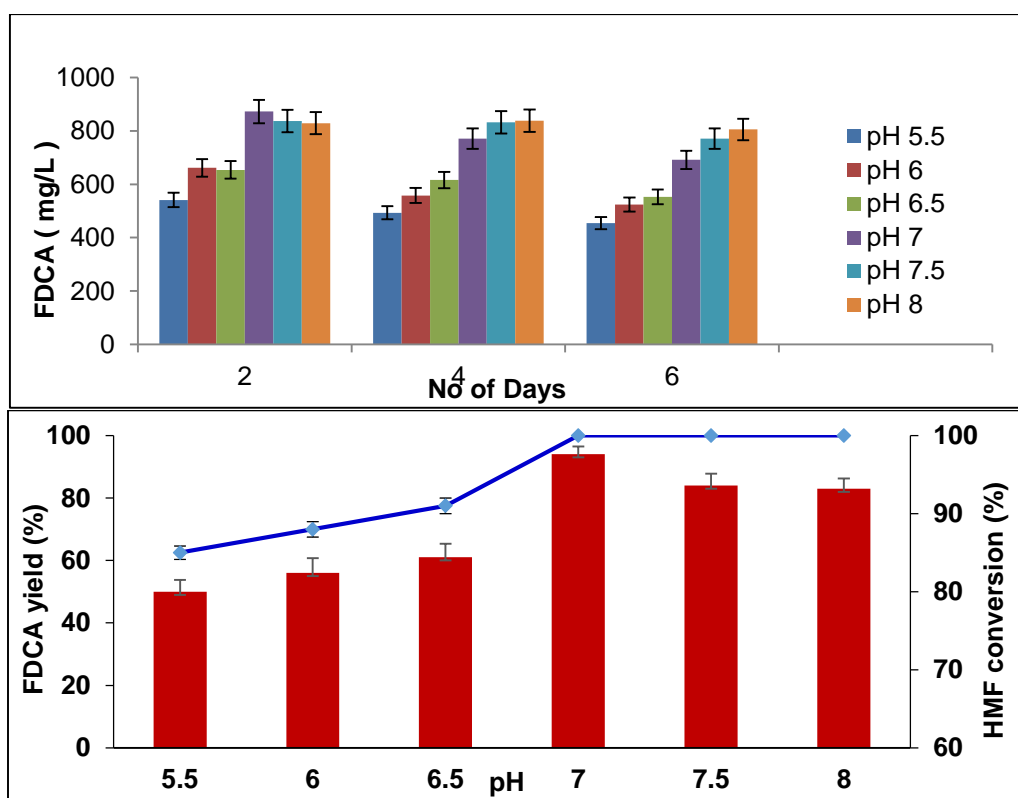
Figure 3.7: Effect of pH; 8 mM HMF, 24th h of inoculum age, 25 mg mL−1 microbial cells, 200 rpm, 30 °C temperature and 50 mL of QMSM.

When the reaction medium was of pH 7.5 and 8.0, the FFCA accumulation was much high. The bio-catalytic oxidation of HMF to FDCA was very much depend on pH and slight variation will result in fluctuation of FDCA yield (Wang et al., 2020). Obtaining a 94%

FDCA yield for a whole-cell bio catalysis using a wild strain from this study was an interesting outcome.

3.3.2E. Effect of HMF concentration on FDCA synthesis

HMF is highly toxic to living cells (Koopman et al., 2010a) and HMF tolerance shows the capability of a microbial strain towards the cell toxicity. Figure 3.8 shows the HMF utilization and yield of HMF derivatives including FDCA.
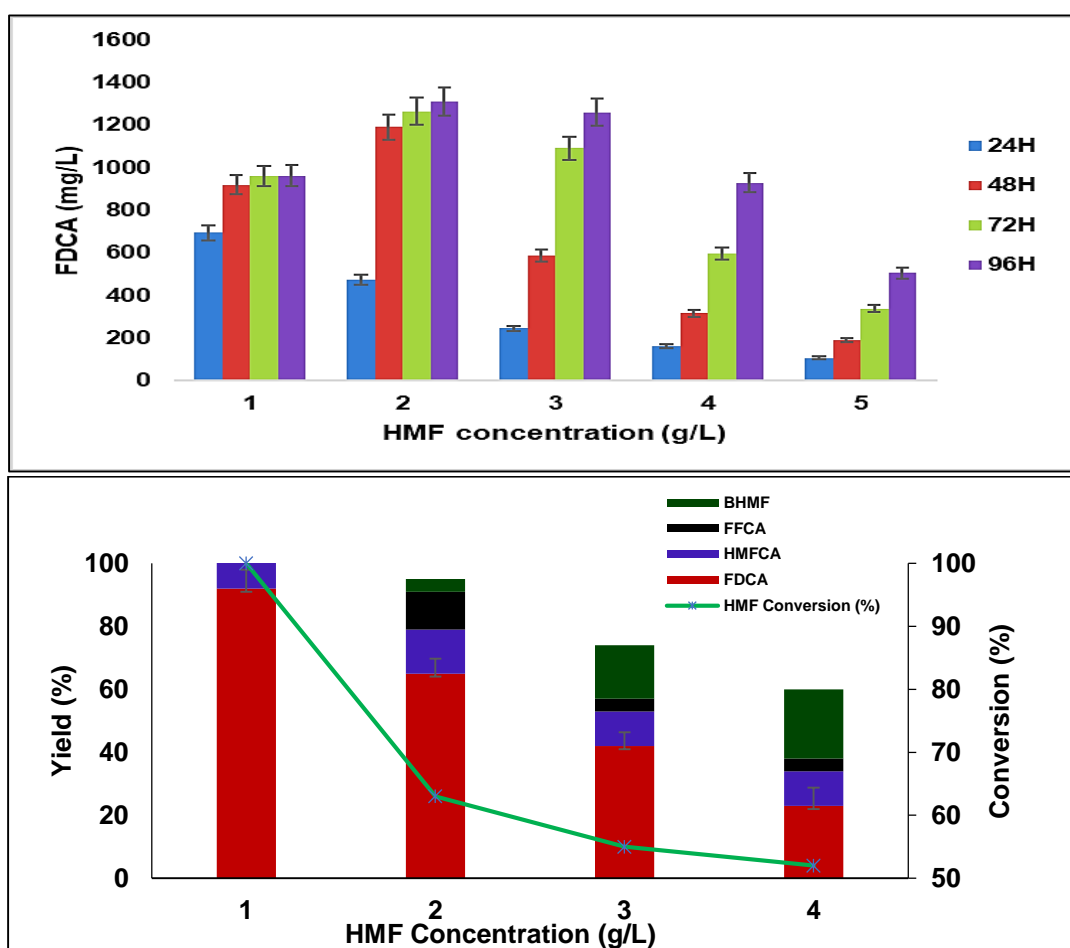
Figure 3.8: Effect of substrate concentration (HMF); 24th h of inoculum age, pH- 7.0 25 mg mL−1 microbial cells, 200 rpm, 30 °C temperature and 50 mL of QMSM.

BHMF yield was increased when substrate concentration was high in order to reduce the substrate toxicity by converting it into a less toxic alcohol. Nonetheless, product yield was pretty low while substrate concentration was 32 mM (4g/L). 65% of FDCA yield (1300 mg/L) was achieved when HMF concentration was 16mM (2 g/L). From HMFCA further oxidation was not happening in almost all HMF concentration except 1 g/L HMF concentration.

3.3.2F. Effect of HMF derivatives

A potential strain has different characteristics; among them wide range of substrate specificity is the key one. In the HMF oxidation pathway there are two routs one is via DFF and other one is via HMFCA (detailed in chapter 1).

HMFCA has two functional groups (alcohol and carboxylic acid) with furan ring. Compared to HMF, HMFCA is less toxic to living cells. *R. qingshengii* C27 strain could not utilize the HMFCA (figure 3.9A).
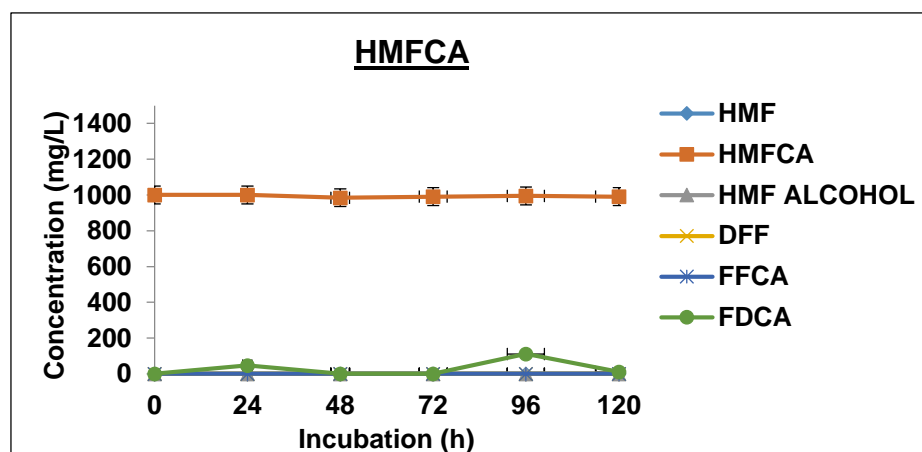
Figure 3.9A: HMFCA as substrate; HMFCA (1 g/L), inoculum age- 24H, pH- 7.0, 25 mg mL−1 microbial cells, 200 rpm, temperature-30 °C and 50 mL of QMSM.

Negligible amount of FDCA was produced after 96 h. With the observation made HMFCA is the safe zone for the strain. Actual reason needs to be investigated through their genome/transcriptome level.

The HMF alcohol (2,5-bis hydroxymethylfuran; BHMF) will produce via HMF reduction. It has two alcohols functional group with a furan ring. In this experiment BHMF was oxidized to HMF and proceeded the general oxidation pathway to form FDCA (figure 3.9B). Because BHMF was utilized within 24 h, HMFCA and FFCA (24th hour) was formed and oxidized to FDCA. The final yield was really impressive (0.93 g/L). HMFCA accumulation was comparatively low and FFCA was completely oxidized.
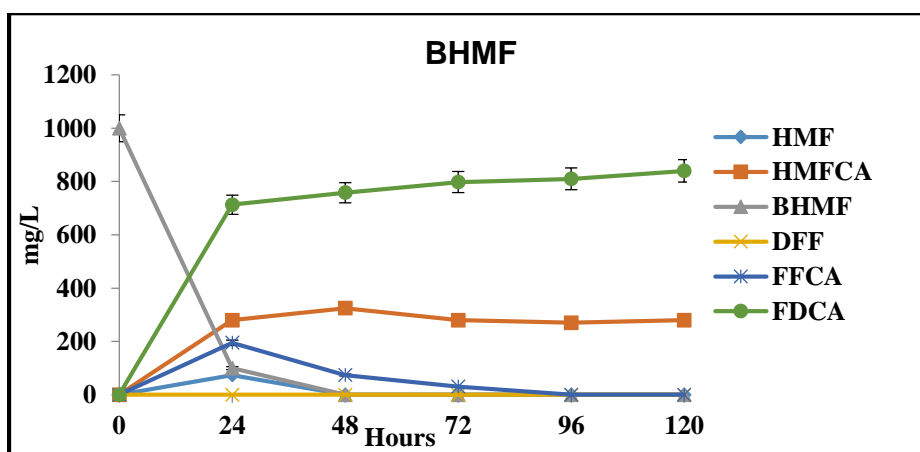
Figure 3.9B: HMF alcohol (BHMF) as substrate; BHMF (1 g/L), inoculum age- 24H, pH- 7.0, 25 mg mL−1 microbial cells, 200 rpm, temperature-30 °C and 50 mL of QMSM.

Already it was found that organism following the route A (HMF to FDCA general oxidation pathway; chapter 1). With the experimental data it was confirmed. The experiments were also conducted DFF as a substrate. Interestingly, the strain could utilize the DFF within 24 h (Figure 3.9C). Diformylfuran consists of two formyl group with furan ring. After 96 h,

FDCA production was 0.8 g/L which is good yield. However, the HMFCA yield was almost 0.5 g/L. The accumulation of other derivatives might be the cause of HMFCA formation (to decrease the toxicity).
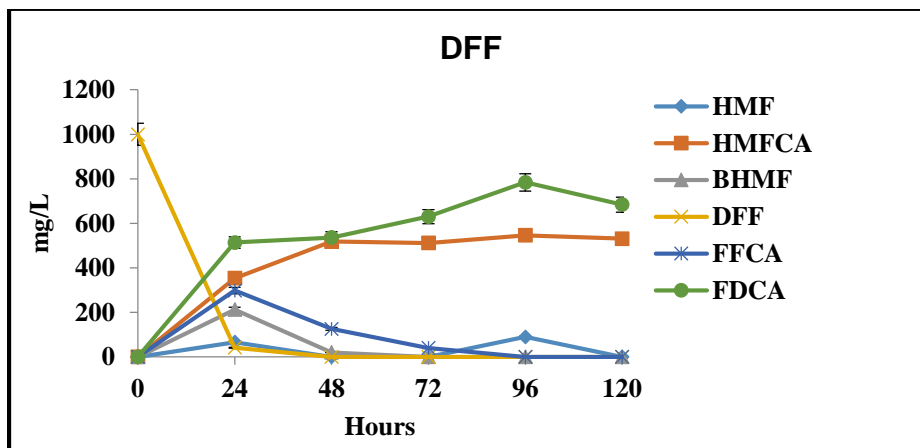
Figure 3.9C: DFF as substrate; DFF (1g/L), inoculum age- 24H, pH- 7.0, 25 mg mL−1 microbial cells, 200 rpm, temperature-30 °C and 50 mL of QMSM.

The FFCA contains formyl group and carboxylic acid with furan ring. It is an oxidation product of either HMFCA/DFF. Substrate (FFCA) was gradually utilized till 96 h (figure 3.9D). Interesting observation was the HMFCA accumulation (0.4 g/L). The result indicated there was a reverse reaction must have occurred i.e., FFCA to HMFCA. The FDCA yield was gradually increased (final yield; 0.8 g/L).
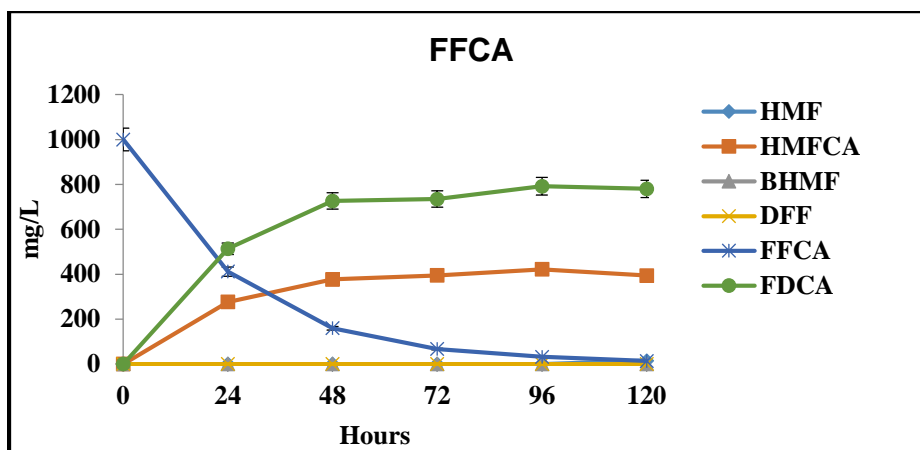
Figure 3.9D: FFCA as substrate; FFCA (1 g/L), inoculum age- 24H, pH- 7.0, 25 mg mL−1 microbial cells, 200 rpm, temperature-30 °C and 50 mL of QMSM.

In this study, it is clearly evident that *R. qingshengii* C27 has the capability to use other furan derivatives. Except HMFCA, all other derivatives were oxidized to synthesize FDCA with a good yield. However, compared to other HMF derivatives it was found that the HMF is the best starting material for the FDCA synthesis because of maximum FDCA yield within 72 hours and comparatively less HMFCA accumulation. Final FDCA yield was 94% with complete HMF conversion (8 mm).

3.4. A comparative study between potent strains on their FDCA production

Both isolates, *Acinetobacter oleivorans* S27 and *Rhodococcus qingshengii* C27 were analyzed and compared based on their FDCA production as well as HMF tolerance. Also the physical parameters were optimized. A comparative study of isolates on FDCA production is given in table 3.4 *R. qingshengii* C27 shows great potential than *A. oleivorans* S27 on their capacity of HMF oxidation. The great advantage of the C27 strain is incubation time is just 96 hours with maximum yield (94%). Moreover, the strain could grow in presence of high HMF concentration (based on initial studies). The range of

substrate is one of the key feature of C27 strain. Hence, more investigation can be conducted with *R. qingshengii* C27 strain to improve the FDCA yield.

Acinetobacter oleivorans S27	*Rhodococcus qingshengii* C27
❖ Gram negative bacteria ❖ Inoculum age- 27 H ❖ Temperature- 30ºC ❖ pH- 6.5 ❖ Incubation time-11 days ❖ FDCA yield- 0.25 g/L	❖ Gram positive bacteria ❖ Inoculum age- 24 H ❖ Temperature- 30ºC ❖ pH- 7.0 ❖ Incubation time- 96 H ❖ FDCA yield- 0.94 g/L

Table 3.4: Optimized conditions of selected FDCA producers.

3.5. Summary and conclusions

In this study, the isolation, screening and identification for 2,5-FDCA was completed. The investigation resulted two potent isolates ***Acinetobacter oleivorans* S27** & ***Rhodococcus qingshengii* C27** for the FDCA production. Based on their FDCA production efficiency ***Rhodococcus qingshengii* C27** was selected for the further research. All the physical parameters were optimized for the whole-cell biocatalysis. The strain also shows remarkable tolerance towards HMF.

In the HMF to FDCA biotransformation, with experimental observations we can assume that:

1. HMF toxicity is the one limiting factor towards FDCA production
2. HMFCA accumulation along with the desired product
3. The bioprocess can be scale-up with the optimized conditions.

With an initial HMF (1 g/L) concentration, the biotransformation of HMF to FDCA was observed. The final yield of FDCA was 0.94 g/L with 100% HMF conversion.

Chapter 4

Biotransformation strategies for the improved production of 2,5-Furandicarboxylic acid using *Rhodococcus qingshengii* C27

4.1. Introduction

The whole-cell biocatalytic studies for FDCA production is still requires intensive research. Each potential strain shows different and unique characteristics. The study on HMF tolerance of isolates are always interesting and this has not been extensively studied. In this context, very few organisms are reported so far. Understanding the fact that the state of microbes (growing cells/resting cells) when it produces the desired product is critical in the whole-cell biocatalysis. Cell immobilization strategies are widely using, especially for bacterial strains. Immobilized cells may facilitate the bioprocess by protecting the cells from environmental stress. Optimization of physical parameters and media components is the first step for the improvement in the production level. Then incorporating different fermentation strategies (biotransformation) would increase the product yield. The biotransformation strategies have a large scope to improve the FDCA production using novel isolate.

The aim of the investigation was to improve the production of FDCA by employing fed batch/continuous biotransformation or combining both with media recycling and the cell immobilization. Also, to validate the HMF tolerance towards the selected strain. We observed the change in the FDCA production when glucose and glycerol (carbon source) were added along with HMF (substrate).

4.2. Materials and methods

4.2.1 HMF tolerance and viability of *Rhodococcus qingshengii* C27

The tolerance of the strain towards HMF was evaluated by inoculating the strain on LB media supplemented with various concentration of HMF (25 mM – 240 mM). After 48

hours of incubation, cells were harvested, serially diluted to 10^4 CFU per ml and plated on LB agar supplemented with 25 mM HMF. The plates were incubated at 30 °C for 144 hours and the cell count was determined. Cells were visualized through light microscope and CFUs (colony forming unit) were determined.

4.2.2 Effect of glucose and glycerol on FDCA production

To understand the impact of other carbon sources on HMF biotransformation by *R. qingshengii* C27, glucose (1%) and glycerol (1%) were separately supplemented in QMSM. The cells were incubated with designated conditions and the percentage of HMF biotransformation and production of HMF derivatives (HMFCA, FFCA, FDCA, BHMF) were evaluated. The production media components and incubation conditions were same as described in chapter 3.

4.2.3 Batch biotransformation using *Rhodococcus qingshengii* C27

The batch biotransformation experiments were carried out in flask level with 50 mL working volume. Cell harvesting and biotransformation conditions are described in chapter 2. Throughout the desired incubation time pH was monitored. The experiments were carried out with 1 g/L of HMF concentration. The FDCA yield and HMF conversion was calculated.

4.2.4 Fed batch biotransformation using *Rhodococcus qingshengii* C27

In the fed batch mode, different substrate feeding strategies were carried out. Strategy 1; HMF was fed at 0h, 24h, 48h and 72h in 0.5 g/L concentration. In strategy 2; HMF was fed at 0h and 48h in 1 g/L concentration. In strategy 3; HMF was fed at 0h,48h and 96h in 1 g/L concentration. In strategy 1 and 2, the total HMF concentration was 2 g/L. In

strategy 3, the total HMF concentration was 3 g/L. In all experiments the incubation conditions are same as mentioned in chapter 2. The fed batch biotransformation experiments were carried out in flask level with 50 mL working volume. Every 24 hour cells from production media were plated and CFU was counted. Also the culture sample was (1 mL) was withdrawn from the production flask (every 24 hours) and measuring the optical density at 600 nm in UVV-Visible spectrophotometer (Shimadzu series UV 1601).

4.2.5 The repeated batch biotransformation and media recycling

The repeated batch biotransformation experiments were carried out in flask level with 50 mL working volume and 25 mg/mL cell concentration. In the repeated batch, the biotransformation was initially run in the batch mode; In cycle 1, HMF (1g/L) was given in 0 hour. After 72 hours, the 50% of production media (QMSM) was replaced with fresh sterile QMSM media fed with HMF (1 g/L). Likewise, 3 to 5 cycles were carried out with total of 3 g/L HMF concentration. In another experiment instead of 50% the complete production media (100%) was replaced with fresh sterile production media fed with 1 g/L HMF concentration. Total of 5 cycles were carried out with total of 5 g/L of HMF concentration. In each cycle the samples were collected and analyzed for FDCA production. The pH was routinely monitored. In every cycle cells from production media were plated and CFU was counted.

4.2.6 FDCA synthesis using immobilized *Rhodococcus qingshengii* C27 cells

4.2.6.1 Calcium alginate beads preparation

The immobilized *Rhodococcus qingshengii* C27 cells were prepared by the modified procedure of Tsai et al., 2021. The encapsulation of *R. qingshengii* C27 cells into a calcium alginate (polymeric matrix) was done. Sodium-alginate (4-8%) and calcium chloride (4%) solutions were prepared and sterilized properly. 150 mg/L^{-1} cell concentration (*R. qingshengii* C27) was mixed with 25 mL sodium-alginate solution. The cell-alginate was mixed properly. Later, the suspension was dropped in to 100 mL calcium chloride solution using a standard 1 mL sterile tips to form beads. To achieve the proper shape and solidify the immobilized cells, the solution was kept steady at 4° C for 12-16 hours. Later, the beads were washed with sterile water and QMSM for two- three times and then it was used for repeated fed batch HMF biotransformation with optimized incubation conditions.

4.2.6.2 *Rhodococcus qingshengii* C27 immobilization by polyurethane foam

Initially, the polyurethane foam (PUF) was cut in to 1 cm cubes. The cells (150 mg/L^{-1} concentration) were inoculated in to QMSM (50mL) with 1 g/L of HMF. The PUF (100 gm) was immersed on to the inoculated medium and kept for designated incubation conditions. The immobilized *R. qingshengii* C27 cells were used for HMF biotransformation with optimized incubation conditions.

4.3 Results and discussion

4.3.1 HMF tolerance of *Rhodococcus qingshengii* C27

HMF is highly toxic to living cells (Koopman et al., 2010a) and HMF tolerance shows the capability of a microbial strain towards the cell toxicity. The cell viability against high concentration of HMF (25 mM-240mM) was investigated and it was found that the *R. qingshengii* C27 strain could tolerate up to 48 h of incubation and observed the optical density value. It was clearly evident that the particular strain has tremendous tolerance towards HMF (Table 4.1). After 48 h of incubation, the cells were plated on LB agar with 25 mM of HMF and LB agar plate (without HMF) (Figure 4.1). The microscopic image of *R. qingshengii* C27 cells were taken before and after incubation (Figure 4.2). At present, very few reports were there on the HMF tolerance (Shi et al., 2019) (Zhang et al., 2017). In the lignocellulosic pretreatment, formation of several inhibitors will be formed including HMF. Therefore, HMF removal from the hydrolysate of lignocellulosic biomass using potent HMF tolerant strain would be a novel approachable way. From the experimental evidences, *R. qingshengii* C27 can be used in HMF detoxification from lignocellulosic biomass. However, tolerance against other lignocellulosic inhibitors has to be studied.

SI No	HMF Concentration (mM)	Number of colonies (CFU/mL)	OD$_{600}$ value
1	25	85	0.56
2	80	27	0.58
3	160	12	0.53
4	240	5	0.56

Table 4.1. HMF tolerance of *Rhodococcus qingshengii* C27; HMF concentrations (25 mM to 240 mM). After 48 h of incubation at 30 °C, cells were harvested, serially diluted to 10^4 CFU per mL and plated on LB agar supplemented with 25 mM HMF.
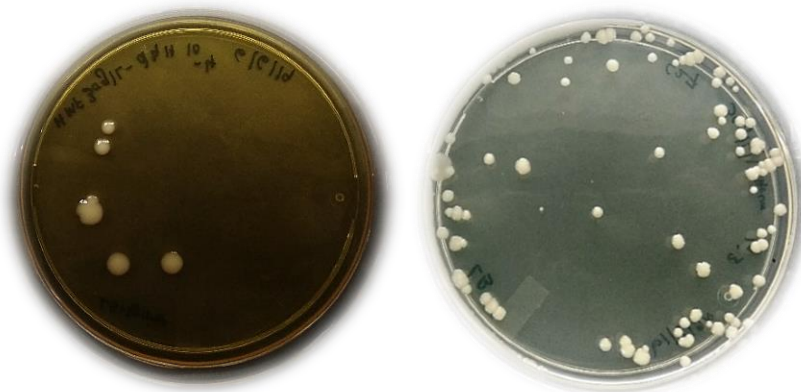
Figure 4.1. The grown *R. qingshengii* C27 cells after incubation with HMF (240 mM) for 48 hours. The cells were harvested, serially diluted to 10^{-4} CFU per mL and plated on LBA+ 25 mM HMF (left side brown colour) and LB agar plate (right side).
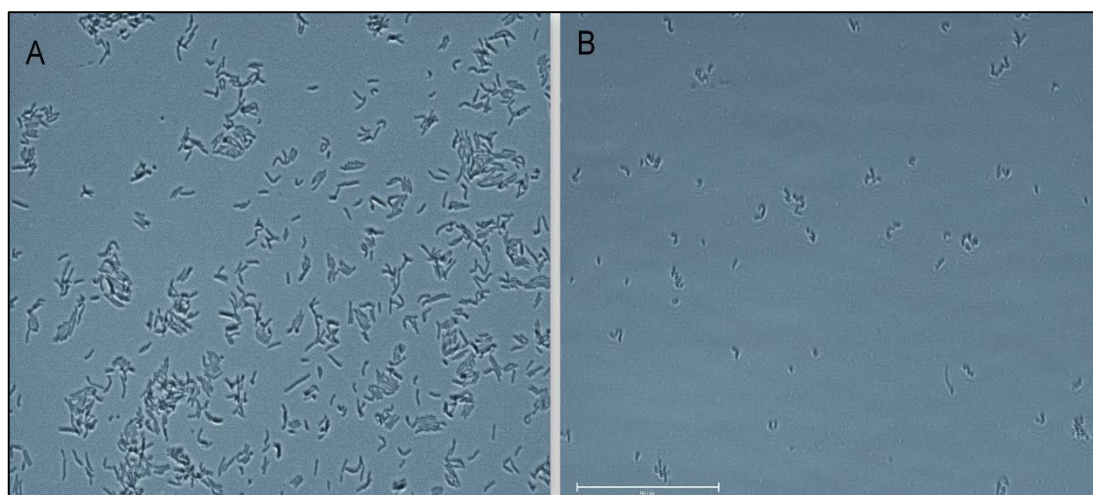
Figure 4.2. Microscopic image of *R. qingshengii* C27 cells in presence of 240 Mm HMF; (A) after 12 h of incubation and (B) after 48 h of incubation

4.3.2 Effect of glucose and glycerol on FDCA production

Biological oxidation of HMF to FDCA was studied on resting and growing cells. The OD was checked routinely to understand the cell growth. Based on the findings, resting cells showed (Figure 4.3) maximum catalytically favourable substrate oxidation towards FDCA

synthesis. Similar result was reported on the resting cells of *Raoultella ornithinolytica* BF60 strain for FDCA synthesis from HMF (Yuan et al., 2018b; Yuan et al., 2018c). In the presence of glucose and glycerol (1% each), the strain could utilize complete HMF within 24h and then oxidised to HMFCA (55% and 50% respectively). Also it was observed that the BHMF (HMF alcohol) formation in presence of glucose and glycerol (36% and 38%). It was found that compared to control (without glucose or glycerol; only HMF), the FDCA production (Figure 4.4) was very unsatisfactory in the presence of glucose and glycerol (9% and 12%). Perhaps, it was because of the presence of other carbon source other than HMF which will initiate the cell metabolism. Simultaneously, the cellular stress got decreased by HMF derivatives formation such as HMFCA and BHMF. Comparatively, both HMF derivatives are less toxic than HMF (Hu et al., 2018, Xu et al., 2020) But, for the efficient transformation of HMF to FDCA, the QMSM was only provided with HMF as substrate.
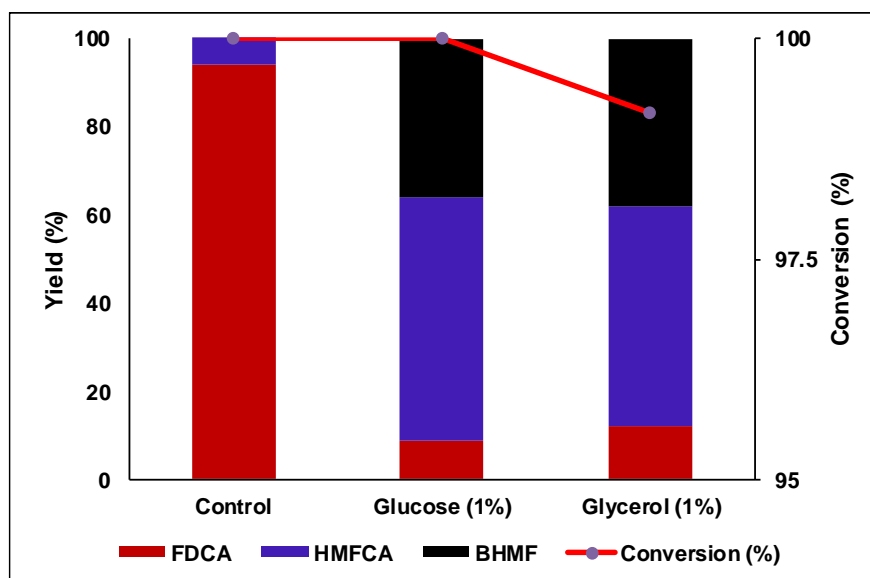
Figure 4.3. Effect of glucose and glycerol on FDCA and HMF derivatives synthesis.
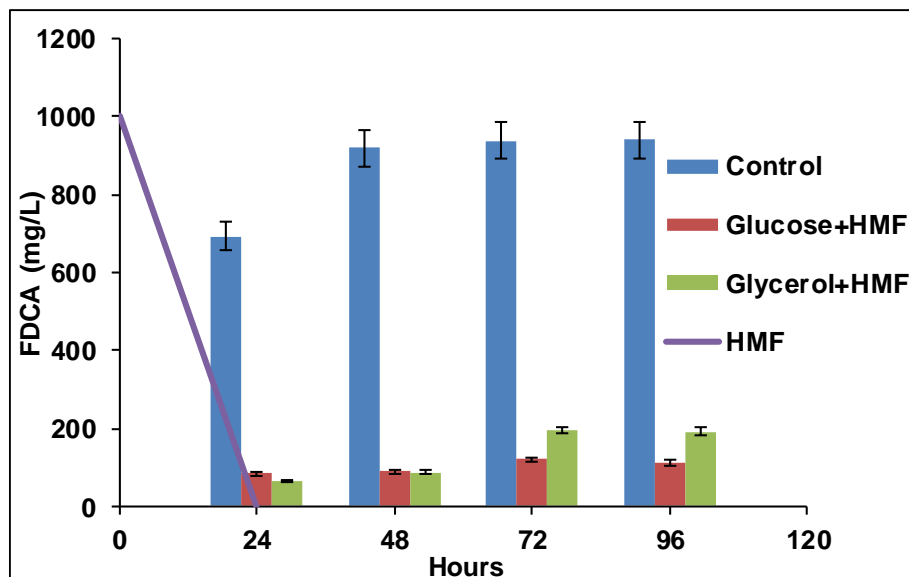
Figure 4.4. Effect of glucose and glycerol on HMF utilization and FDCA

synthesis

4.3.3. Batch HMF biotransformation

All the parameter optimization was completed in batch level HMF biotransformation. Under the optimized conditions, *R. qingshengii* C27 strain could convert the HMF (1 g/L) to FDCA with 94% yield (Figure 4.5). In batch level, the yield percentage was decreased while increasing the HMF concentration due to the substrate inhibition towards the cells. HMF utilization was studied in different HMF concentration (detailed in chapter 3, section 3.3.1B). From the batch level studies, it was observed that the substrate inhibition towards cells. The fed-batch strategy would overcome the substrate toxicity towards the cells compared to batch mode. From the observations, we understood that the strain can be viable up to 13-15 days. So the study also suggests the repeated batch strategy would improve the FDCA yield.
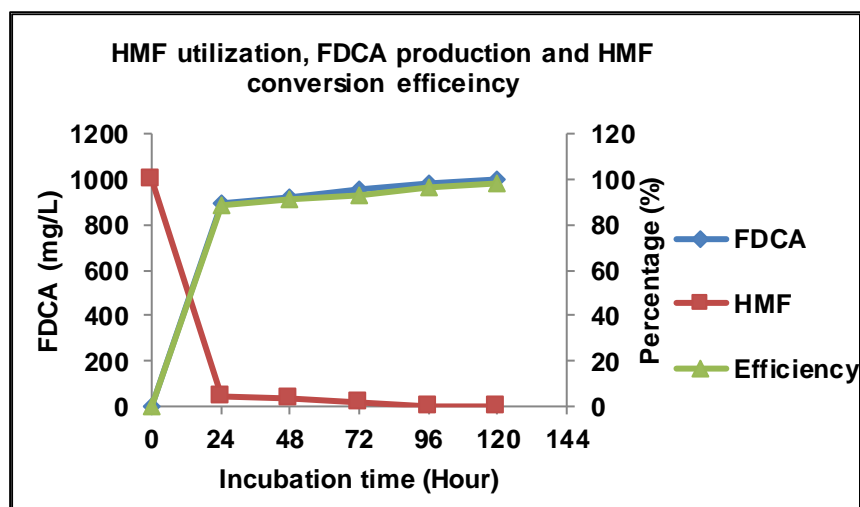
Figure 4.5. Batch level FDCA production efficiency

4.3.4. Fed batch HMF biotransformation

The fed batch experiments were carried out in different strategies. Compared to batch, the fed batch biotransformation could increase the HMF utilization and FDCA yield. In strategy 1, HMF was given at 0h, 24h, 48 and 72 h in 0.5 g/L concentration. Through the fed batch biotransformation, total of 1530 mg/L FDCA was obtained from 2000 mg/L HMF (Figure 4.6A). The experiment result suggests fed batch strategy is getting better yield (76.5%) than batch biotransformation with respect to the higher HMF concentration. However, it was observed the HMF accumulation was started after 48 hours. After 72 hours, it was found almost 600 mg/L of HMF was unutilized. It was possibly due to the not only the substrate also the product inhibition. It was evident that the product inhibition towards the cells because the pH was coming down from 7.0 to 3.14. This fact was due to the formation of HMF derivatives like HMFCA, FFCA and FDCA in the production media.

In strategy 2, the initial HMF concentration was 1 g/L. After complete HMF utilization again HMF was fed at 48th hour. The FDCA yield was improved while compared to strategy 1. Interestingly, the maximum FDCA yield was observed at 72 hours. The final yield was 1604 mg/L of FDCA from 2000 mg/L of HMF (Figure 4.6B). However, the HMF accumulation was same as strategy 1. There was no FFCA after 24 hours, because strain could transform it in to FDCA.

The extended version of strategy 2 was performed in strategy 3 where HMF was fed at 0 h, 48 h and 96 h. But, there was no interesting result observed. The strain could utilize the complete HMF up to 2000 mg/L. However, rest of the HMF was not completely utilized (after 96 hours) (Figure 4.6C). HMF toxicity was so high and HMF alcohol (BHMF) was formed along with HMFCA to reduce the toxicity. From these experiment results it was observed that *R. qingshengii* C27 cells could not transform more than 2000 mg/L of HMF at a time. In a recent report by Hsu et al., described the fed batch strategy for FDCA production where 545 mM of FDCA was produced within 72 hours using engineered *Pseudomonas putida* S12 whole-cell biocatalyst. They also overcome the substrate inhibition by increasing the inoculum density, since they have used growing cells of *P. putida* S12 (Hsu et al., 2020). We have experimented with higher density inoculum but could not overcome the substrate toxicity. The reason might be the only resting cells of *R. qingshengii* C27 was involved in the FDCA production not with the growing cells. With the observation made the depletion of nutrients and the accumulation of other HMF derivatives (HMFCA, BHMF) in the production media might be the cause of decrease the product yield. So the results suggest other strategies like immobilization or media recycling. The cell concentration in each fed-batch strategies were monitored. Table 4.2

shows the biomass concentration and FDCA yield of strategy 2 (maximum FDCA yield was obtained) The gradual decrease in the CFU value was obtained because of HMF toxicity and lack of other carbon source for their growth.
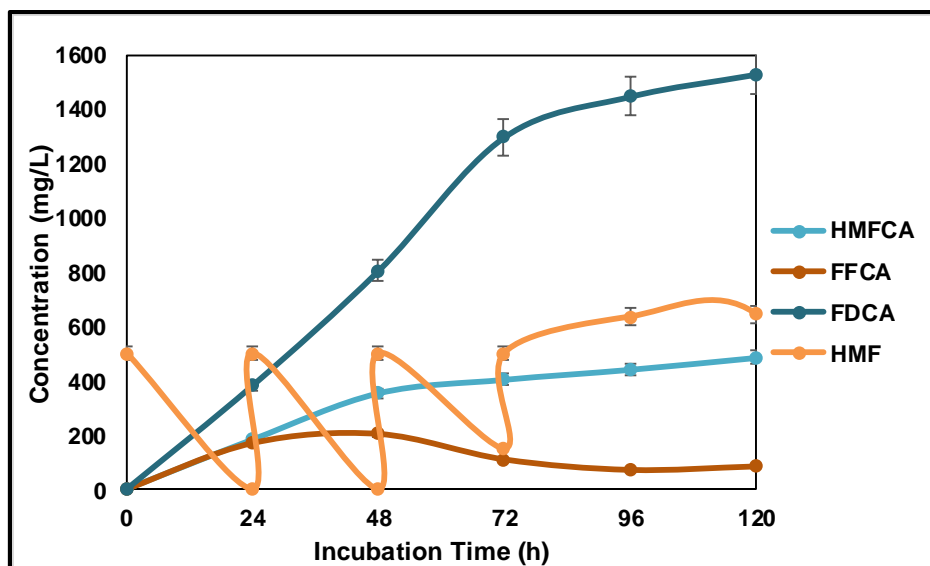
Figure 4.6A. Strategy 1- Fed batch biotransformation
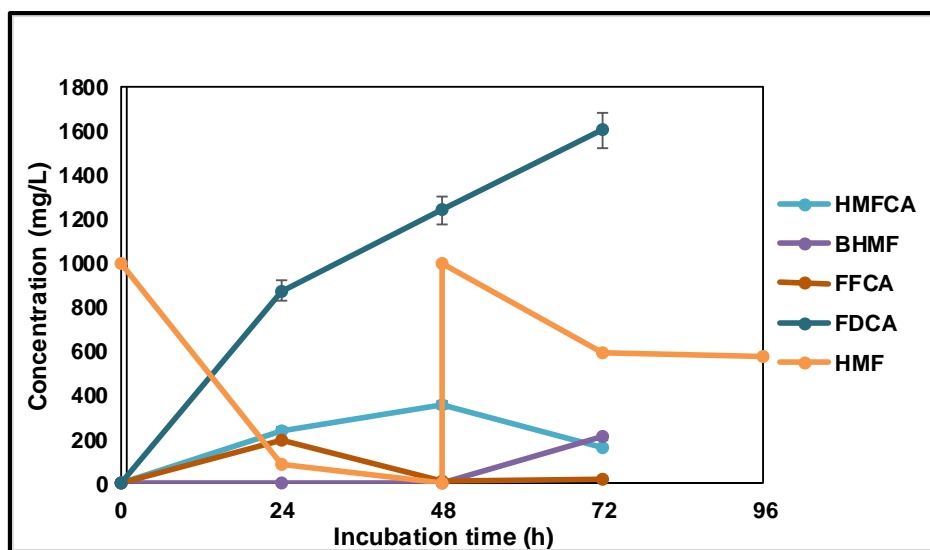
Figure 4.6B. Strategy 2- Fed batch biotransformation
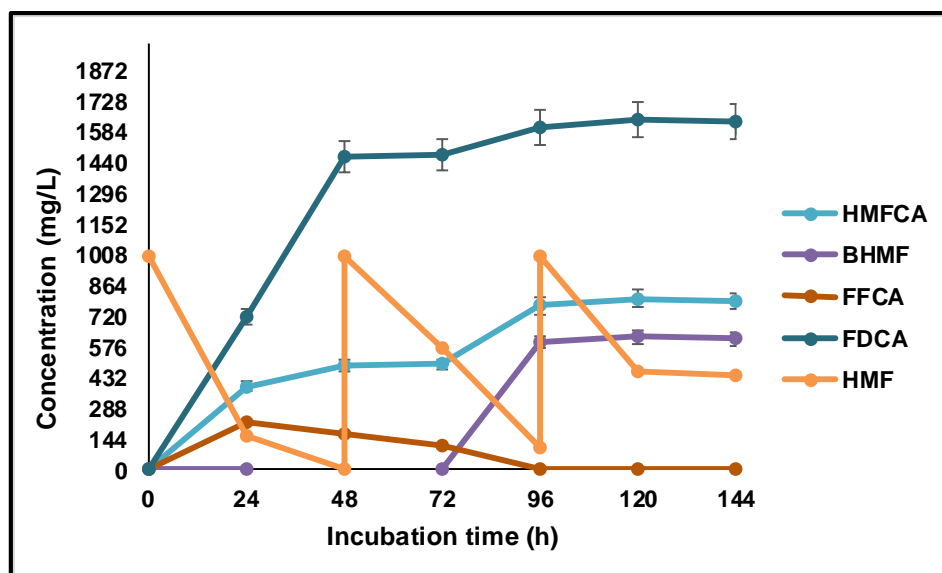
Figure 4.6C. Strategy 3- Fed batch biotransformation

Incubation time (h)	Biomass (CFU/MI)	FDCA (g/L)
24	141	0.874
48	117	1.239
72	89	1.604
96	78	1.588
120	45	1.474
144	27	1.459

Table 4.2. The biomass concentration and FDCA yield of strategy 2; CFU/mL (10^{-5}) count and FDCA (every 24 hours of incubation till 144 h). The starting OD during the production was 0.6 at 600 nm.

4.3.5. Repeated batch HMF biotransformation

The repeated batch HMF biotransformation was carried by replacing the spent production media (QMSM) with fresh sterile media at 72 hours of every cycle. Compared to batch and fed batch biotransformation, repeated batch biotransformation was observed

to increase the total HMF concentration (3 g/L) and FDCA yield (80%). There was no report on repeated batch HMF biotransformation using wild strains. In this study, 1 g/L of HMF was given in every cycle. Every 72 hours 50 % of the production media was replaced. The repeated batch biotransformation was carried out in 3 cycles with 72h intervals.

From the observations, the FDCA yield was satisfying compared to batch and fed batch experiments. The FDCA yield was good at first cycle then gradually decreased in the cycle 2 and 3 (Figure 4.7A). However, the unutilized HMF was observed in the 3rd cycle. Also the BHMF (HMF alcohol) formation indicated the cells converted the HMF to reduce the substrate toxicity. The depletion of nutrients in the production media might be the reason for decreasing FDCA yield because we have recycled with 50% of media. After utilizing the fresh nutrients in the cycle 2 and cycle 3, it might have not sufficient for the further HMF biotransformation. Since we are using resting cells for the biotransformation, the strain had to utilize the given HMF as early as possible to maintain the cellular environment and reduce the toxicity. After 3 cycles, 2402 mg/L of FDCA was produced from 3000 mg/L of HMF (Figure 4.7B). The complete HMF conversion was observed in the cycle 1. Later, the HMF conversion was decreased in the cycle 2 and 3. From the experimental results, the overall FDCA yield was 80% and the HMF conversion efficiency was 81.66%. The performance of strain was significantly improved from batch and fed batch experiments.
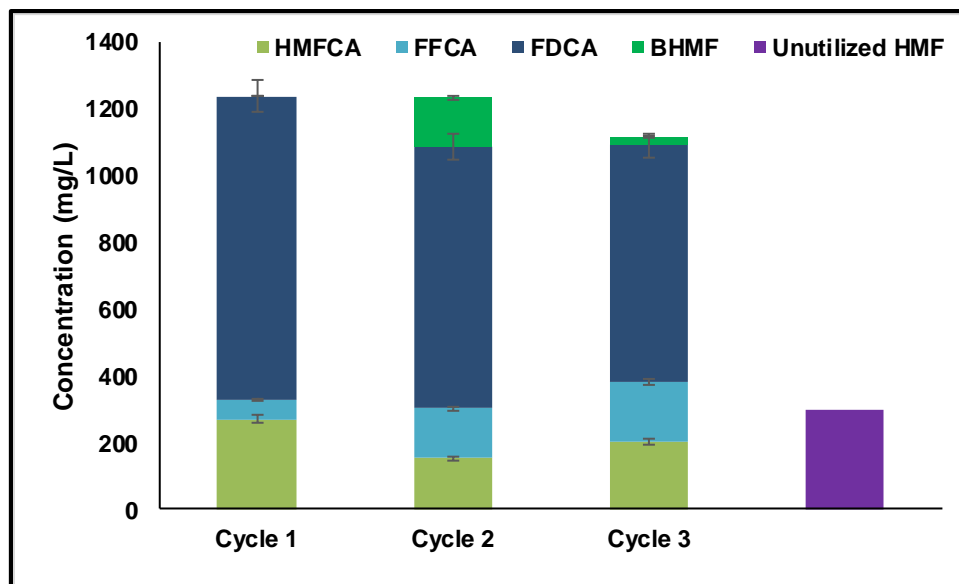
Figure 4.7A. Repeated batch biotransformation; 50% of media replacing, HMF and HMF derivatives production in each cycles.
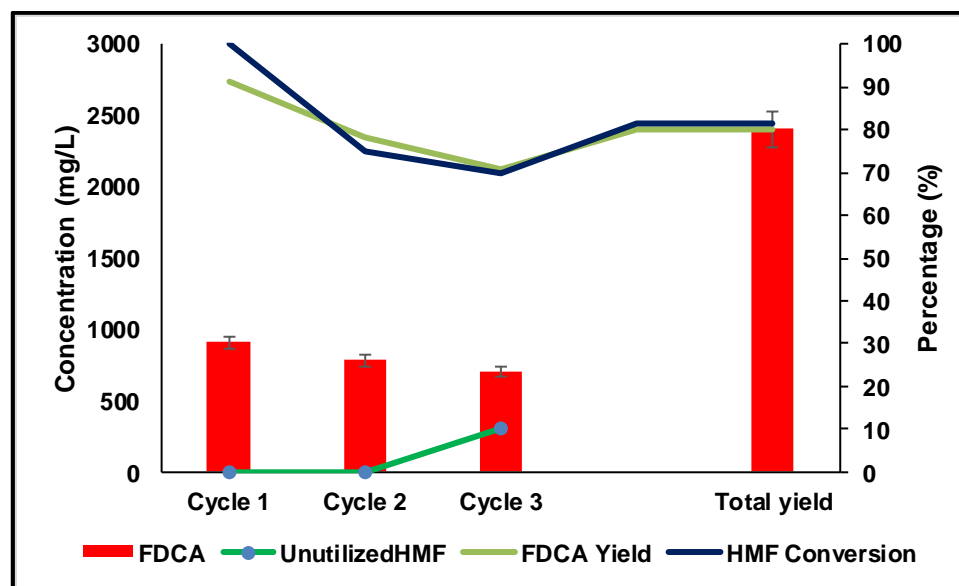
Figure 4.7B. Repeated batch biotransformation; 50% of media replacing, FDCA production in each cycle, total FDCA yield, unutilized HMF and HMF conversion.

In another experiment, the production media was completely replaced with sterile fresh media. Other conditions were same as the previous experiment. Interestingly, the strain could perform up to 5 cycles. It was clear that there was no substrate inhibition happened because of routine replacement of production media. In the first three cycles, FDCA yield was more than 90% (Figure 4.8A). There were no other HMF derivatives formed except HMFCA until 3 cycles. Even though after 3 cycles, negligible amount of BHMF and FFCA was formed. From cycle 4 to cycle 5, the performance of *R. qingshengii* C27 was decreasing because the cells were reaching to death phase. It is worth mentioning that the tolerance of *R. qingshengii* C27 was quite incredible because not only it could utilize the given HMF up to 15-17 days (5 cycles) also achieved almost 95% HMF conversion efficiency (Figure 4.8B). Compared to previous experiment with 50% media replacing, there was 16% of increment in FDCA yield up to 3 cycles.
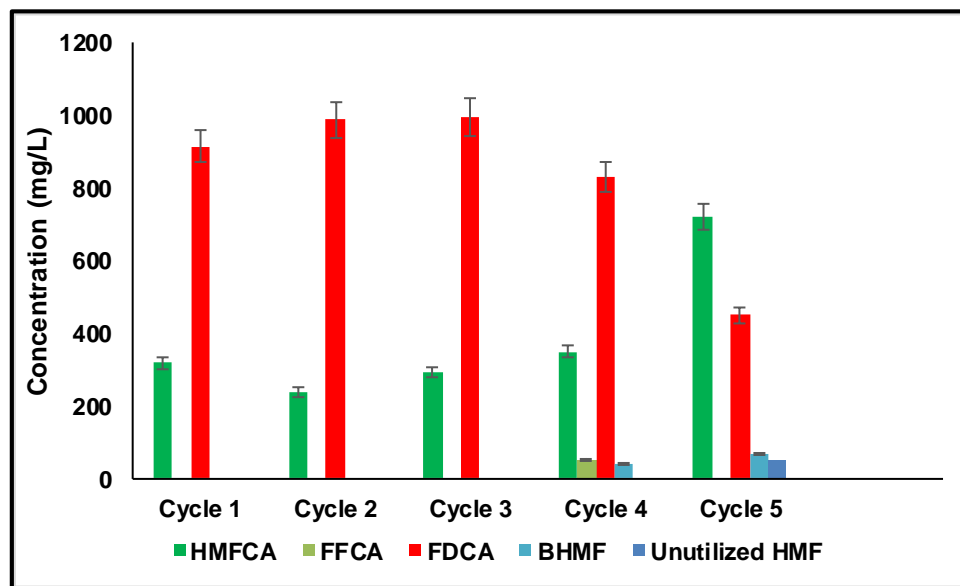
Figure 4.8A. Repeated batch biotransformation; HMF derivatives including FDCA production in each cycle, Total HMF was given in each cycle and unutilized HMF after 5th cycle.
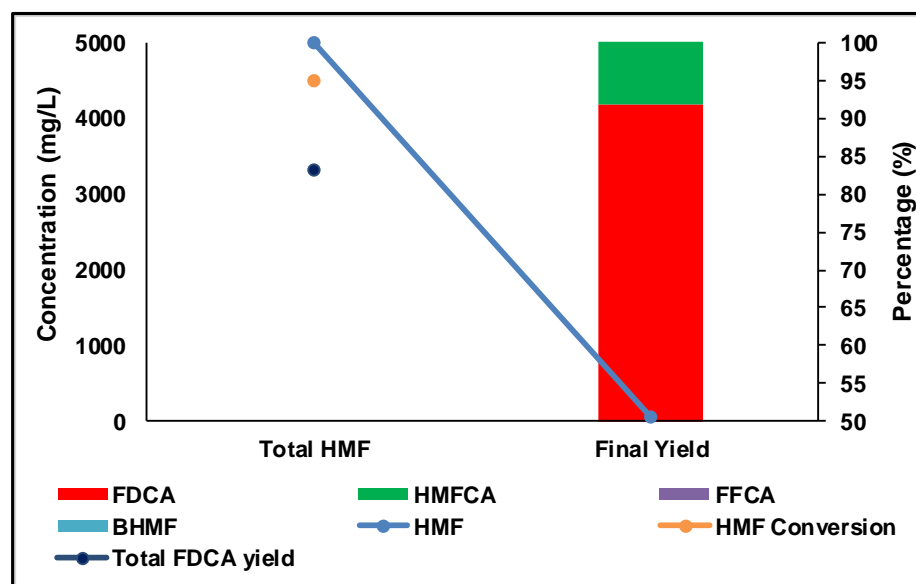
Figure 4.8B. Repeated batch biotransformation; Total HMF derivatives including FDCA production after 5 cycles, total HMF given (5 g/L), FDCA yield and HMF conversion.

The total FDCA yield was 4178 mg/L from 5000 mg/L HMF concentration after 5 cycles. Compared to all the previous studies on improvement strategies for FDCA, this is the highest FDCA yield (4.1 g/L) from higher concentration of HMF (5g/L). There is no report till today on FDCA production using wild strain as whole-cell biocatalyst. Most of the recent studies were introducing HMF oxidase gene and hmfH (encoding oxidoreductase gene) for the improved FDCA yield Yuan et al., 2018a; Yuan et al., 2018b; Yuan et al., 2018c, Lin et al., 2020).

4.3.6. Immobilization of *Rhodococcus qingshengii* C27

Immobilization experiments were studied under optimized conditions. Production media was replaced in the regular intervals (same as previous studies; section 4.3.5). The *R. qingshengii* C27 cells were immobilized by calcium alginate beads (Figure 4.9A) and HMF biotransformation was performed. Un-immobilized cells were considered as control

(same as in section 4.3.6). From the experimental observations, the immobilized cells could produce FDCA from given HMF up to 3 cycles. However, compared to control FDCA yield was pretty low (Figure 4.9B & Figure 4.9C). The major hurdle facing with immobilization was the bead strength. Beads were started to disintegrate in the end of 3rd cycle. To overcome this hurdle, we have increased the concentration of sodium alginate and calcium chloride. Unfortunately, HMF was converted to HMFCA and further oxidation was not occurred when we increased the sodium alginate concentration. Media components might not have consumed by the strain eventually it affected the strain performance on further oxidations. The maximum yield obtained under optimized conditions of sodium alginate and calcium chloride was at 4% concentration. A recent study on immobilized *Burkholderia cepacia* H-2 cells, the strain could transform 2000 mg/L of HMF in presence of other lignocellulosic inhibitors. However, the FDCA yield was very low (Tsai et al., 2021). Through cell immobilization, total of 1830 mg/L FDCA was synthesized from 3000 mg/L of HMF concentration by 3 cycles. The unutilized HMF was around 510 mg/L and FFCA and HMFCA accumulation was observed. Other immobilization strategies have to be implemented for the better FDCA yield.

The PUF mediated immobilization was also performed. From the observations, the consecutive oxidations from HMF to FDCA were not happened compared to control and also HMFCA accumulation was high. Mass transfer of nutrients in to the strain is the key factor of bioprocess. In this study, it is clearly evident that the HMF oxidations was not happened properly in immobilized cells as compared to un-immobilized cells (control). In fact, the main disadvantage of cells without immobilization on HMF biotransformation is the requirement of an additional centrifugation step between each cycles to separate the

cells from the used production media and keep back the cells for the biotransformation with replaced media.

Figure 4.9A. Immobilized *R. qingshengii* C27 by calcium-alginate beads.
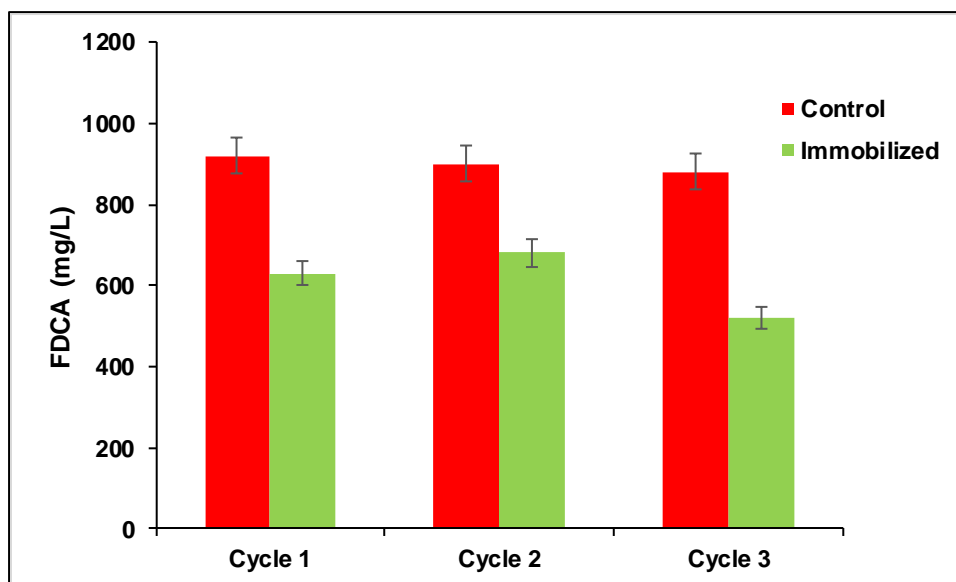
Figure 4.9B. HMF biotransformation; control vs immobilized cells (calcium-alginate beads), FDCA production in each cycles.
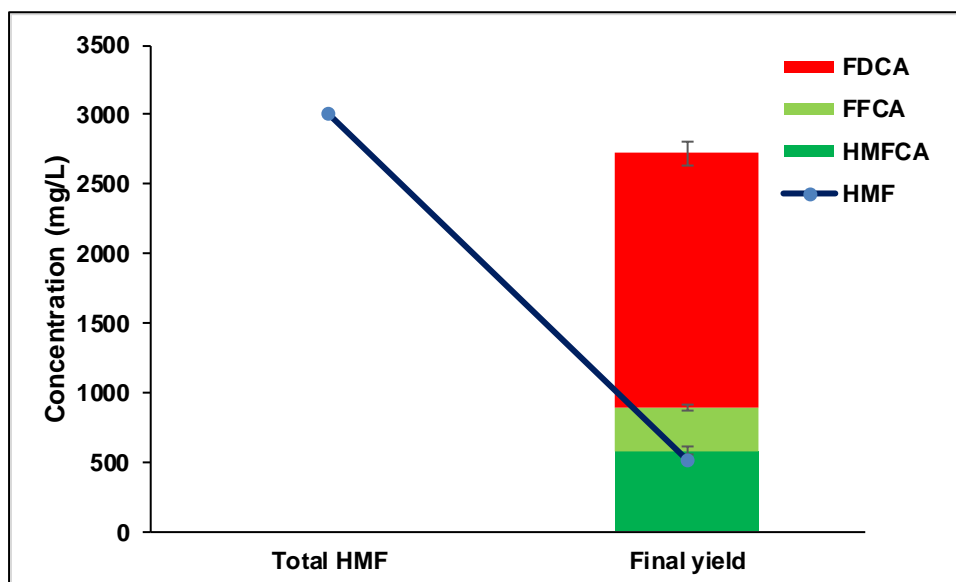
Figure 4.9C. HMF biotransformation; immobilized cells, the total HMF derivatives including FDCA yield (mg/L), the HMF given (mg/L).

4.4. Conclusion

The HMF tolerance of *Rhodococcus qingshengii* C27 was studied. It was observed that the strain could tolerate up to 48 hours in presence of high HMF concentration (240 mM). It was confirmed that only the resting cells of *R. qingshengii* C27 could produce FDCA not with the growing cells.

In this research, the different strategies were investigated for the improved FDCA yield. From the batch biotransformation, 94% of FDCA yield was obtained from 1 g/L of HMF concentration. Through fed batch strategy, 1.604 g/L of FDCA from 2.0 g/L of HMF was obtained. It was found that repeated batch biotransformation could improve the FDCA yield. Through the repeated batch, FDCA yield was 4.178 g/L from 5.0 g/L of HMF. The overall FDCA yield was 83.5%.

Chapter 5

Biotransformation of sorghum syrup derived HMF to FDCA; Process demonstration in fermenter

5.1. Introduction

In biocatalysis, there are mainly two methods are known, enzyme based and whole-cell based. In case of FDCA production, the biocatalytic approaches have been emerging with novel whole-cell biocatalysts. The ideal whole-cell biocatalysts should have some basic characteristics.

1. The capability to produce metabolites in minimal media

2. Enormous amount of enzyme.

3. Robustness.

4. Metabolically active.

Once the biocatalyst is selected, then the reaction conditions and different strategies for improving the product yield need to be optimized. Generally, these experiments will be carried out in a flask level. At the end, the process has to be demonstrated in a fermenter level. In the present study, we had selected the biocatalyst and optimized all the physical parameters under flask level. Also different strategies were investigated for the improved production of FDCA.

In this study, the fermenter level FDCA production was attempted. We used the sorghum syrup derived HMF for the biotransformation. The main objective of the study was to demonstrate the complete process of sorghum syrup derived HMF to FDCA.

5.2. Materials and methods

5.2.1 Sorghum syrup derived HMF to FDCA; flask level studies

5.2.1.1. Sorghum syrup derived HMF biotransformation

In this experiment, the sorghum syrup derived HMF was used for the batch biotransformation (in flask level). For the confirmation, the standard HMF was used as control. All the reaction conditions were same as given in chapter 3.

5.2.1.2. HMF to FDCA biotransformation in 1 L flask level demonstration

The pre-fermenter studies were completed in flask level (1 L), fed batch biotransformation was carried out in 1 L of working volume. HMF (1 g/L) was fed with an interval of 72 hours. The total 2 g/L of HMF was given. Overall HMF utilization and FDCA yield was analyzed.

5.2.2. Fermenter studies

The bioreactor studies were carried out in 2 L Infors Minifors reactor using QMSM production media (1 L working volume). The pre-inoculum and inoculum (25 mg/mL) were prepared with 5% of unutilized glucose and unreacted fructose from the sorghum syrup. All the media components were same as described in chapter 3. Same working volume of control was kept in flask level also. The reaction conditions were maintained at 30° C, 7.0 pH and stirrer speed at 200 rpm. High purity air was sparged into the fermenter at a flow rate of 1 vvm. The fed-batch strategy was obtained for fermenter level biotransformation. The initial HMF concentration was 1 g/L in the fermenter. After 72 h and 144 h, HMF (1 g/L) was added to the production media. Total of 3 g/L HMF concentration was given. The final FDCA yield and total HMF conversion were analyzed.

Also the culture sample was (1 mL) was withdrawn from the fermenter (every 24 hours) and measuring the optical density at 600 nm in UVV-Visible spectrophotometer (Shimadzu series UV 1601).

5.3. Results and discussion

5.3.1.1. Sorghum syrup derived HMF biotransformation

The batch experiments were carried out in flask level. Apart from sorghum syrup derived HMF, the standard HMF was used as control. In both experiments HMF concentration was 1 g/L. After 72 hours, the *Rhodococcus qingshengii* C27 cells could transform the standard and sorghum syrup derived HMF to FDCA. Interestingly, the FDCA yield was almost same when compared between sorghum syrup derived HMF (SS-HMF) and standard HMF on their biotransformation (Figure 5.1). This result indicated that the minor impurities presented in the sorghum syrup derived HMF was not inhibited the biotransformation and also the performance of the strain was not affected.
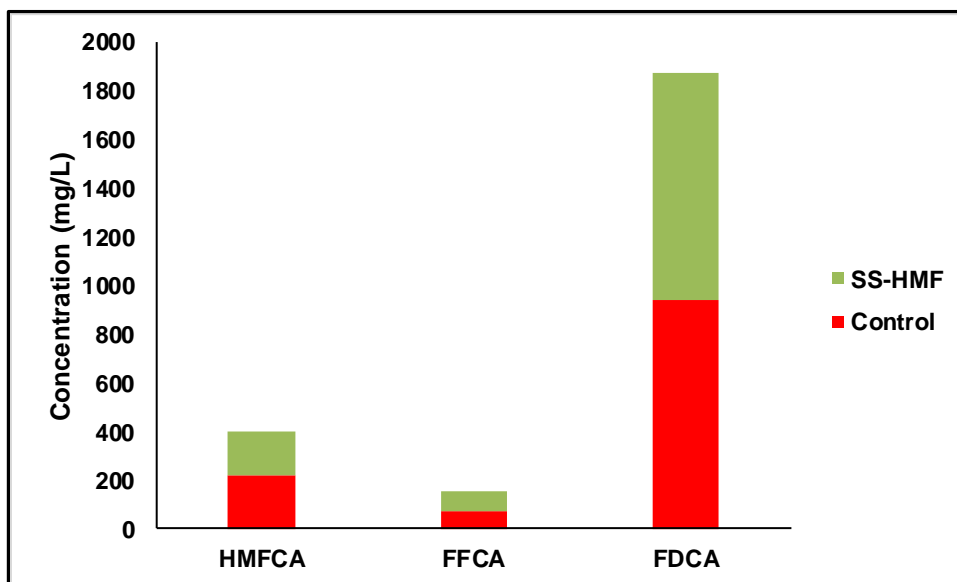
Figure 5.1. Comparison between sorghum syrup derived HMF (SS-HMF) and standard HMF (control) on biotransformation.

5.3.1.2. HMF to FDCA biotransformation; 1 L flask level demonstration

In flask level, demonstration was successfully completed with total of 1671 mg/L FDCA from 2000 mg/L HMF in 144 hours (Figure 5.2). 92% of total HMF conversion was observed with 91% FDCA yield with respect to HMF utilized. The final HMFCA accumulation was around 405 mg/L.
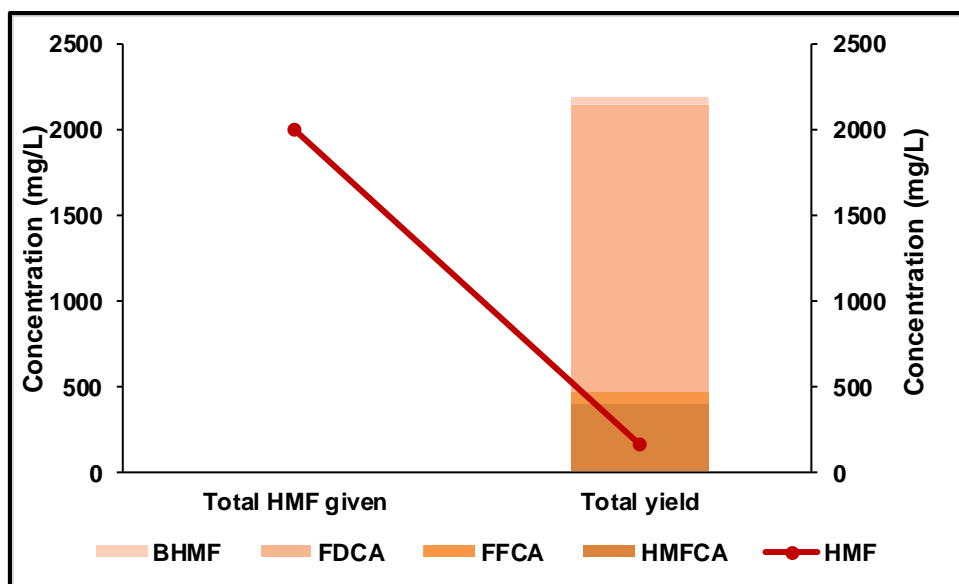
Figure 5.2. Fed batch HMF biotransformation- 1L flask level.

5.3.3. Fermenter studies

The fed batch strategy was performed in bioreactor after the flask level trials. The biotransformation was carried out in 2 L Infors Minifors bioreactor with a working volume of 1 L production media supplemented with initial HMF concentration (1 g/L). Compared to flask level, the initial HMF was started to utilize only after 24 hours in fermenter. The strain might take time to adapt the fermenter conditions. However, 70% of HMF was utilized within 48 h. After 72 h, not only the complete HMF utilization but also the FDCA yield was nearly 99%. It possibly due to the purified air was sparged into the production media throughout the process. After 72 h, 1 g/L HMF was fed to fermenter. The HMFCA accumulation was observed along with BHMF formation. It might be due to the substrate inhibition. After 144 hours, the FDCA yield was almost 85%. HMF (1 g/L) was fed at 144th hour. The strain could not utilize the HMF more than 30%. Still the FFCA was completely oxidized to FDCA. The pH was monitored throughout the biotransformation. The initial pH

(7.0) was reduced to pH 3.16 at 9th day. It must be due to the presence of FDCA and HMFCA. The initial OD_{600} value was 0.6 (at late exponential phase of cell harvesting) which is corresponded to 25 mg/mL. The biomass (cell concentration) was gradually decreased. In the end (9th day) of fermenter the OD_{600} value was observed and CFU was reduced (Table 5.1). This result was clearly indicating the cells were at its death phase. The major reason was there were no other carbon source for their metabolism only HMF was presented and it was biotransformed to FDCA. The first report on fed batch strategy was reported by Koopman et al. They have produced 30.1 g/L FDCA through fed batch strategy by using engineered *Pseudomonas putida* S12. Moreover, they had given glycerol as carbon source (Koopman et al., 2010b). In Recent studies of Yuan et al reported FDCA production using engineered *Raoultella ornithinolytica* BF60 strain. They had conducted series of research on the mentioned strain for the FDCA production in higher concentration including fed batch and pulse-substrate feeding strategies (Yuan et.al., 2018b).

From the total of 3000 mg/L of HMF concentration 2100 mg/L FDCA was obtained in fermenter level biotransformation. 23% of HMF was unutilized. Also the HMFCA and BHMF was 475 mg/L and 135 mg/L respectively (Figure 5.3). The study demonstrated the fermenter level HMF biotransformation (figure 5.4) which could replicate the flask level experiment results on FDCA production.
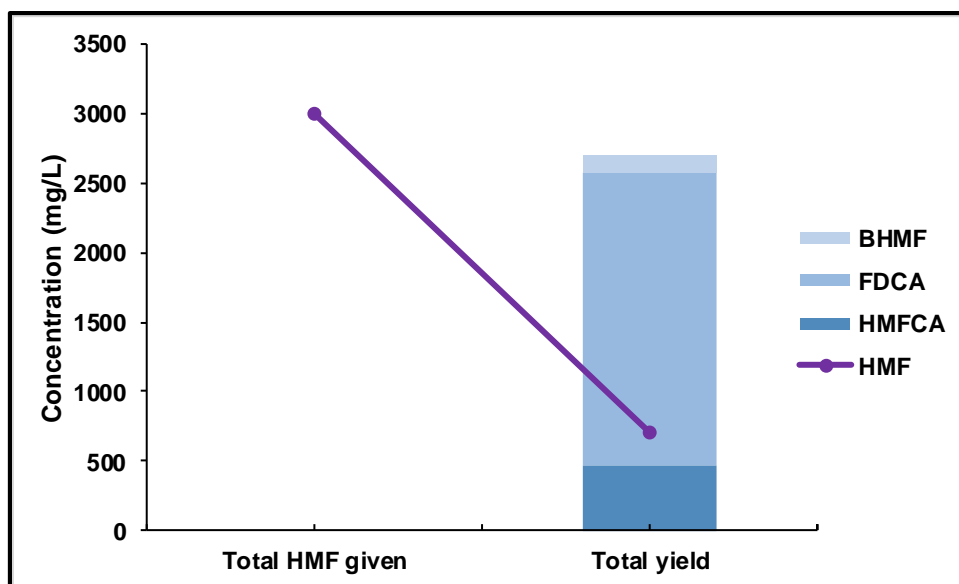
Figure 5.3. Fed batch biotransformation in fermenter level; total HMF given and total yield of FDCA, HMFCA and BHMF in mg/L concentration.

Incubation time (h)	Biomass concentration (CFU/mL)	FDCA (g/L)
24	126	0
48	109	0.505
72	90	0.988
96	74	1.403
120	63	1.853
144	38	2.106
216	08	1.587

Table 5.1. Biomass concentration: CFU/mL (10^{-6}) count and FDCA production in fermenter (every 24 hours of incubation till 9th day). The starting OD during the production was 0.6 at 600 nm.
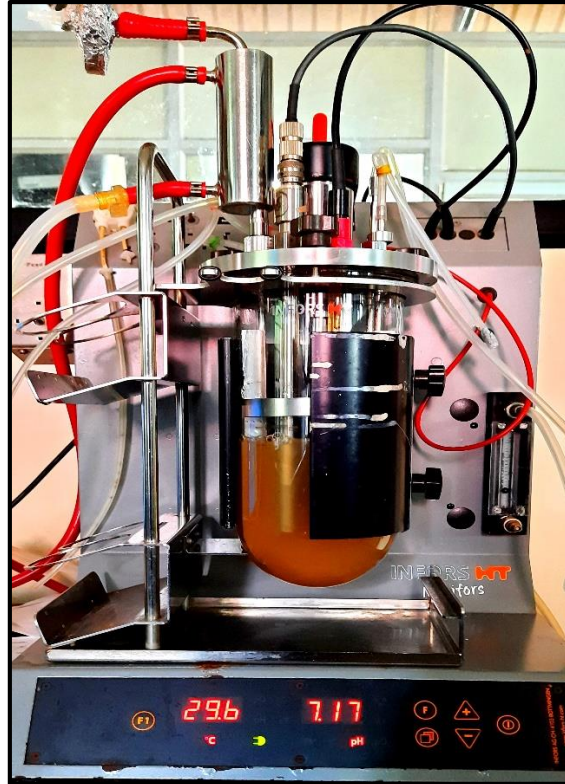
Figure 5.4. Fermenter level HMF biotransformation.

5.4. Conclusion

The complete process of sorghum syrup derived HMF to FDCA biotransformation was confirmed in flask level. In fermenter (1L), fed batch biotransformation was performed. In this study total of 2.1 g/L of FDCA was obtained from 3.0 g/L of HMF concentration.

Chapter 6

Separation and purification of FDCA

6.1. Introduction

The polyethylene 2,5-dicarboxylate also known as polyethylene furanoate (PEF) has great physical and mechanical properties compared to polyethylene terephthalate (PET). PEF can be produce by FDCA polymerization with ethylene glycol (EG). It has various application including in polyester industry (detailed description given in chapter 1). Commercial production of PEF bottles has been started by companies like Avantium, Novozymes. The characteristics of these particular polymers depend on purity of the monomers and raw materials. If the quality of the polymer is up to mark, then it would go for commercialization.

The biological production of FDCA address the limitation of existing chemical processes like the high energy cost, toxic raw materials and high cost of catalysts. One of the challenges in biological production of FDCA is the product purification. To be precise in whole-cell biocatalytic production of FDCA, chances of impurities are high. Normally the downstream processing of FDCA starts with the removal of microbial cells from the fermentation/biotransformation broth by centrifugation/filtration/flocculation. Then removal of other impurities like proteins, nucleic acids, salts etc. by boiling and centrifugation. Then separation of FDCA and finally purification by vacuum distillation etc. Koopman et.al described the FDCA recovery and purification by acid precipitation followed by tetrahydrofuran (THF) mediated solvent extraction. However, the purity was coming down when titre increased (Koopman et al., 2010a). Downstream processes of FDCA still needs intensive research for the better recovery methods. Economic viability is another important aspect for the downstream process.

In the present study, the FDCA was produced from the HMF using *Rhodococcus qingshengii* C27. After HMF biotransformation, the unutilized HMF, HMFCA, FFCA in addition to FDCA was presented in the broth. This work aims to selective separation of FDCA from fermentation broth using functionalized diethanol-polystyrene resin.

6.2. Materials and methods

6.2.1. Chemicals

The chemicals used in this study like, Chloromethylated polystyrene (CMPS), N, N-dimethyformamide (DMF) and Diethanolamine (DEA) were procured from Merck, India. NaOH and $NaHCO_3$ were purchased from SRL, India.

6.2.2. Synthesis of functionalized PS-DEA

Before the reaction, CMPS resin was conditioned by washing with deionized water follwed by methanol wash. The resin was then kept in hot air oven at 50⁰ C for 6 hours for complete methanol evaporation. CMPS was ready for the amine functinalization. The PS-DEA was synthesized by standard protocol by Khot et al (Khot et al., 2014). 5 g of CMPS, 2.62 g of DEA and 20 mL of DMF were taken for the reaction. The reaction was conducted at 90⁰ C for 10 hours. After 10 hours of reaction, the resin was separated from the reaction mixture. The resin was washed with deionized water twice followed by methanol wash. The resin was dried in oven at 50⁰ C. The dried and activated PS-DEA was used for adsorption studies.

6.2.3. Characterization of PS-DEA resin

The CMPS resin and dried and activated PS-DEA resin was weighed before the further characterization to understand the amine concentration (initial confirmation).

6.2.3.1. FTIR spectroscopy

The amine functionalization of PS-DEA was characterized by Fourier Transform Infrared Spectroscopy (FTIR) using IRTracer-100, Shimadzu instrument with MCT detector. The FTIR frequencies were analyzed and compared with CMPS, PS-DEA and PS-DEA after adsorption. The C-Cl stretching band reduction of CMPS was monitored to understand the loading of amines in the PS-DEA.

6.2.3.2. Surface area characterization and Pore size distribution

Nitrogen adsorption and desorption experiments were conducted by the BET analyzer for the surface area and pore size distribution. The CMPS and PS-DEA resins were analyzed.

6.2.4. Recovery and purification of FDCA using Diethanol amine functionalized polystyrene (PS-DEA)

The functionalized resin namely PS-DEA was studied for the adsorption of FDCA from the fermentation broth. The fermentation broth was subjected to centrifugation @13000 rpm for 10 min. The Supernant was further boiled and centrifuged to remove the residual proteins. Batch adsorption of FDCA was carried out in a stoppered conical flask by suspending predetermined quantity of PS-DEA resin in the broth.

Preliminary experiments suggested good adsorption affinity of standard FDCA on the PS-DEA resin among other HMF derivatives. The suspension was kept in constant temperature shaker at 30°C with an agitation speed of 200 rpm. The adsorption was studied for different time intervals and was quantified by HPLC. After adsorption, 1N NaOH and NaHCO$_3$ were used to desorb the adsorbed components on the resin. Desorbed components were analyzed by HPLC. FDCA was recovered by neutralizing the pH followed by concentration of water in rotavac. After the evaporation, the residue obtained was reconstituted in methanol to remove the salt formed during the neutralization process. The purified FDCA was analyzed in HPLC and confirmed by NMR (^{1}H NMR (500 MHz, CDCl3)) using Bruker NMR spectrophotometer.

6.2.5. Kinetic adsorption and desorption study

The kinetic study was done with standard HMFCA and FDCA with 1 g. The PS-DEA was taken with 1 g. It was diluted in 10 mL of water. Adsorption study was conducted with specified time. The sample was collected (5 min, 10 min, 15 min, 30 min, 45 min and 60 min). For desorption 10 mL of 1N NaOH was used. The samples were taken at different interval of time (15 min, 30 min, 45 min, 60 min, 75 min and 90 min). All other parameters were same as previous studies.

6.2.6. Direct adsorption using PS-DEA resin on repeated batch biotransformation

In this experiment, we have used the PS-DEA resin directly in the HMF biotransformation. In the repeated batch process, HMF was given in every cycle and production media was replaced (procedure described in chapter 3). After every cycle (72 hour) we have suspended predetermined quantity of PS-DEA to the HMF biotransformation broth and

incubated under designated conditions for 1 hour. After 1 hour, the adsorbed PS-DEA was removed and continued the repeated batch biotransformation with predetermined HMF concentration. The adsorbed FDCA along with other HMF derivatives were analyzed through HPLC.

6.3. Results and discussion

6.3.1. PS-DEA characterization

The PS-DEA resin was synthesized as described in the materials and method section (Figure 6.1). The loading of the amine on the resin takes place with the simultaneous formation of HCl as shown in figure. The weight of CMPS before the reaction was 5 g. After the amine attachment the weight of PS-DEA was 7.78 g. That confirmed the attachment of diethanol amine. To further confirm the observed result, we have performed the FTIR and surface area and pore size distribution.
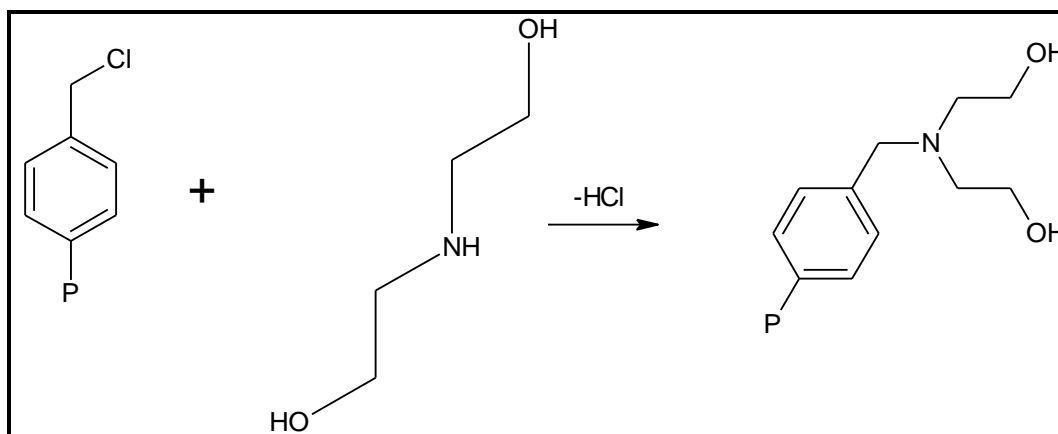
Scheme 6.1; PS-DEA resin synthesis (P= polymer)
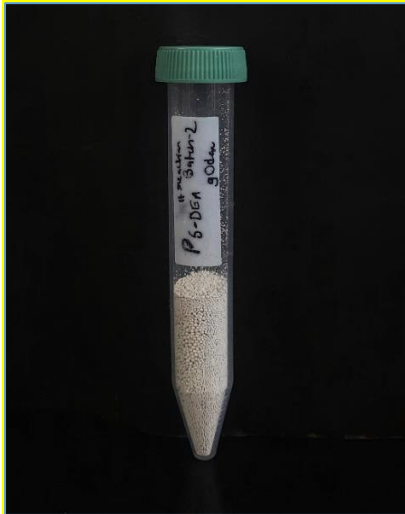
Figure 6.1. The synthesized PS-DEA.

6.3.2. FTIR studies on PS-DEA

The FTIR spectra was analyzed in CMPS, PS-DEA and PS-DEA after adsorption. It was clearly evident that the significant change in the PS-DEA resin compared to CMPS the amine group attachment was confirmed.
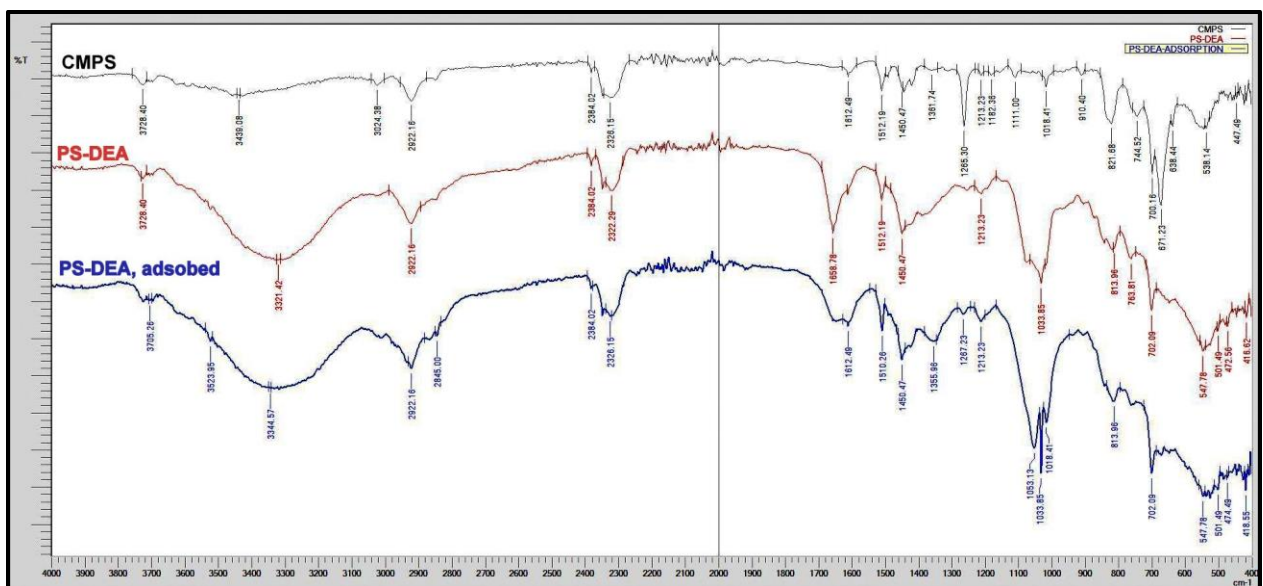
Figure 6.2. FTIR spectra of CMPS, PS-DEA and PS-DEA adsorption.

FTIR spectra provided valuable insights on the functionalization of CMPS resin with DEA. DEA gets attached to CMPS resin by displacement of chloride group with simultaneous linking of nitrogen atom of DEA with CMPS resin. A peak observed at 671 cm^{-1} in the CMPS resin corresponds to C-Cl stretching (660-680 cm^{-1}). The absence of these peak in the PS-DEA resin indicates the complete displacement of chloride ion from CMPS as HCl with simultaneous loading of amine in the CMPS resin. At 3400, 1033 and 800 cm^{-1} peaks were seen in PS-DEA which corresponds to the amine attachment (Figure 6.2).

6.3.3. Surface area characterization and Pore size distribution on PS-DEA

Chemical bonding of a molecule on the resin affects the surface properties of the resin. In order to evaluate the performance of PS-DEA resin, surface area and pore size of the resin was checked. It was observed that BET surface area of the CMPS reduced from 22.28 m^2/g to 16.92 m^2/g after functionalizing it with diethanol amine. However pore size increased from 345 Å to 419 Å. Higher pore size for PS-DEA resin indicate greater adsorption tendency for larger molecules like FDCA in comparison to HMF. There was slight reduction in the pore volume of CMPS resin from 0.171 cm^3/g to 0.166 cm^3/g after funtionalizing it with diethanol amine.

6.3.4. Kinetic adsorption and desorption studies

The adsorption and desorption studies were investigated. The figure 6.3 shows FDCA was adsorbed 0.83 g within 5 minutes compared to HMFCA (0.32 g). In case of FDCA the maximum adsorption was observed at 30 min (96%). However, the HMFCA was adsorbed maximum at 15 min (0.72 g). Comparing both FDCA and HMFCA adsorption, FDCA has 24% of high adsorption than HMFCA. It is possibly due to the stong interaction

of weak PS-DEA resin and strongly charged FDCA. Compared FDCA, HMFCA less stronger than FDCA (only one carboxylic acid group).
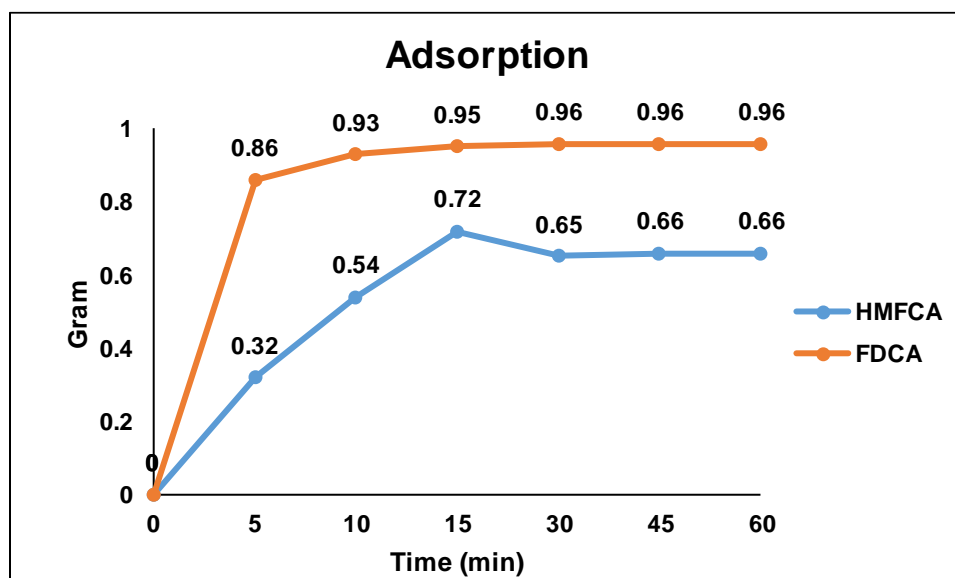
Figure 6.3. Adsorption of standard HMFCA (1g) and FDCA (1g), PS-DEA (1g).

The desorption was carried out with 1N of NaOH. The investigation revealed that desorption rate of HMFCA was high than FDCA desorption. From the adsorbed 0.72 g of HMFCA, 0.66 g of HMFCA was desorbed in the 1N NaOH within 30 min. Hence, FDCA desorption was maximum of 0.86 g within 30 minutes (Figure 6.4). HMF was not used for the adsorption and desorption studies because from the preliminary experiments with fermenter broth, the HMF adsorption was negligible. These kinetic studies suggest that PS-DEA resin mediated adsorption was good for the separation and purification of FDCA from the biotransformed broth.
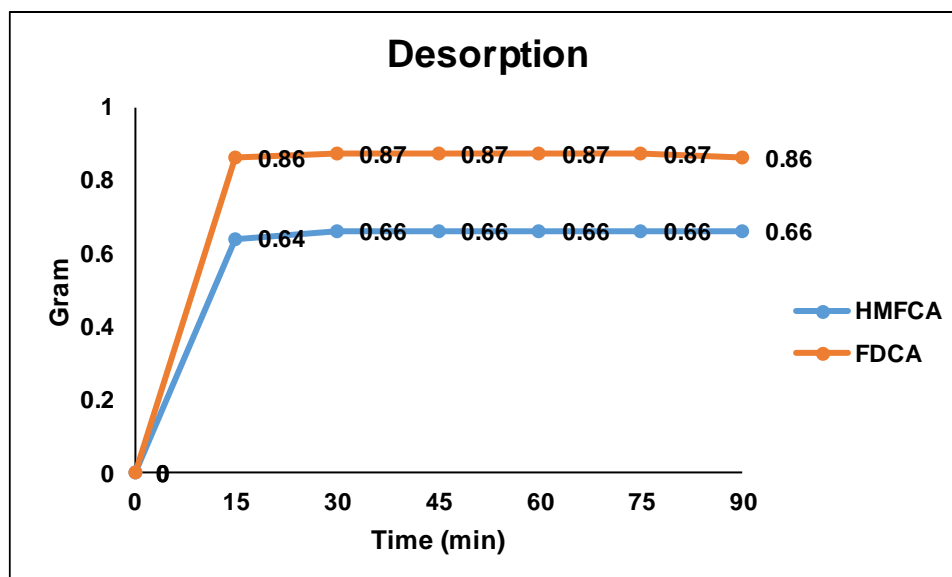
Figure 6.4. Desorption of standard HMFCA (1g) and FDCA (1g) in 1N NaOH.

6.3.5. Recovery and purification of FDCA

Batch adsorption and desorption was carried out for the recovery of FDCA using PS-DEA resin (Figure 6.5). PS-DEA resin being a weak base can interact with FDCA by charged interaction exerted by the lone pair of nitrogen atom on DEA with carboxyl group of FDCA. Beside this acid- base interaction, van der Waals interaction can also be expected between the two aliphatic hydroxyl groups of DEA with two carboxyl group of FDCA. Preliminary batch adsorption experiments of standard model compounds on DEA resin indicated high adsorption of FDCA and HMF acid. Hence the acid–base interaction between the resin and the acid seems to be predominant and strong.

The HPLC profile of fermentation broth suggested percentage of FDCA, HMF acid and HMF as 80%, 18%, 23% respectively. Our experimental findings suggested that nearly 94% of FDCA was adsorbed on the resin followed by 73 and 26% of HMF acid and HMF respectively after 1 hour. Adsorption of HMF is probably due to hydrogen bonding

interaction between the aliphatic hydroxyl groups of the HMF with aliphatic hydroxyl group of the resin. Hence van der waals interaction also on play leading to adsorption of other components. However, it was observed that FDCA was strongly adsorbed to the resin as it was not eluted out using water, methanol or butanol as a desorbing solvent. Hence NaOH and $NaHCO_3$ solution was used for desorption. Comparing the results of desorption, it was clear that NaOH mediated desorption was better (Table 6.1). Nearly 92% FDCA was desorbed using 1N NaOH along with 32 and 4% desorption of HMF acid and HMF, respectively after 1 hour. The desorption rate of FDCA was maximum at 50^0 C. The temperature was not further increased as it might affect the physical properties of the PS-DEA resin. Hence recovery of FDCA from the fermentation broth was successfully carried out using PS-DEA resin. Literature data widely reports the recovery of FDCA by organic solvent extraction. The significant feature of the synthesized PS-DEA was the reusability. The resin was actively reused three times continuously for FDCA adsorption from biotransformed broth. To best of our knowledge, we believe that this is the one of the pioneer report on the recovery of FDCA using amine functionalized polystyrene.
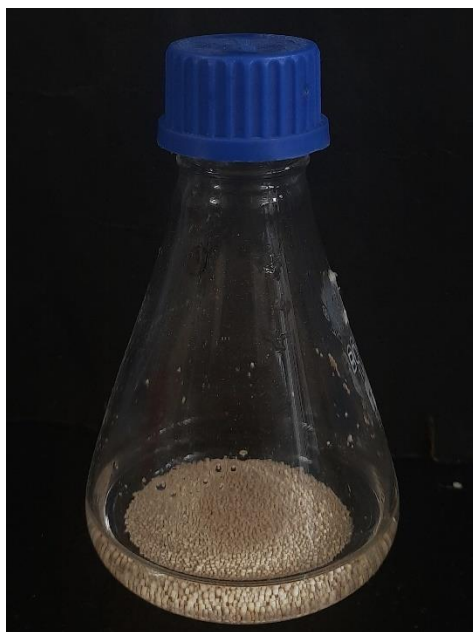
Figure 6.5. PS-DEA adsorption.

(%)	HMF	HMFCA	FDCA
Adsorption	26	73	94
NaOH desorption	4	32	92
NaHCO₃ desorption	38	35	80

Table 6.1. PS-DEA mediated adsorption (%) and desorption (%).

6.3.6. Characterization purified FDCA

The recovered FDCA was purified (Figure 6.4) by concentrating the water in rotavac followed by reconstitution with methanol for removal of salts. HPLC analysis indicated a single peak in chromatogram (Figure 6.5). The structural similarity of purified FDCA with that of standard was confirmed through ¹H NMR (Figure 6.6). However, some additional

peaks were seen in the NMR spectra which might be HMFCA or coming form D_2O. However, the two COOH protons were seen in the spectra (4.03 & 6.9). It was matching with the NMR spectra of standard FDCA obtained commercially.
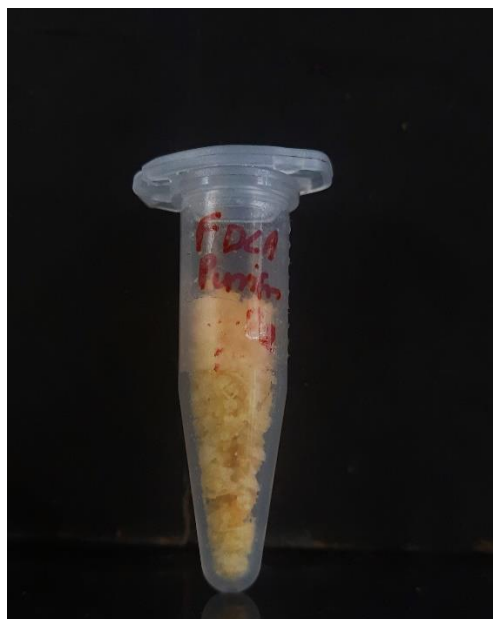
Figure 6.6. The purified FDCA.
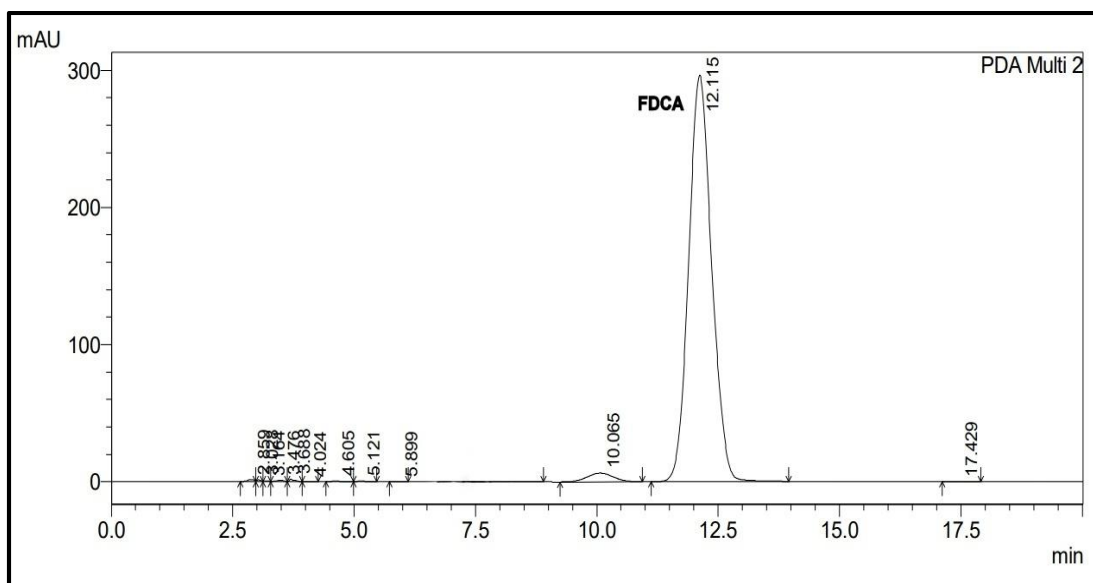
Figure 6.7. HPLC chromatogram of purified FDCA.
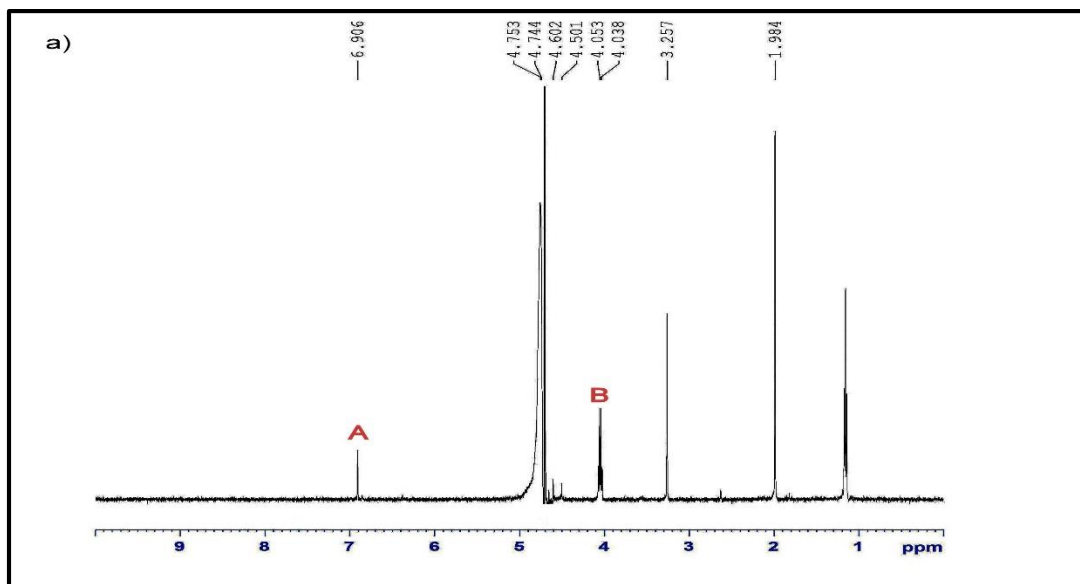
Figure 6.8 a. NMR spectra of Purified FDCA from transformation broth.
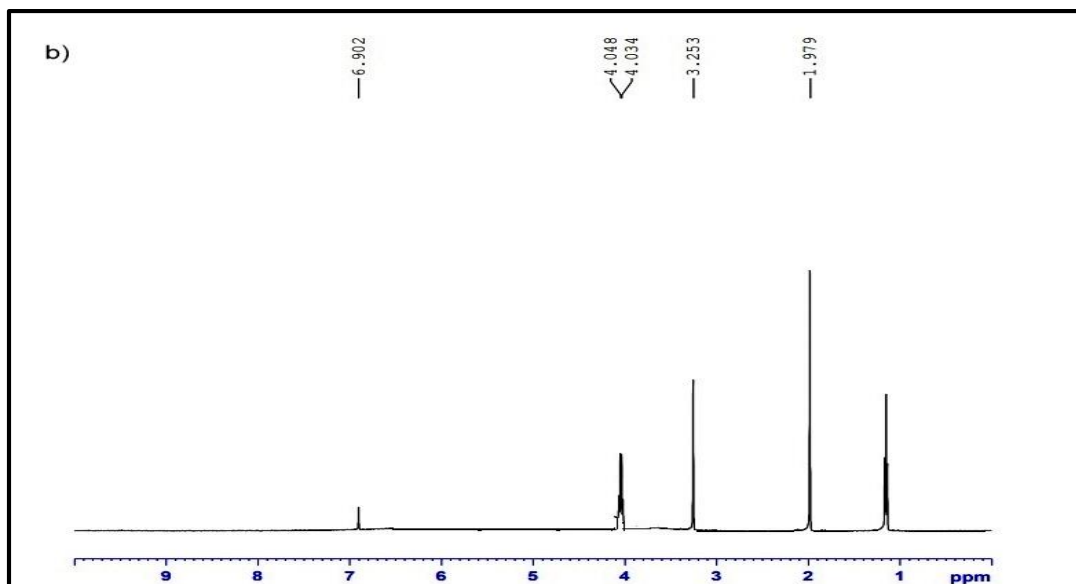
Figure 6.8 b. NMR spectra of analytical standard FDCA .

6.3.7. Effect of direct adsorption using PS-DEA resin

In this experiment, PS-DEA resin was used directly in HMF biotransformation. After every

cycle, the adsoprtion rate of products were analyzed and calculated. HMF accumuation

in each cycle was less than 5%, therefore HMF adsoprtion was not considered in this experiment. In cycle 1, the FDCA and HMFCA rate was 57% & 17% respectively. However, FDCA adsoprtion rate was decreased in other cycles. The adsorption rate was reduced to half in the cycle 3 compared to cycle 2. Unfortunately, the FDCA yield was started to decrease after the first cycle compared to control. The resin might be inhibiting the performance of *Rhodococcus qingshengii* C27 strain, but the actual reason for this is unknown. Moreover, the presence of other proteins in the production media might have reduced the FDCA adsorption rate.
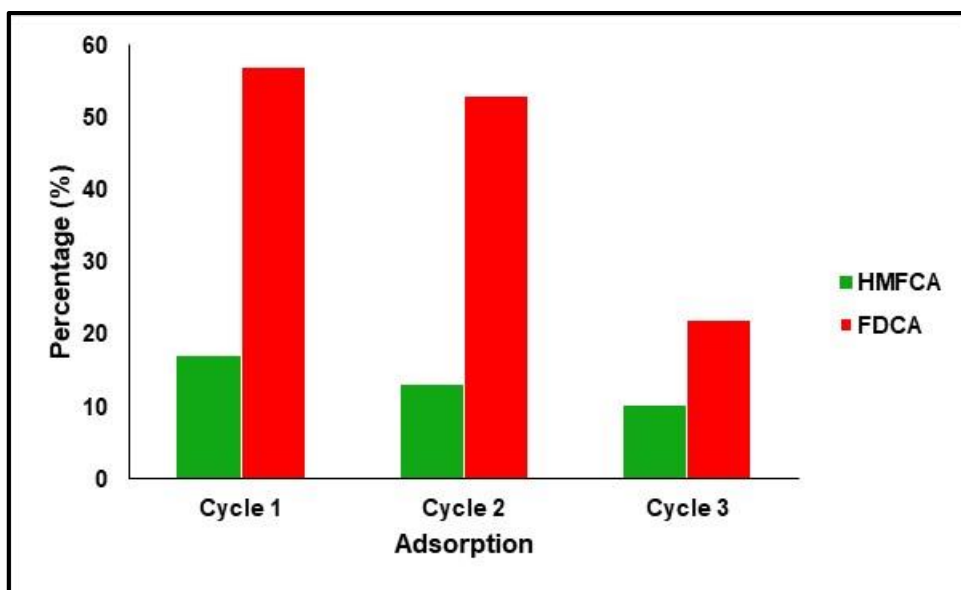
Figure 6.9. Direct adsorption using PS-DEA and FDCA production**.**

6.4. Conclusion

Polystyrene-Diethanolamine (PS-DEA) resin was synthesized. A novel resin mediated approach for FDCA purification from biotransformation broth was performed. In this study, the synthesized PS-DEA resin was characterized by FTIR and surface area analyzer. The PS-DEA resin showed 94% adsorption for FDCA. Adsorption results indicated stronger chemical interaction of FDCA with the PS-DEA resin. NaOH rendered higher desportion performance compared to $NaHCO_3$. Thus FDCA was recovered using PS-DEA resin with 94% purity. The purified FDCA was characterized through HPLC analysis and by ^{1}H NMR and comparable results were obtained as that of standard FDCA.

Chapter 7

Environmental impact assessment of sorghum biomass to FDCA process using Comparative Life Cycle Analysis (LCA)

7.1. Introduction

Biobased products have been known for several decades. Most of the industries have started promoting 'greener approaches' for the sustainable future. However, sustainability of a process or product can be achieved by a comprehensive system analysis which includes technical feasibility, resource sustainability, techno economic analysis (TEA), environment risk assessment (ERA), life cycle analysis (LCA) and policy analysis. Among them life cycle analysis is the most important model to assess environmental impact of the products, processes and services. Compared to other methods like water footprint method, ecological footprint method, LCA accounts of different environmental impacts including GHG emissions.

There are two types of LCA; attributional LCA (aLCA) and consequential LCA (cLCA). The aLCA will tell the 'total' emissions from the process during the life cycle of the product. Therefore, industries are more interested on attributional LCA. The cLCA will tell about the 'change' in total emissions from the process during the life cycle of the product. Hence it is more interested to policy makers because cLCA involves more assumptions like consumers/competitors behaviour etc. There are other variants in LCA; cradle to grave, cradle to cradle, cradle to gate and well to wheel. Each variant has its own purpose and advantage. Cradle to grave involves LCA for production, manufacturing, use and disposal. Cradle to cradle has same phases as cradle to grave, but additionally it involves recycle. The cradle to gate variant involves cradle to factory gate impact assessment. It is more important to understand the environmental friendliness of the process. Finally, well to wheel variant is a specific LCA for the transportation sector.

LCA has four distinct stages; goal definition and scoping, life cycle inventory, life cycle impact assessment and life cycle interpretation. The goal and scope of the LCA study should be well defined because purpose of the study has to be consistent. Life cycle inventory is the toughest task of entire LCA study. It requires complete data collection. All the inputs and output information should be validated. In the life cycle impact assessment, resulting several indicators for the different impact categories. Moreover, the aggregate inputs and outputs of the product system should be considered for the impact categories. At last in the life cycle interpretation, the completeness, sensitivity and consistency will be evaluated. Also the conclusions, limitations and recommendations of the complete process can be presented.

FDCA production involves several steps such as sorghum plant cultivations, sorghum syrup production, HMF synthesis, FDCA production and purification. The main objective of the study is to understand the environmental impact of the complete process of biomass to FDCA. All the collected data was validated (energy, raw materials etc.).

7.2. Materials and methods

7.2.1. Goal definition and system boundary

The main goal of the study was to quantify the environmental impacts of sorghum biomass to FDCA production. The LCA study's functional unit was the production of "1 Kg of FDCA" at the factory gate. The "cradle to gate" approach was chosen for the processes where the processes from sorghum cultivation up to the production of purified FDCA. The complete process of sorghum to FDCA synthesis was segmented into four sub systems (SS).

SS1 Sorghum plant cultivation: The data of land preparation, water irrigation, fertilizers and chemicals were collected. The cultivated sorghum stalks were crushed for juice preparation.

SS2 Sorghum syrup production: The juice was extracted from the sorghum stalks and sorghum syrup was produced by concentrating the extracted juice (Ratnavathi et al., 2016).

SS3 HMF synthesis and FDCA production: HMF was produced from sorghum syrup through Seralite SRC 120 cationic resin mediated dehydration. All the input data was calculated and validated. sorghum syrup derived HMF biotransformation to FDCA using *Rhodococcus qingshengii* C27. HMF synthesis and FDCA production processes were made as one subsystem because of the unutilized sugars (glucose and fructose) of HMF synthesis were directly used for the *R. qingshengii* C27 enrichment/boosting. Therefore, the inventory data of HMF synthesis and FDCA production were given in subsystem 3.

SS4 FDCA purification: The produced FDCA was then purified and recovered using PS-DEA mediated adsorption. The system boundary with processes (sorghum to purified FDCA) are given in scheme 7.1.
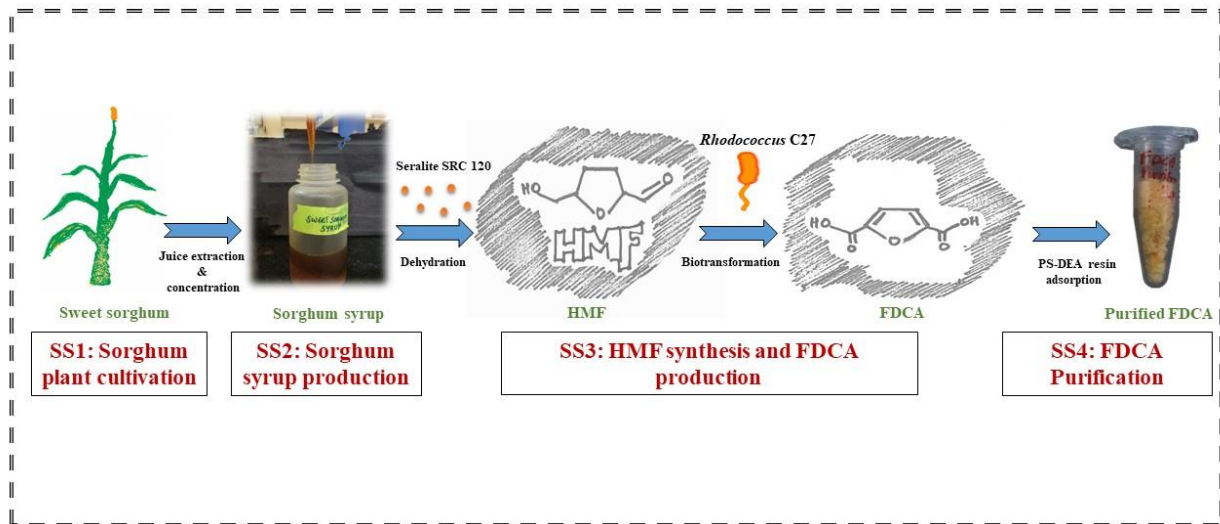
Scheme 7.1. System boundaries of sorghum biomass to FDCA production.

7.2.2. Data inventory

In this study, the data of sorghum plant cultivation and sorghum syrup production was collected from Ratnavathi et.al., 2016 (ICAR- Indian institute of millet research) and Tamilnadu agritech portal (https://agritech.tnau.ac.in). All the remaining inputs of sorghum syrup to FDCA was validated. The detailed inventory data was given in Table 7.1. The database was used for the LCA study was ecoinvent version 3.2, Switzerland.

SS1 Sorghum cultivation		SS2 Sorghum syrup production	
Inputs		Inputs	
Sorghum seeds	12.5 Kg/ha	Sorghum stalks	9350 Kg
Captan	25 g	Electricity	0.25 kWh
Azospirillum	600 g	LPG	75.64 L
Chlorpyriphos	50 mL		
NPK fertilizer	120:40:40 Kg		
Atrazine	500 g		
Electricity	2.238 kWh		
Water	4500000 L		
SS3 FDCA production		SS4 FDCA purification	
Inputs		Inputs	
Sorghum syrup	15 Kg	FDCA	1 Kg
Seralite SRC 120	15 Kg	PS-DEA	100 g
Chloroform	7.5 L	Sodium hydroxide	40 g
Sodium chloride	750 g	Methanol	100 mL
R.qingshengii C27	25 Kg	Electricity	0.1 kWh
LB broth	27.5 Kg	Water	1 L
QMSM components	5.13 Kg		
Electricity	8.9 kWh		
Water	2500 L		

Table 7.1. Inputs used in the sorghum cultivation to FDCA purification.

7.2.3. Life cycle impact assessment

The method for the environmental impact assessment of the current study was TRACI 2.1 (Tool for the Reduction and Assessment of Chemical and other environmental Impacts) developed by Environmental Protection Agency (EPA). TRACI 2.1 has 10 impact categories (environmental and human health related).

7.2.4. Software

The openLCA v 1.10.3 software was used for the LCA study. SuperPro 12.01.1002 software was used to calculate the standard power consumption for different process (fermenter, centrifuge etc.).

7.2.5. Assumptions and limitations

The quality of inventory data is critical for the environmental impact study. In case of sorghum plant cultivation and sorghum syrup production data was based on secondary database. In the HMF to FDCA route, other derivatives like HMFCA, BHMF were not taken into the study because they were negligible in quantity.

7.3. Results and discussion

7.3.1. Environmental impacts on sorghum to FDCA process

Environmental impacts were studied based on purified '1 Kg of FDCA' using TRACI 2.1 method. TRACI has 10 different impact categories with specific units are given in Table 7.2. Seyed et. al studied LCA of biodiesel production using TRACI 2.1 impact method. They were mainly focused on GHG emissions on production of camelina in different regions (Seyed et.al., 2017). TRACI method is widely accepted especially for the environmental impact assessment. The availability of process input data was the challenging factor for inventory analysis. The data variation in the sorghum plant cultivation especially in the water usage and electricity consumption. The water was used in the sorghum plant cultivation was rain water and electricity was used for milling purpose (Ratnavathi et al., 2016).

LCIA category	Unit/Kg
Eutrophication	kg N-eq
Acidification	kg SO2-eq
Fossil fuel depletion	MJ surplus
Global warming	kg CO2-eq
Carcinogenesis	CTUh
Ozone depletion	kg CFC-11-eq
Non carcinogenics	CTUh
Smog	Kg O3 eq
Ecotoxicity	CTUe
Respiratory effects	kg PM2.5-eq.

Table 7.2. Life cycle impact assessment (LCIA) categories.

The model graph of product system was made through OpenLCA software. This product system involves all the inputs and outputs from sorghum plant cultivation, sorghum syrup production, FDCA production and FDCA purification (Figure 7.1). All the unit processes were linked with supply chains in the product system.
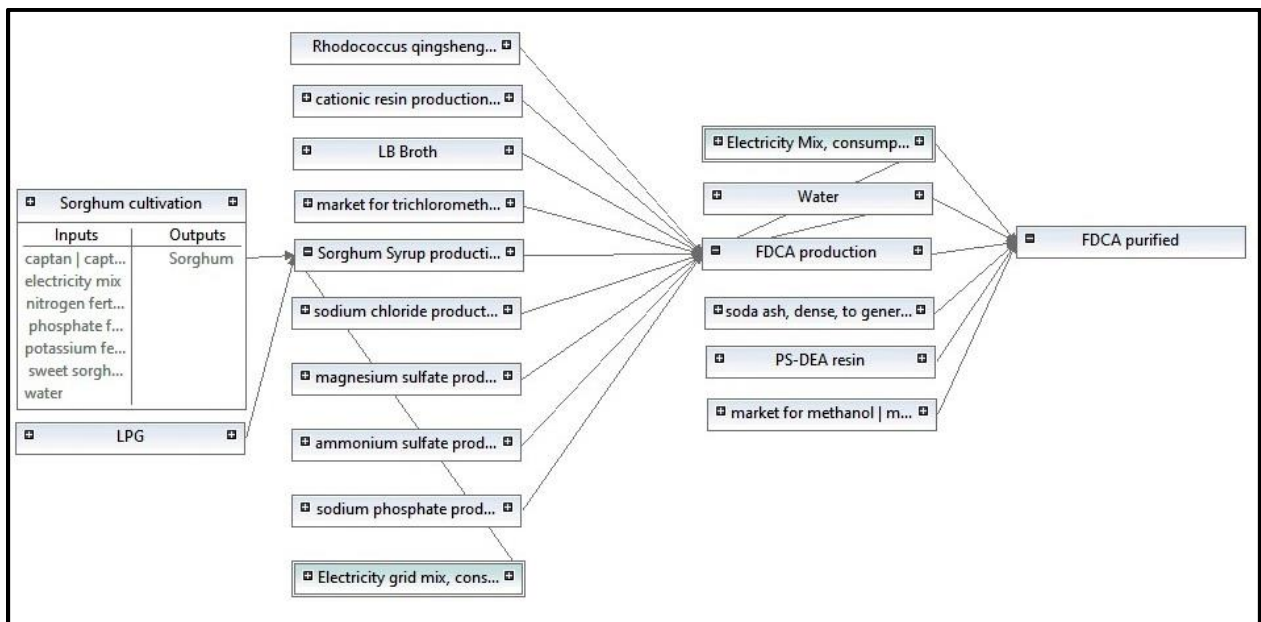
Figure 7.1. FDCA product system- model graph.

The environmental impact analysis was done with 10 impact categories. The complete results are given in table 7.3. In all categories we considered 5% above contributors. The LCIA category includes seven environmental impacts and three health related impacts.

LCIA category	Unit/Kg	LCIA result
Eutrophication	kg N-eq	0.22817
Acidification	kg SO2-eq	0.349
Fossil fuel depletion	MJ surplus	108.01
Global warming	kg CO2-eq	72.404
Carcinogenesis	CTUh	7.23031E-6
Ozone depletion	kg CFC-11-eq	0.00548
Non carcinogenics	CTUh	2.27344E-5
Smog	Kg O3 eq	3.365
Ecotoxicity	CTUe	703.181
Respiratory effects	kg PM2.5-eq.	0.006320

Table 7.3. Life cycle impact analysis results.

7.3.1.1. Global warming

In this study, total of 72.404 Kg of CO_2-eq was observed for the 1 Kg of purified FDCA. The major contributors for the carbon dioxide were polystyrene, chloroform, electricity and others (Figure 7.2). Polystyrene was the major contributor of CO_2; it was used in the PS-DEA resin synthesis for the FDCA purification. As mentioned in chapter 6, the PS-DEA resin can also be reused for the FDCA purification. Chloroform was the next contributor of CO_2; it was mainly used in the HMF purification process. However, the used chloroform can be recycled through distillation and can be reutilized. The electricity usage was unavoidable in the bioprocess (Matthew et.al., 2021) because the major electricity was used for the fermenter. Moreover, organism is aerobic so 'aeration number' had to be considered in the electricity consumption. Eerhart et.al., 2012 was the first to study LCA

on FDCA production. They have used the cradle to grave method for the comparison between non-renewable energy use (NREU) and GHG emissions for the PEF with PET (1 tonne each) production. The study resulted PEF can reduce 40-50% of NREU and 45-55% of GHG emissions. However, the CO_2 emission was low in the current process. High fructose corn syrup was used for the FDCA production. The analysis resulted 0.63 tonne of CO_2 (Eerhart et.al., 2012).
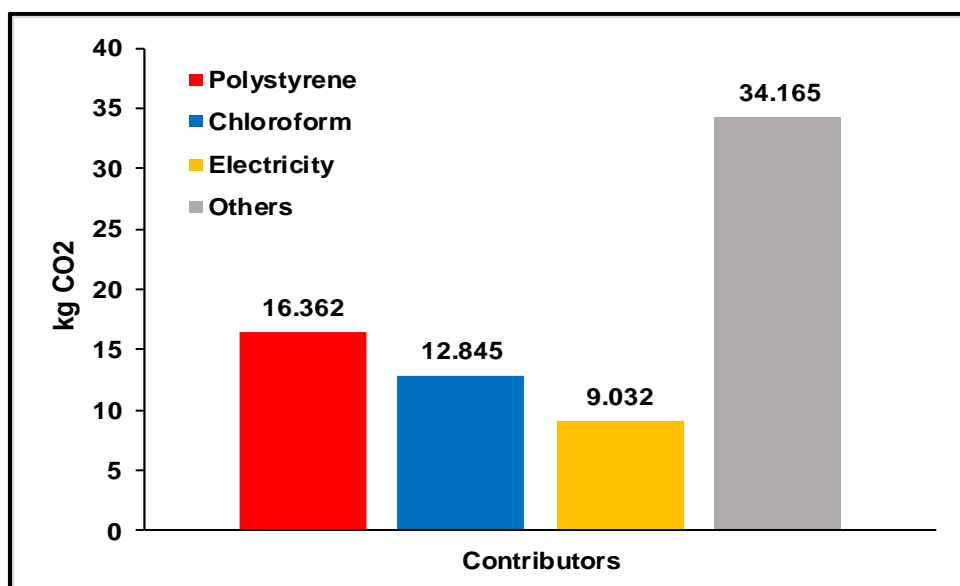
Figure 7.2. Global warming contributors (kg CO_2).

7.3.1.2. Fossil fuel depletion

In the complete bioprocess the total fossil fuel depletion was 108.01 MJ surplus. The major contributors were polystyrene, natural gas, petroleum production and others (from all process together) (figure 7.3). Polystyrene production has contributed 45% of total fossil fuel depletion. Still the energy value was not high compared to the results by bello et. al. where they have 52% fossil depletion contributors in the FDCA purification and separation process (Bello et.al., 2019). The natural gas and petroleum gas were used for

the sorghum syrup production process to concentrate the sorghum juice. Sorghum juice was continuously heated for overnight to avoid fermentation. Therefore, the LPG use cannot be avoided in the process.
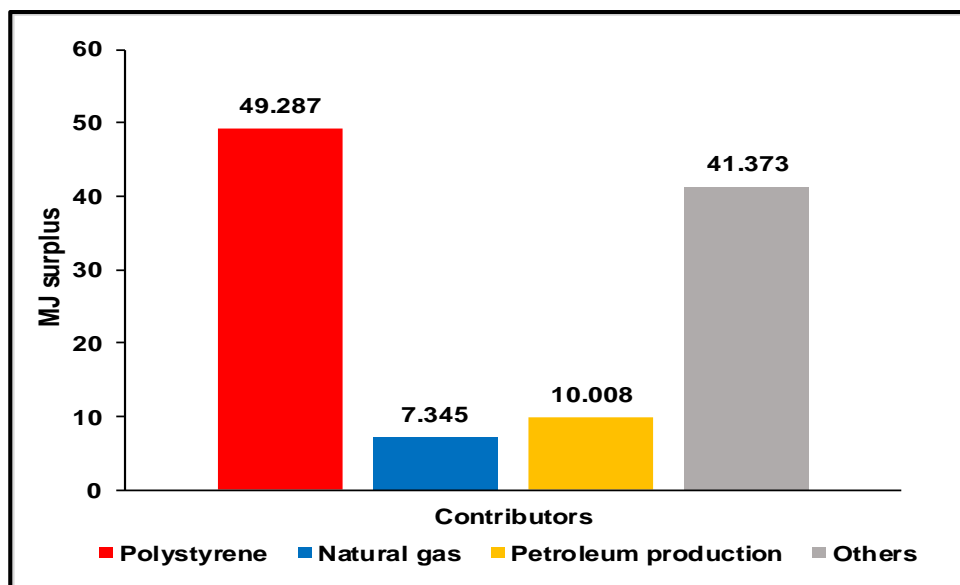
Figure 7.3. Fossil fuel depletion contributors (MJ surplus).

7.3.1.3. Acidification

In this impact analysis, acidification result was 0.349 Kg SO_2 eq (figure 7.4). Compared all other categories it is very negligible in quantity. The result was almost same as camelina biodiesel production in different regions in the northwestern USA (Seyed et al., 2017). The major contributors were sulphuric acid, polystyrene, electricity mix, heat production, natural gas production and others (from all unit processes).

7.3.1.4. Ecotoxicity

Most of the LCA studies were focused on eco toxicity especially in the aquatic system. In general, the major contributors are zinc, copper, antimony, nickel etc. In case of sorghum to FDCA process, the major contributors were sodium phosphate, cationic resin, chloroform, LB broth and others. The total of 703.18 CTUe (Comparative Toxic Units ecotoxicity) was quantified from this study. These all contributors were come under FDCA production and purification process. The contribution tree graph was given in figure 7.5. A recent study on LCA analysis on ecotoxicity was conducted on marine plastic impacts on aquatic biota (Yibo et.al., 2022). Also LCA study of FDCA production from lignocellulosic biomass was analyzed 47% of marine ecotoxicity, which is very critical for the environment (Bello et.al., 2019).

7.3.1.5. Smog

The combination of smoke and fog will be termed as smog; it is a mixture of pollutants which will be primarily made up on ground level. In this study we quantified the smog (3.365 Kg O3 eq). The major contributors were polystyrene production, electricity mix, sorghum production and others (Figure 7.6). There are no reports on smog quantification in LCA analysis of FDCA. As mentioned earlier the polystyrene was used in the FDCA purification and electricity for FDCA production. Other impact categories such as eutrophication and ozone depletion were quantified 0.228 Kg N eq and 0.0054 Kg CFC-11-eq respectively.
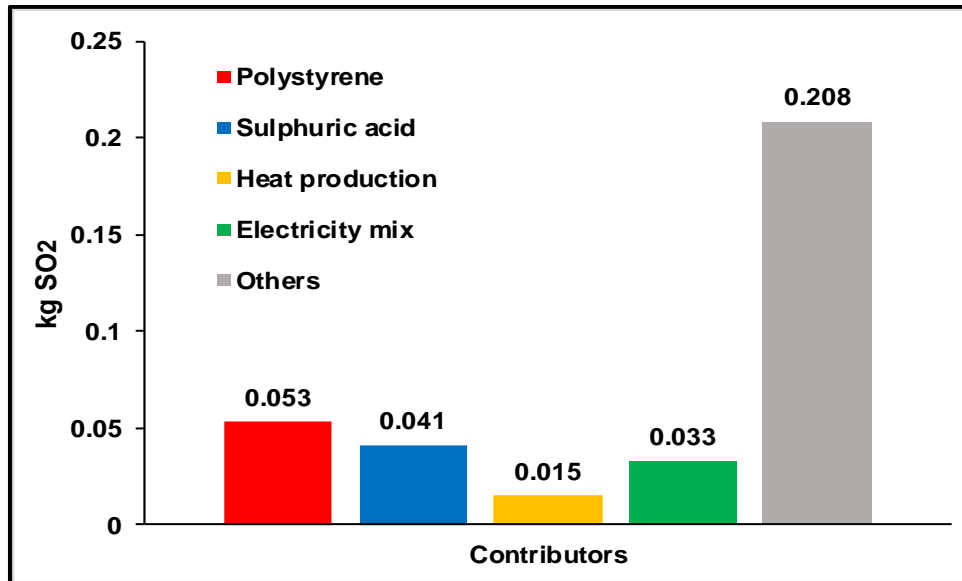
Figure 7.4. Acidification contributors (Kg SO_2 eq).
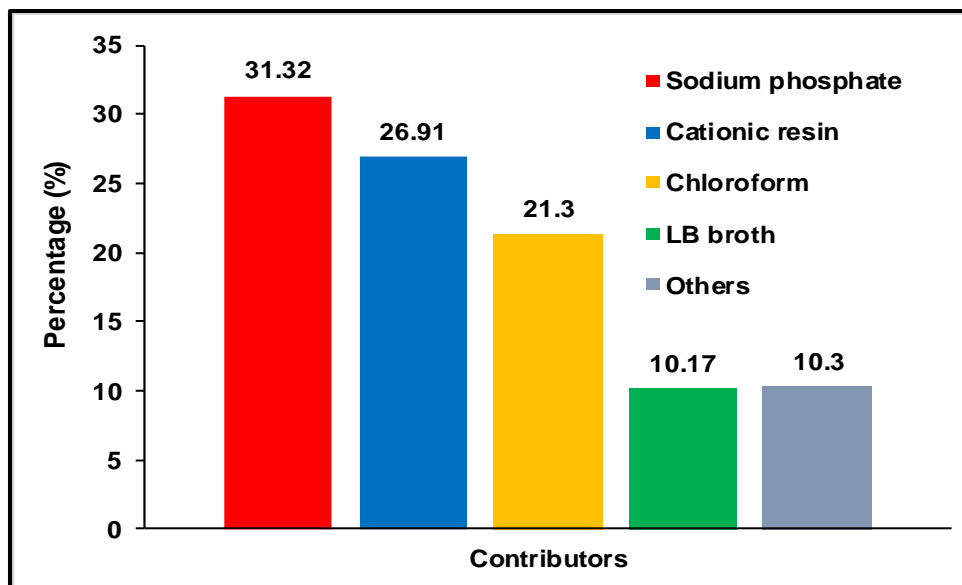
Figure 7.5. Contribution tree of ecotoxicity (%).
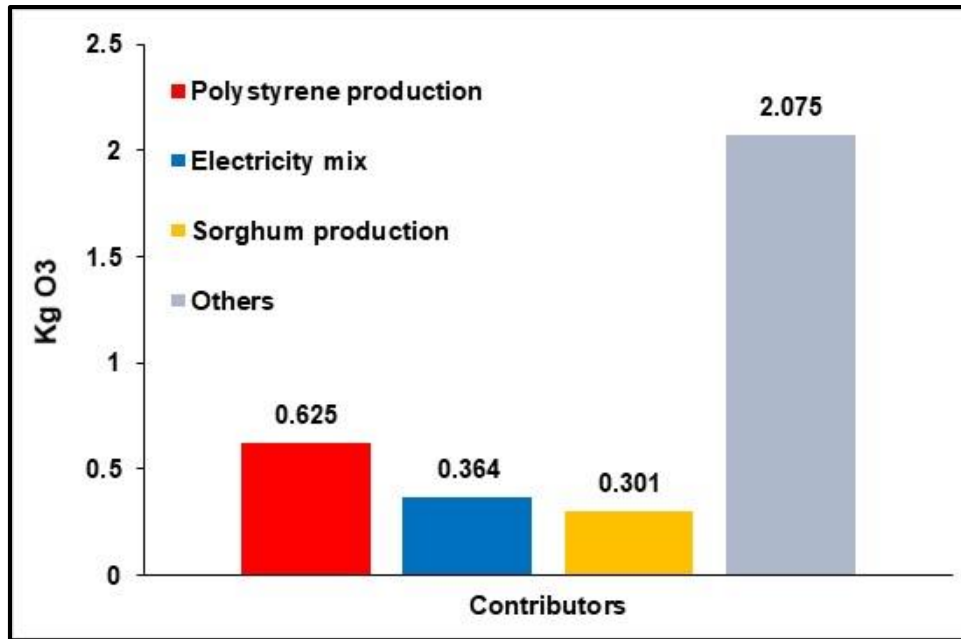
Figure 7.6. Contributors for the smog (Kg O3 eq).

7.3.2. Human health impact on sorghum to FDCA production

There are mainly three human health related impact categories such as carcinogenics, non carcinogenics and respiratory effects. The carcinogenics and non carcinogenics were quantified as 7.23031E-6 CTUh (comparative toxic unit for human toxicity impacts) and 2.27344E-5 CTUh respectively. In both cases the major contributors were from FDCA production and purification. Sodium phosphate production, chloroform and cationic resin were the main contributors (Figure 7.7). Apart from carcinogenics, the processes such as sorghum syrup production and FDCA production (LB broth) were contributed in the non carcinogenics. The respiratory effect from the sorghum to FDCA process was 0.0063 Kg PM 2.5 eq (Fine particulate matter) and concluded that the process is not causing respiratory effects.
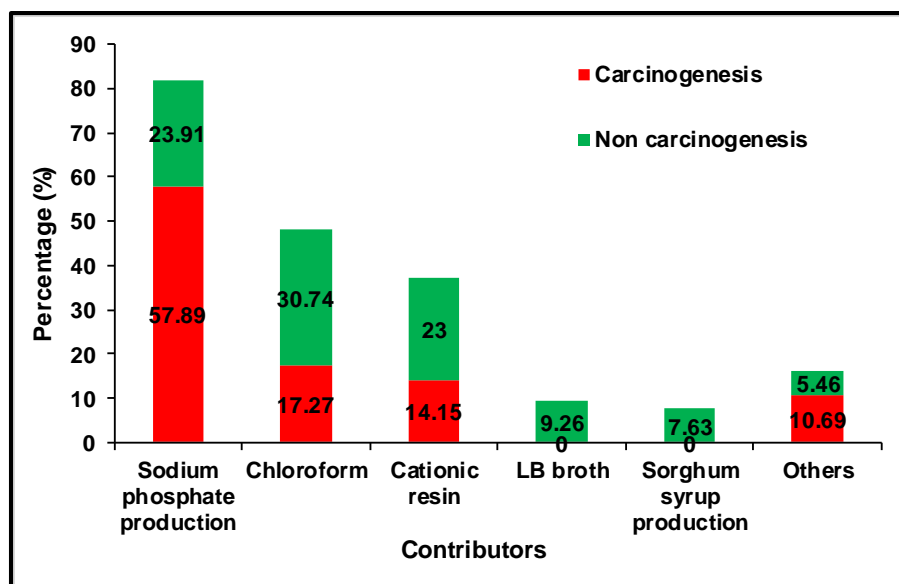
Figure 7.7. Contribution tree of carcinogenics and non carcinogenics (%).

7.3.3. Comparative study on contributors of environmental impacts

The major contributors in all environmental impact categories were studied and compared based on their contribution percentage (Table 7.4). Most important five impacts were selected such as global warming (GW), fossil depletion (FD), ozone depletion (OD), eco toxicity (EC) and carcinogenesis (CG). The major contributors were polystyrene, chloroform, cationic resin, electricity and sodium phosphate (figure 7.8). Most of the contributors were from HMF and FDCA purification (chloroform and polystyrene). The major electricity usage was for fermenter, electrical heating (HMF synthesis) and centrifugation. A recent study by Kim et. al., resulted electricity has 30% contribution on fossil fuel depletion for biomass to FDCA synthesis (Kim et al., 2020). Among all contributors, chloroform was the most contributing factor for all environmental impacts. Especially, the 99% contribution in ozone depletion. However, as mentioned earlier the chloroform can be recovered after the process and reutilized in future. The sodium

phosphate was the next contributor; it was mainly used in the FDCA production (QMSM). The sodium phosphate contributes 57.89% in the carcinogenesis, still it is not directly involved in the sorghum to FDCA process (sodium phosphate production process). The polystyrene was contributed in the global warming and fossil depletion. We used polystyrene in the PS-DEA (polystyrene Diethanolamine) resin synthesis for FDCA purification. As mentioned earlier the resin can be reused. The cationic resin was used for HMF synthesis (sorghum syrup derived sugar dehydration). It contributing to the ecotoxicity and carcinogenesis (26.91% and 14.15% respectively).

The comparative study clearly indicating that environmental impacts can be reduced by changing raw materials used in the HMF purification and FDCA purification. Also a direct comparison studies between two process cannot be performed due to variations in the methodology of each process.

Contributors	GW (%)	FD (%)	OD (%)	ET (%)	CG (%)
Poly styrene	22.5	45.5	0	0	0
Chloroform	17.7	7.0	99	21.3	17.27
Cationic resin	6	6.0	0	26.91	14.15
Electricity	12.4	0	0	0	0
Sodium phosphate	8.0	6.92	0	31.32	57.89

Table 7.4. Environmental impact contribution (percentage).
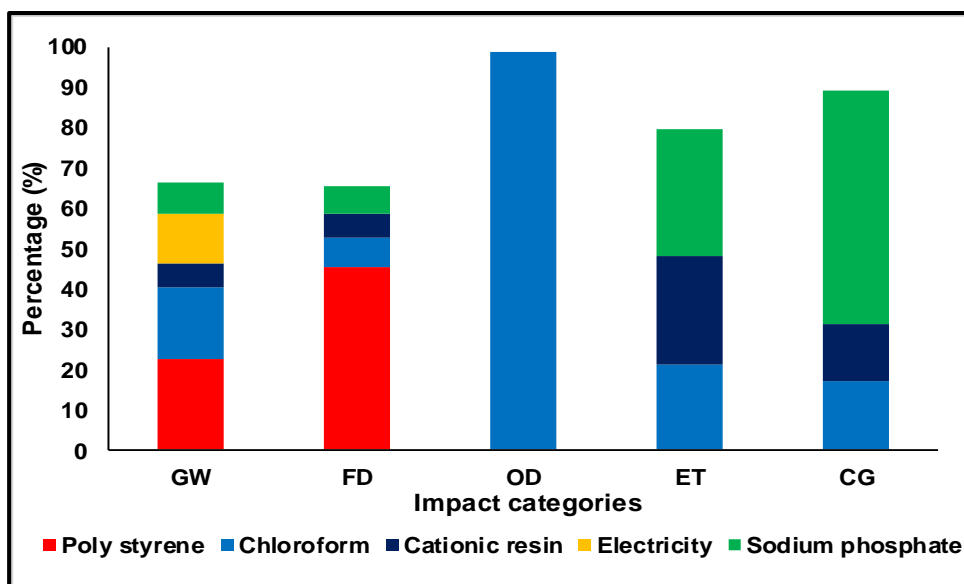
Figure 7.8. Environmental impacts and major contributors (%).

7.4. Conclusion and future perspectives

The environmental impact assessment of complete process from sorghum biomass to FDCA production was studied using LCA (functional unit; 1 Kg of purified FDCA). The different environmental impact categories were quantified. In this research, the TRACI 2.1 method was used to quantify the environmental impacts and results showed for GHG emission was 72.40 Kg of CO_2. The fossil fuel depletion and acidification were 108.1 MJ surplus and 0.349 Kg SO_2 respectively. The ecotoxicity was 703.18 CTUe. Based on the LCIA analysis, the major contributors for the environmental impact categories were chloroform, polystyrene, cationic resin etc. Most of the contributors were came from HMF and FDCA purification processes. However, the complete process was found to be sustainable based on the results.

The research on FDCA synthesis from biomass should be continued based on environmental aspects. Especially, solvents and resins for HMF and FDCA purification

has to be replaced to with more environmental friendly substances. Also The uncertainty analysis has to be done because of data variability would vary the environmental impacts. The findings of this study could results to enhance the bio-based process of FDCA in future.

Chapter 8

Summary and conclusions

8.1. Summary and conclusion

The technologies and policies for renewable fuels and platform chemicals are demanding because of greenhouse gas emissions, shifting market prices and dwindling fossil fuel sources. Many of the value added chemicals are currently made from petroleum can be made from biomass. Among them 2,5-Furandicarboxylic acid (FDCA) has huge potential market especially in the polyester industry. The sustainable process for FDCA provided a platform to switch from polyethylene terephthalate (PET) to polyethylene furanoate (PEF). Compared to chemical based FDCA, biobased FDCA production has gained worldwide attention. The most suitable process is through biotransformation (bioprocess); as environmental friendly microorganisms mediate the process as biocatalysts.

The objectives and experimental aspects of the work carried out in this study reflects the dedication towards the development of a biobased process for FDCA production from sorghum syrup.

The significant finding of the study were given below;

- The HMF synthesis from sorghum syrup

The sorghum syrup was selected as biomass for the HMF production based on high amount of sucrose content. In a single step, sucrose was hydrolyzed in to fructose and glucose and the fructose was dehydrated to synthesize HMF using seralite SRC 120 (strong cationic resin). All the reaction parameters were optimized and finally HMF was produced from sorghum syrup with 69% yield. Sucrose component of sorghum syrup was chemo-catalytically converted to HMF in water albeit with low conversion and selectivity.

72 mg of HMF was obtained from 1 ml of sorghum syrup. The synthesized HMF was recovered and purified using chloroform extraction. Moreover, the unreacted sugars (majorly glucose) was utilized for the cell enrichment as booster. The main objective of the study was the complete utilization of biomass.

- Isolation and screening for FDCA producing microbes;

Isolation was a continuous process; we have isolated more than 300 strains. Based on their FDCA production 10 strains were screened. From the selected strains, two potential isolates were selected for the further study (*Acinetobacter oleivorans* S27 and *Rhodococcus qingshengii* C27). All the physical parameters were optimized. Comparatively, *Rhodococcus qingshengii* C27 strain shows high FDCA yield (0.94 g/L) and good HMF tolerance than *Acinetobacter oleivorans* S27 (0.25 g/L). Thus *Rhodococcus qingshengii* C27 was selected for the further research.

- Physical parameters optimization and development of whole-cell biocatalyst;

Rhodococcus qingshengii C27 was isolated from Sikkim, India. The strain showed high HMF tolerance up to 240 mM. The physical parameters were optimized (cell age: 24 h, pH: 7.0, temperature: 30⁰ C). The media was modified specifically for *R. qingshengii* C27 and termed as Qingshengii mineral salt media (QMSM). Through batch mode biotransformation, 0.94 g/L of FDCA was produced from 1.0 g/L of HMF with complete HMF conversion.

- Biotransformation strategies for the improved production of FDCA;

Effect of other carbon sources like glucose and glycerol were studied. It was confirmed that only resting cells were involved in the HMF to FDCA biotransformation not with the

growing cells. After batch biotransformation, fed-batch strategies were performed. Compared to batch mode, fed-batch biotransformation was improved the FDCA yield, 1.604 g/L of FDCA was produced from 2.0 g/L of HMF concentration. Since resting cells were involving in the FDCA production, the repeated batch biotransformation was performed with the production media replacing. In this study, we observed 4.178 g/L of FDCA was produced from 5.0 g/L of HMF with 83.5% of FDCA yield.

- Sorghum syrup derived HMF to FDCA process demonstration in fermenter;

The sorghum syrup derived HMF biotransformation was successful in the flask level (94% FDCA yield). In fermenter, total of 3.0 g/L of HMF was fed and total of 2.1 g/L of FDCA was obtained.

- Separation and purification of FDCA;

Separation of FDCA form the fermenter broth was carried out using PS-DEA functionalized resin mediated adsorption (94%). The PS-DEA resin was synthesized and characterized by FTIR and surface are- pore size distribution. Adsorbed FDCA was desorbed by 1 N of sodium hydroxide. FDCA was recovered by neutralizing the pH followed by concentration of water in rotavac. After the evaporation, the residue obtained was reconstituted in methanol to remove the salt formed during the neutralization process. Thus FDCA was recovered using the synthesized resin with 94% purity. The purified FDCA was analyzed in HPLC and confirmed by NMR.

- Life cycle assessment (LCA) of complete process of Sorghum to FDCA;

The environmental impacts of whole process were analyzed by comparative LCA. The method followed was 'Cradle to gate'. 'The purified 1 Kg of FDCA' at the factory gate was

used as functional unit. The open LCA software was used for the analysis. The TRACI 2.1 impact assessment method was used. The complete process was assessed and quantified. The complete process was found to be sustainable based on the quantification of GHG emissions, fossil fuel depletion, acidification, smog, ecotoxicity. The uncertainty analysis was also done for the data variability.

So with the experimental results we can conclude that a bio-based process with optimized upstream and downstream processes for 2,5-Furandicarboxylic acid production from sorghum syrup. The environmental impact analysis of the complete process was successful and understood the changes in future for the sustainable process.

8.2. Significance of the study

FDCA as a monomer, is well known for the synthesis of polyethylene furanoate (PEF) by polycondensation reaction. Also FDCA has several applications including polyesters. Hence FDCA is a potential value added chemical with an industrial significance. In this study, the focus is on developing an efficient bio-based process for the FDCA production using novel isolate *Rhodococcus qingshengii* C27. Significant findings of the study as follows.

I.   Selection of a potent FDCA producer *Rhodococcus qingshengii* C27, with substrate (5 g/L) and product (4.1 g/L). Strain shows high HMF tolerance (240 mM).

II.   Complete utilization of sorghum syrup biomass by converting fructose to HMF via strong cationic resin mediated dehydration and unutilized glucose was used for the cell enrichment/boosting.

III. A novel functionalized resin PS-DEA was synthesized and characterized for the FDCA recovery with 94% adsorption rate.

IV. Environmental impact assessment of the complete study was performed by LCA analysis. The major contributors of environmental impacts were from HMF and FDCA purification processes. Therefore, these process requires more research towards green approach for the sustainable process.

8.3. Future perspectives of the study

The novel isolate *Rhodococcus qingshengii* C27 can be used as an industrial host for the FDCA production. FDCA production can be improved by identification and overexpression of key gene(s) involved in the HMF to FDCA pathway. Repeated batch HMF biotransformation can be improved in a membrane bioreactor. HMF synthesis can be improved by a reusable strong solid catalyst which can effectively convert sucrose to HMF. Technoeconomic analysis (TEA) would give a new dimension for economic feasibility.

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ANNEXURE I- Media Composition

1. Luria Bertani Medium (LB) gL^{-1}	
Tryptone	10
NaCl	10.0
Yeast Extract	5.0
Agar (for solid medium)	15.0
Adjust the pH to 7.0 by 1 N HCl/ NaOH, and sterilized by autoclaving	

2. Potato Dextrose Agar (LB) gL^{-1}	
Potatoes	4.0
Dextrose	20.0
Agar (for solid medium)	25.0
Final pH 5.6 ± 0.2 and sterilized by autoclaving	

ANNEXURE II-List of instruments & software

Instruments	Model and Country
Autoclave	Labline, India
Balance	Mettler Toledo, Mumbai, India
Centrifuge	Kubota 7780, Japan; Eppendorf, Germany; MICRO CL 17, Thermo Fisher Scientific, India
Cold room	Rinac Pvt. Ltd, India
Deep freezer	Elanpro, India; Haier, China
DNA sequencer	3500 Genetic Analyzer, Applied Biosystems, Hitachi, Japan
Electrophoresis unit	Bio-Rad, USA
Fermenter	Infors Minifors, Switzerland
Fluorescence spectrophotometer	Infinite M200 PRO microplate reader, Switzerland
Gel documentation	ChemiDoc, Biorad, USA
Hot air Oven	Kemi Instruments, India
HPLC	Shimadzu, Japan
Incubator	Infors Ht, Switzerland
Laminar air flow chamber	Labline, India
Nanodrop spectrophotometer	ND1000, Thermo Fisher Scientific, India
pH meter	Eutech, Thermo Fisher Scientific, India
PCR machine	Bio-Rad, USA; Eppendorf, Germany
Sonicator	Vibra cell, Sonics and materials Inc., USA
Thermostat	Eppendorf, USA
UV-Vis Spectrophotometer	UV-160A, Shimadzu, Japan,
Software	**Version**
Minitab	Minitab Inc. Ver. 17.1.0
OpenLCA	v 1.10.3
SuperPro	12.01.1002

ANNEXURE III-AcSIR course work

SI No.	Level 100	Course No. and Title	Status
1.	BIO-NIIST-1-0001	Biostatistics	Completed
2.	BIO-NIIST-1-0002	Bioinformatics	Completed
3.	BIO-NIIST-1-0003	Basic Chemistry	Completed
4.	BIO-NIIST-1-0004	Research Methodology, communication/ ethics/ safety	Completed
	Level 200		
1.	BIO-NIIST-2-4101	Biotechnology and Instrumentation	Completed
2.	BIO-NIIST-2-4102	Protein Sciences and Proteomics	Completed
3.	BIO-NIIST-2-4104	Basic Molecular Biology	Completed
	Level 300		
1.	BIO-NIIST-3-4101	Seminar Course	Completed
2.	BIO-NIIST-3-4104	Bioprocess Technology	Completed
3	BIO-NIIST-3-4106	Enzymology and Enzyme Technology	Completed
	Level 400		
1.	BIO-NIIST-4-0001	Project proposal	Completed
2.	BIO-NIIST-4-0002	Review	Completed
	Level 800		
1.	BIO-NIIST-4-0003	Project work	Completed

Abstract of the thesis

Name of the Student:	**Godan T K**	Registration No :	**10BB19J39003**
Faculty of study :	**Science**	Year of submission :	**2022**

AcSIR Academic Centre/CSIR Lab :	CSIR-NIIST, Thiruvananthapuram
Name of the supervisor (s) :	Dr. Binod P

Title of the thesis:	Bioprocess designing for the synthesis of 2,5 Furandicarboxylic acid and its life cycle assessment

The 2,5- Furandicarboxylic acid (FDCA) is one of the top 12 value added chemical which can be synthesized from the biomass. In this study, the two-step process of chemo-biocatalytic approach was adapted for the conversion of sorghum syrup derived fructose to FDCA. Sucrose in the sorghum syrup was chemo-catalytically hydrolysed to glucose and fructose followed by the dehydration of fructose to 5-hydroxymethyl furfural (HMF) using seralite SRC120 (cationic resin) as heterogeneous solid acid catalyst with 69% of HMF yield. Based on the FDCA yield, *Rhodococcus qingshengii* C27 was selected for the further study. Through batch biotransformation resulted 94% FDCA yield (72 hours, 30 °C, pH 7.0) with complete HMF conversion. Later FDCA production was improved by the repeated batch, FDCA yield was 4.178 g/L from 5.0 g/L of HMF. The overall FDCA yield was 83.5%. The sorghum syrup derived HMF to FDCA was demonstrated in the fermenter level. The FDCA was recovered and purified using PS-DEA resin mediated adsorption. Finally, the environmental impacts of complete process of sorghum to FDCA was studied using life cycle assessment. This is the first report on the conversion of biomass derived sugars to FDCA using a highly HMF tolerant novel biocatalyst, *R. qingshengii* C27 with promising green chemistry operations. The discussed process is a state of the art approach for FDCA production from biomass.

List of publications

Publications emanated from the thesis

1. **Tharangattumana Krishnan Godan**, Rajesh, R.O, Phukon C. Loreni, Amit Kumar Rai, Dinabandhu Sahoo, Ashok Pandey, Parameswaran Binod. 2019. Biotransformation of 5-hydroxymethylfurfural by *Acinetobacter oleivorans* S27 for the synthesis of furan derivatives.

   Bioresource Technology, 288, 88-93, doi: 10.1016/j.biortech.2019.02.125

2. **Tharangattumana Krishnan Godan**, Leena Devendra, Maria Paul Alphy, R O Rajesh, Narisetty Vivek, Parameswaran Binod. 2022. Chemo-catalytic synthesis of 5-Hydroxymethyl furfural from sorghum syrup derived fructose. Fuel (under revision).

Other publications

3. Rajendran Omana Rajesh, **Tharangattumana Krishnan Godan**, Amit Kumar Rai, Dinabandhu Sahoo, Ashok Pandey, Parameswaran Binod. 2019. Biosynthesis of 2,5-furan dicarboxylic acid by *Aspergillus flavus* APLS-1: Process optimization and intermediate product analysis. Bioresource Technology, 284, 155-160, doi: 10.1016/j.biortech.2019.03.105

4. R O Rajesh, **Tharangattumana Krishnan Godan**, Ashok Pandey, Parameswaran Binod. 2018. Whole cell based biocatalytic production of 2, 5- furan dicarboxylic acid. Indian Journal of Experimental Biology (IJEB). 56, 493–497

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6. Phukon C. Loreni, Rounak Chourasya, Megha Kumari, **Tharangattumana Krishnan Godan,** Dinabandhu Sahoo, Parameswaran Binod, Amit Kumar Rai. 2020. Production and characterisation of lipase for application in detergent industry from a novel *Pseudomonas helmanticensis* HS6. Bioresource Technology. 309, 123352, doi: 10.1016/j.biortech.2020.123352.

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9. Mohammed RafiqKhan, **Godan Tharangad Krishnan**, Ranjini K, Suresh SN**,** Umadevi pongiya, Yalaga rama rao. 2014. Micronucleus assessment as a biomarker and susceptibility to DNA damage in workers occupationally exposed to pesticides. Biomedical research and therapy.1(3):78-84, ISSN 2198-4093.

10. Mohammed RafiqKhan, Ranjini K, **Godan T K**, Suresh SN**,** Umadevi pongiya, Yalaga rama rao.2014. Pharmacognostic Study and Phytochemical Investigation of Lycopersicon esculentum (Tomato) Flower Extracts. Research Journal of Pharmaceutical, Biological and Chemical Sciences. 5(3):1691-1698, ISSN: 0975-8585.

Under preparation

1. **Tharangattumana Krishnan Godan**, Ganti Suryanarayana Murthy and Parameswaran Binod ; Life cycle assesment of FDCA production from sorghum biomass.

2. **Tharangattumana Krishnan Godan**, R O Rajesh and Parameswaran Binod ; The improved FDCA production using *Rhodococcus qingshengii* C27.

Conference proceedings

1. Production of 2,5-Furandicarboxylic acid from sorghum syrup by chemo catalytic dehydration and whole-cell biotransformation" presented in international conference, BREECH 2021 held on December 1-4, CSIR-IIP, Dehradun, India (2021), **Tharangattumana Krishnan Godan**, Leena Devendra, Maria Paul Alphy, R O Rajesh, Narisetty Vivek, Raveendran Nair Dileep, Phukon C. Loreni, Amit Kumar Rai, Ashok Pandey, Parameswaran Binod.

2. "An Efficient Whole-cell Biocatalytic Production of 2,5-Furandicarboxylic Acid by a novel Strain *Acinetobacter Oleivorans* S27" presented at BioSD-18 held at CSIR-IICT, Hyderabad on November 21-25,2018.

Corrigendum

Corrigendum to "Biotransformation of 5-hydroxymethylfurfural by Acinetobacter oleivorans S27 for the synthesis of furan derivatives" [Bioresour. Technol. 282 (2019) 88–93]

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The authors would like to apologise for any inconvenience caused.

Biotransformation of 5-hydroxymethylfurfural by *Acinetobacter oleivorans* S27 for the synthesis of furan derivatives

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ARTICLE INFO

ABSTRACT

Hydroxymethylfurfural (HMF) is an industrially important chemical which is a starting material in the production of plenty of platform chemicals. In this study, a complete biotransformation of HMF was achieved using a novel isolate, *Acinetobacter oleivorans* S27. This strain could tolerate up to 3000 mg/L of HMF concentration and convert to other furan derivatives. The conversion products includes high-value chemicals like 5-hydroxymethyl-2-furancarboxylic acid (HMFCA), a known interleukin inhibitor and 2,5-furan dicarboxylic acid (FDCA), an alternate of terephthalic acid in polyester industries. The biotransformation efficiency was found to be 100%, as there is complete conversion of HMF to other chemicals. Most importantly, it is an environmental friendly process for the production of furan derivatives.

1. Introduction

The use of lignocellulosic biomass as a feedstock for the fermentative production of biofuels and platform chemicals is one of the major topics of research by the scientific community. To reduce the use of fossil resources by increasing the availability of renewable feedstock is the major research interest. Fermentable sugars could be released from lignocellulosic biomass using different approaches over the years. Disruption of crystalline structures of the cellulose fibers and removal of lignin can be done by efficient biomass pretreatment (Taherzadeh and Karimi, 2008). For that dilute acid hydrolysis is one of the effective and accepted methods for large-scale applications. During acid pretreatment several inhibitors are generated including furanic aldehydes which inhibit the efficiency of pretreatment. Among these inhibitors furfural and hydroxymethylfurfural (HMF) are more dangerous for the further fermenting organisms. HMF is having an aldehyde functional group which shows toxicity and can decrease the fermentative productivity based on the nature of lignocellulosic hydrolysate (Almeida et al., 2009; Palmqvist et al., 2000; Thomsen et al., 2009). Growth and metabolism of microorganisms, which are involved in the fermentation processes also inhibited by these furanic aldehydes (Klinke et al., 2004). Prevention of inhibitors is must for the better biomass pretreatment as

well as the increase of product yield. It is evident that by-product formation cannot be prevented completely in an economical way especially for a industrial scale of production (Wierckx et al., 2011). For the removal of inhibitors, different other separation techniques like solvent extraction and biological detoxification (Mussatto et al., 2004) has to be followed. Several studies indicate that furanic aldehydes can be degraded using microorganism. Some microbes can tolerate the high concentration furfural and HMF (Petersson et al., 2006). These discoveries made a great impact on the area of microbes, which are involved in the furfural and HMF degradation. The degradation pathway and enzyme involved in degradation of HMF by *Pseudomonas putida* strains Fu 1 and F2 has been proposed (Koenig et al., 1989, 1990). Followed these, research specifically on HMF and furfural degradation enabled the molecular identification and characterisation of *Cuprivadus basilensis* HMF 14. Furfural and HMF metabolizing pathway in *Pseudomonas putida* also have been proposed (Koopman et al., 2010a,b).

Microorganisms are known for the furanic aldehyde degradation. Interestingly, in bacteria most of them belong to gram negative community and aerobic. Because of the toxicity of furanic compounds, microbes evolved several types of defensive mechanisms including oxidation and/or reduction to carboxylic acids and furanic alcohol forms (Wierckx et al., 2011). Even though HMF is a toxic substance, it

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can be used as a platform chemical for the production of variety of top value added products such as 5-hydroxymethyl 2-furancarboxylic acid (HMFCA), 2,5-Furandicarboxylic acid (FDCA) and 2,5-diformylfuran (DFF). Each derivatives of HMF has its own property as a capable biochemical. Precisely FDCA is considered as a biomass derived chemical. FDCA can replace terephthalic acid, which is a petrochemical widely used as a precursor for the production of polyesters. In addition, polyethylene furanoate (PEF) is a pure biopolymer which can completely replace the polyethylene terephthalate (PET). In this scenario FDCA is considered as one of the top 12 value added chemicals synthesized from biomass (Werpy and Peterson, 2004). Currently FDCA is mainly produced by chemical routes and it needs stoichiometric amount of temperature, pressure and organic solvents which leads to high energy consumption and also huge environment pollution. HMFCA is formed by oxidisation of formyl group of HMF. It is a platform chemical which is used in polyester production. It is a known interleukin inhibitor and had an antitumor activity (Hirai et al., 1984; Braisted et al., 2003; Munekata et al., 1981). HMFCA production is also mainly with chemical approaches using various noble metal catalysts (Davis et al., 2011, 2014; Casanova et al., 2009).

To switch from chemical approaches to biological, more precisely invention of bio-catalytic oxidation has always a great attention (Hollmann et al., 2011). It has lots of advantages including environment friendly, high selectivity compared to chemical approach and formation of less impurities. Several metabolic engineering approaches are going on for the efficient synthesis of FDCA. In *Pseudomonas putida* S 12, hmfH gene was introduced which is isolated from *Cuprivadus basiensis* HMF14 for the bio-catalytic production of FDCA (Koopman et al., 2010a,b). Recently, a series of work carried have been out in *Raoultella ornitholytica* BF60 towards the better production of FDCA from HMF (Hossain et al., 2017; Yuan et al., 2018a,b,c). In the case of bio-catalytic production of HMFCA very few reports are there in best of our knowledge. Among this whole-cell bio-catalytic oxidation of HMF to produce HMFCA using *Comamonas testosteroni* SC1588 has been reported by Zhang et al. (2017). They got approximately 100% conversion of substrate. Additionally HMFCA production could be improved while addition of histidine with fine pH tuning at higher concentration of substrate (Zhang et al., 2017). In the present study, a novel strain *Acinetobacter oleivorans* S27 was isolated from high altitude soil samples of Sikkim Himalaya in India. This isolate is able to grow in presence of HMF and could able to transform HMF to FDCA and HMFCA. By statistical optimization approach, this organism could able to convert 100% HMF to FDCA and HMFCA. To our knowledge, globally this is the first report of *Acinetobacter* sp. involved in the HMF degradation and synthesis of top value added HMF derived platform chemicals like FDCA and HMFCA.

2. Materials and methods

2.1. Isolation and enrichment of 5-HMF to 2,5-FDCA biotransforming microbes

Isolation of microorganisms was carried out from soil samples collected from 25 different locations of Sikkim State in India with altitude ranging from 1120 to 4272 m above sea level. To isolate the potential microorganisms for the production of FDCA from 5-Hydroxymethyl furfural, the collected soil samples were pre-cultured on minimal salt solution (MSS composition: g/l: MgSO$_4$·7H$_2$O, 0.2 g; CaCl$_2$·2H$_2$O, 0.002 g; KH$_2$PO$_4$, 0.5 g; K$_2$HPO$_4$, 0.5 g; NH$_4$Cl, 0.5 g; Trace elements solution 10 mL/l, Trace element composition mg/l: FeSO$_4$·7H$_2$O, 300 mg; MnSO$_4$·H$_2$O, 50 mg; CoCl$_2$·6H$_2$O, 34 mg; Na$_2$MoO$_4$·2H$_2$O, 34 mg; ZnSO$_4$·7H$_2$O, 40 mg; CuSO$_4$·5H$_2$O, 50 mg) with varying concentrations of HMF (500 mg/l–2500 mg/l) for 24 h. This was followed by spread plating of pre-cultured samples following standard serial dilution procedure on four different media (MSS agar, nutrient agar, potato dextrose agar and yeast extract peptone dextrose agar)

supplemented with different concentration of HMF (500 mg/l–2500 mg/l). The plates were incubated for a maximum of 48 h at 28 °C and 37 °C. Morphologically distinct isolates were streaked on fresh plates for obtaining single colony. Individual pure cultures were screened further for the production of FDCA.

2.2. Molecular identification of bacterial strain

Genomic DNA was isolated using known traditional method for gram negative bacteria. 16S rRNA fragment amplification was done by PCR using universal primers 27F and 1492R (5′-AGAGTTTGATCMTG GCTCAG-3′ and 5′-GGTTACCTTGTTACGACTT-3′ respectively). The amplified 16SrRNA gene was purified by QIA quick gel extraction kit (Qiagen). Later purified PCR products were sequenced (ABI 3130 automated DNA sequencer). Sequence has been deposited in GenBank under accession No.MK359024.

2.3. Preparation of whole-cell biocatalyst

For the preparation of pre-inoculum, *Acinetobacter oleivorans* S27 were grown at 30 °C for 12 h on a rotary shaker (200 rpm) in a Luria-Bertani (LB) medium (100 mL). From the pre-inoculum 1% (v/v) were transferred to freshly prepared LB medium (100 mL) and kept for 27 h for incubation with same conditions. After 27 h of incubation, cells were harvested by centrifugation at 12,000 RPM for 15 min at 4 °C. Cells were washed twice with sterilized water and phosphate buffer (pH 8) to remove complete media components. The cell pellet was then transferred to mineral salt media (50 mL) supplemented with 500 mg/L HMF as a sole carbon source. The production media is kept for further analysis at 30 °C on rotary shaker (200 rpm). The supernatant from the reaction mixture was withdrawn in every 24 h and analysed using high performance liquid chromatography (HPLC).

2.4. Analytical methods

HMF and its derivatives (HMFCA, HMF alcohol, DFF, FFCA, FDCA and furoic acid) were analysed using gradient HPLC (Shimadzu, Japan) with ultraviolet detector on a C-18 column (250 mm × 4.4 mm × 5 μm) at 40 °C temperature. The two mobile phase solvents were A-acidic water (0.02 N H2SO4 and deionized water) and B-Acetonitrile. Initially start with mobile phase A(100%) for 1 min, then 94% of A and 6% of B for next one min. From 2nd min to 15th min, 98% of A and 2% of B and final 5 min running with 94% of A and 6% of B. The flow rate for all the compounds were 1.0 mL/min and total running time was 20 min. The UV wavelength for the detection of HMF and FFCA was 280 nm, HMFCA and furoic acid was fixed at 254 nm and FDCA, HMF Alcohol and DFF at 263 nm, 225 nm and 290 nm respectively. The OD600 was measured using UV/Vis spectrophotometer (UV-1601 Shimadzu, Japan) at 600 nm.

2.5. Process development by optimization of parameters

All the parameters were optimized using 50 mL reaction mixture. For the optimization studies, priority was given to FDCA production. To optimise the temperature, the reaction was run at variable temperature at 30 °C and 37 °C with pH 7 and 200 rpm. For pH optimization, reaction mixtures were prepared with variable pH ranges (6, 6.5, 7, 7.5, 8 and 8.5) at 30 °C and 200 rpm. The substrate concentration is an important parameter to be optimized and various concentration of HMF such as (g/L) 0.5, 1, 1.5, 2, 2.5 & 3 were maintained at 30 °C, pH at 6.5 and 200 rpm. Inoculum age is an importance parameter especially in a bio-catalytic process and for the inoculum age optimisation, reaction conditions were 30 °C, pH 7.0 and 200 rpm with variable hours. Initially experiment was started from 6th hour to 30th hour, and later came to know the production happens between 24 h and 28 h. Hence this variable was kept in ranges from 24 h to 28 h. The supernatant was

Table 1
RSM experimental design.

Experiment order	Inoculum age (h)	Pre-inoculum size (mL)	pH
1	30	5	7.5
2	24	2	7.5
3	30	2	5.5
4	27	3.5	6.5
5	27	6.02	6.5
6	27	3.5	6.5
7	27	3.5	8.18
8	27	0.98	6.5
9	27	3.5	6.5
10	27	3.5	6.5
11	24	2	5.5
12	27	3.5	6.5
13	27	3.5	6.5
14	24	5	7.5
15	24	5	5.5
16	30	2	7.5
17	30	5	5.5
18	27	3.5	4.82
19	21.95	3.5	6.5
20	32.05	3.5	6.5

recovered by centrifugation at 12,000 rpm for 15 min for HPLC analysis. All experiments were repeated for three times and standard deviation (SD) was calculated.

2.6. Response Surface Methodology (RSM)

Further optimization of the whole-cell biotransformation process using *Acinetobacter oleivorans* S27 was done by statistical optimization methods. Response Surface Methodology (RSM) studies were conducted using Minitab software version 15.1.10. Three parameters such as inoculum age, pre-inoculum size and pH were selected with respective ranges for Central Composite Design (CCD). The experimental design contained 20 runs as given in Table 1.

2.7. Chemicals

Analytical grades of HMF and its derivatives HMFCA, HMF Alcohol, DFF, FDCA, FFCA and furoic acid standards were purchased from Sigma-Aldrich, India. H_2SO_4 for analytical grade was purchased from Fluka Pvt Ltd, and HPLC grade methanol and acetonitrile solvents were purchased from Merck (India).

3. Results and discussion

The soil samples collected from different altitude of Sikkim Himalaya regions of India were used for isolation of microorganisms. A total of 324 microorganisms were isolated from different soil samples which were able to grow in media supplemented with different concentration of HMF. The isolated cultures were further screened for FDCA production and pure culture glycerol stock was maintained at −80 °C for future use.

3.1. Isolation of Acinetobacter oleivirans S27

More than 10 strains which are growing in the presence of HMF were selected initially for the FDCA production. From the selected list of strains, S27 (given code number) strain could grow in HMF up to 3000 mg/L concentration and showed more FDCA production compared to other strains. After 16s rRNA gene sequencing, the organism was identified as *Acinetobacter Oleivorans*. Sequence were deposited in GenBank with an accession No. (MK359024). The *Acinetobacter oleivorans* S27 was found to be a gram-negative and oxidase negative. It shows good activity on FDCA production when HMF is giving as a sole
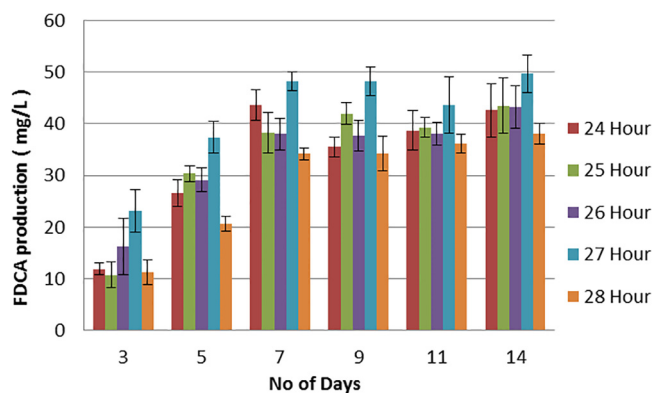

Fig. 1. Optimization of inoculum age.

carbon source.

3.2. Effect of inoculum age, temperature, pH and substrate concentration

The optimization of parameters of whole-cell biocatalyst has a significant role in improving the production of HMF derivatives. A best model of whole-cell biocatalyst should have some good features such as genetically flexible, fast growing capability in a simple media, enzyme production in fair amount and downstream process should be compatible with current system of approaches (Klatte et al., 2014). Among all HMF derivatives, FDCA got a special interest because of its wide applications. Hence the focus of this study was majorly on FDCA production and biotransformation parameters were optimized. The optimization was started with inoculum age because the time of cell harvesting phase has a strong influence in the production level. Most of the enzymes, which are involved in the biotransformation, will be produced in a specific phase. Moreover studies suggested late exponential phase is the crucial time for the production (Hossain et al., 2017). It was found that maximum conversion happens between 24 and 28 h (Fig. 1) and 27th hours were found to be optimum. Single parameter optimization for incubation temperature resulted 30 °C (Fig. 2). When temperature increases up to 37 °C, less activity was noted and most probably this is happening because of the enzyme denaturation. Increase in temperature will not make much difference and also in the case of gram negative *Acinetobacter* species 30 °C was reported as optimum temperature in case of diesel oil degradation (Kang et al., 2011). For pH optimization studies wide range of pH (6–8.5) were selected and results showed that pH 7.5 was optimum (Fig. 3). Tuning of pH had an important role in the production level. Because of the other derivatives which include alcohol and acids, pH of the reaction mixture becomes acidic. Additionally pH was maintained throughout the incubation period. The study shows that the optimum substrate concentration ranges from 500 mg to 3000 mg (Fig. 4). The strain could utilize 90%
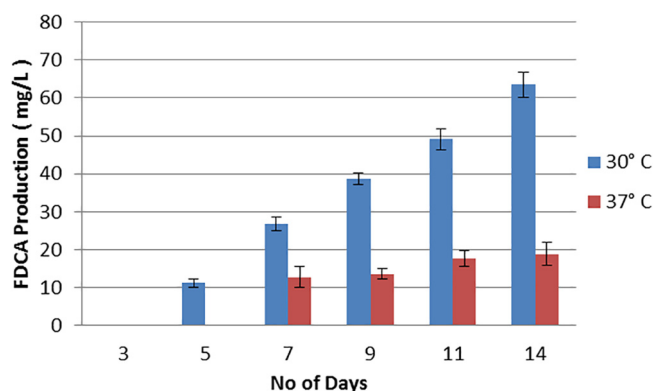

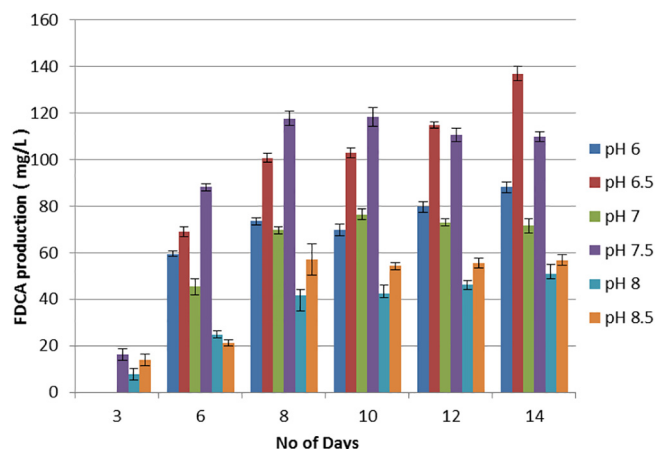**Fig. 2.** Optimization of incubation temperature.
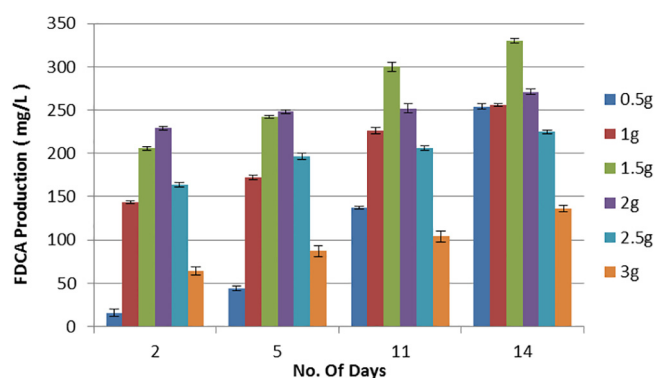
Fig. 3. Optimization of pH.

Fig. 4. Optimization of HMF concentration.

the HMF up to 2000 mg/L concentration (data is not shown). It was found FDCA yield was almost 51% when the initial HMF concentration of 500 mg/L. Higher than 2000 mg/L of HMF concentration, the reaction got inhibited because of the toxicity to the organism.

3.3. Experimental design and analysis of RSM

The result of 20 experiments based on three important parameters (inoculum age, pre-inoculum size and pH) for the biotransformation of HMF into major value added products like HMFCA and FDCA were analysed using Minitab software version 15.1.10.

3.3.1. Synthesis of HMFCA/HMFCA

The biodegradation capability of our strain is quite remarkable. Within 12 h of incubation almost full substrate was utilized. Interestingly from the 24th hour of incubation HMFCA production has started. It was the indication that first oxidation of HMF had been started. Focusing on HMFCA production from the RSM based experiments result (Fig. 5a–c) shows maximum yield (approximately 85%) was observed when pH ranges between 6.5 and 8.2. Recently a work on the synthesis of HMFCA using *Comamonas testosteroni* shows that HMFCA yield was increased when pH of the buffer between 7 and 8 (Zhang et al., 2017). In all the cases excellent substrate utilization (approximately 100%) happened. The relation between inoculum age and pre-inoculum size was crucial for the HMFCA yield (27 h and 3.5 mL respectively). Oxidation of HMFCA resulted in the low yield of FDCA in these cases and furoic acid was produced. The results indicate the steps involved in HMF biotransformation to its derivatives is somehow similar to the *Raultella ornitholytica* BF60 involved HMF biodegradation (Yuan et al., 2018a,b,c).
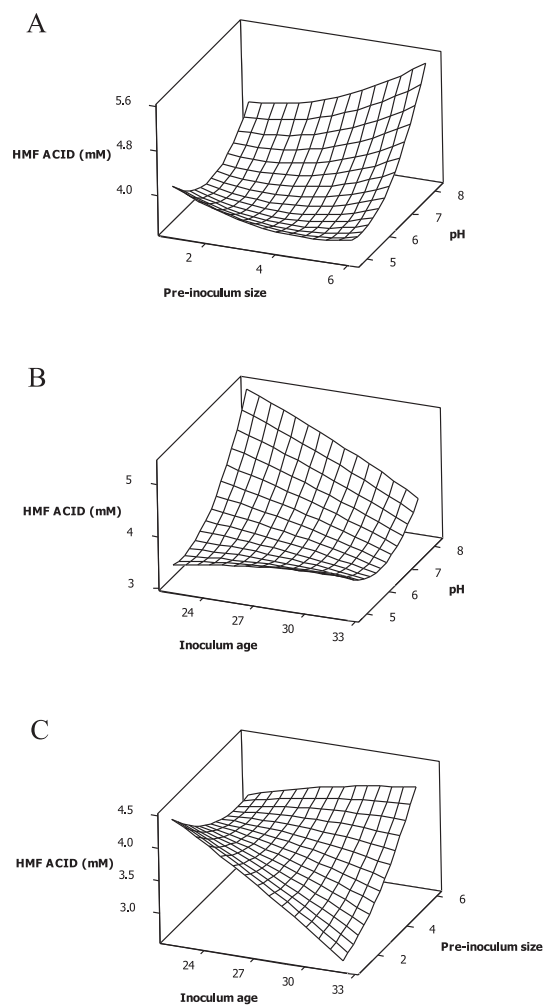

Fig. 5. Surface plot of HMFCA optimization A) pH vs Pre-inoculum; B) pH vs inoculum age; C) pre-inoculum size vs inoculum age.

3.3.2. Biosynthesis of FDCA

The biocatalytic production of FDCA involves three subsequent oxidation steps (Wierckx et al., 2015). Results showed that the particular strain had no inhibition on given HMF concentration because it could degrade the toxic HMF to less toxic carboxylic acids and alcohols. The conversion of HMF to HMFCA was a comparatively faster than HMFCA to FDCA. Availability of oxygen for the further oxidation may be the reason and also compared to HMF, the strain may more tolerant towards HMFCA because it is less toxic than aldehyde. Here FDCA was produced more while giving high pre-inoculum size (5 mL). It should be noted that while pH is changing towards acidic (5.5–6.5) FDCA yield got increased. In the same time initial pH was decreased and showed slightly alkaline towards as incubation time is increasing (Fig. 6a–c). This result was quite similar with the previous work of *Methylobacterium radiotolerance* mediated FDCA production from HMF in algal acid hydrolysate (Yang and Huang, 2017). More precisely, in the present work, in run number 15 (Table 1) complete HMF degradation has occurred and interestingly approximately 65% yield of FDCA was obtained. Moreover, pre-inoculum size was high and pH was at 5.5, and from this, it can be concluded that HMF toxicity could be decreased by increasing the cell concentration. To connect with the result of optimum pH for biotransformation, several fungal enzymes like aryl alcohol oxidase and recombinant galacto oxidase can be oxidized the HMF into HMF derivatives with the pH range of 5.5–7. This indicates the action of enzymes also would prefer slightly alkaline conditions (Karich et al., 2018). The study also revealed that *Acinetobacter*
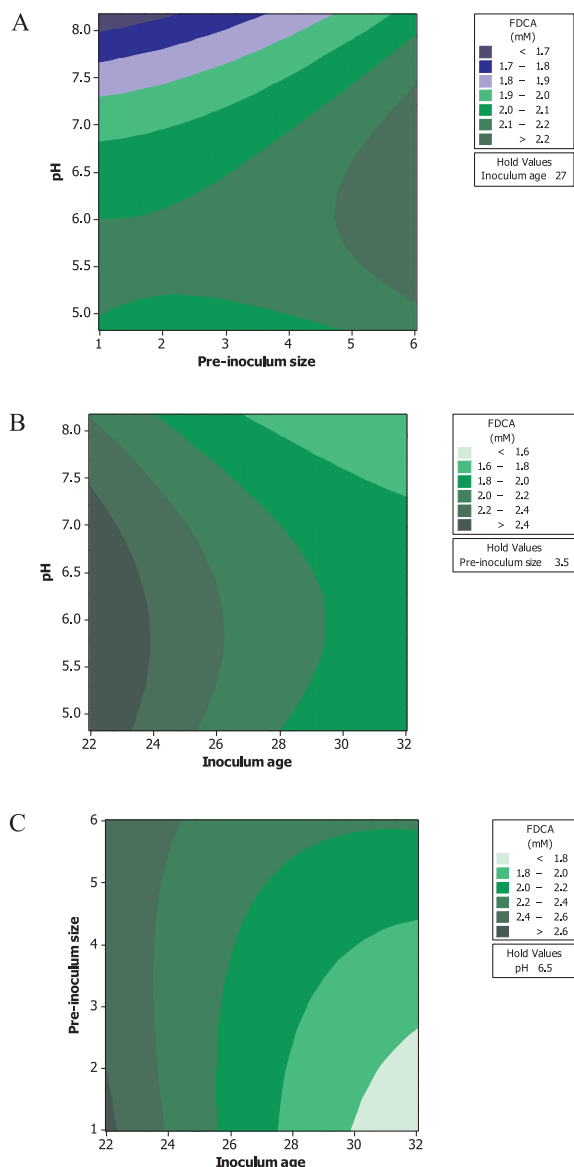
Fig. 6. Contour plot of FDCA production A) pH vs pre-inoculum size B) pH vs inoculum age; C) pre-inoculum size vs inoculum age.

oleivorans S27 strain has comparatively wide range of pH tolerance. Actual relation between pH tolerance and FDCA production is to be further investigated.

4. Conclusion

The present work describes the biotransformation of HMF by *Acinetobacter oleivorans* S27. The isolated strain transformed HMF fully into furan derivatives like FDCA and HMFCA, which are known for alternate of terephthalic acid and a building block of polyester industry respectively. The bacterial strain could be used industrially for the bio-based production furan derivatives to minimize the usage of fossil fuel reserves. It can be concluded that the strain *Acinetobacter oleivorans* S27 is novel in the conversion of HMF and there are potential to exploit this strain for the large-scale synthesis of chemicals like HMFCA and FDCA.

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