Whole cell and cell-free synthesis of 2,5-furandicarboxylic acid from pineapple waste

by

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A thesis submitted to the
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Under the supervision of
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CERTIFICATE

This is to certify that the work incorporated in this Ph.D. thesis entitled, **"Whole cell and cell-free synthesis of 2,5-furandicarboxylic acid from pineapple waste"**, submitted by **Mr. Rajesh R.O.** 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 degreeor 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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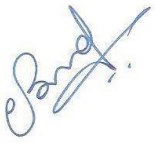

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Dedicated to my family and friends

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Rajesh R.O.

CONTENT

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
Tab	Table
FTIR	Fourier transform infrared spectroscopy
g/L	Gram per liter
GHG	Greenhouse gases
HMF	5-Hydroxymethylfurfural
HMFCA	5-Hydroxymethyl-2-furancarboxylic acid/ HMF acid
HPLC	High Performance Liquid Chromatography
L	Liter
M	Molar
meq	milliequivalent
mg	Milligram
mM	Millimolar
MSM	Mineral salt medium
NAD^+	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide hydrate
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
nm	Nanometer
v/v	Volume per volume
g/g	Gram/gram
vvm	Volume per volume per minute
w/v	Weight per volume
MSM	Mineral salt medium
AAO	Aryl alcohol oxidase
ALDH	Aldehyde dehydrogenase
PaoABC	Periplasmic aldehyde oxidase
MO	Methanol oxidase
HRP	Horse Radish Peroxidase
AO	Alcohol oxidase
GAO	Galactose oxidase

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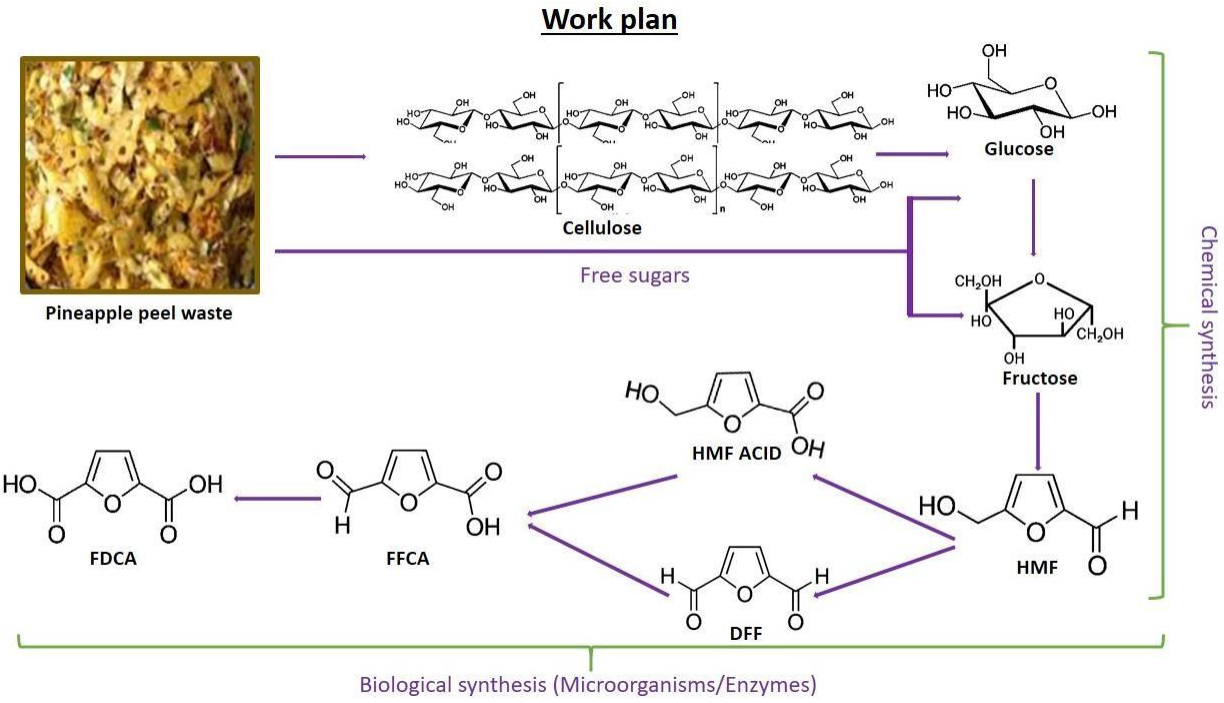
OBJECTIVES

Aim

To develop a method for the production of 2,5-furandicarboxylic acid (FDCA) using renewable resources

Objectives

- Development of a chemo-catalytic process for the conversion of fructose/glucose/lignocellulosic biomass to 5-hydroxymethyl furfuraldehyde (HMF)
- Isolation and screening of microorganisms capable of converting HMF to FDCA
- Development of a fermentation technique for the production of FDCA using positive isolates
- Media engineering and optimization of fermentation for maximizing the FDCA yield from HMF
- Evaluation of various fermentation strategies for FDCA production
- Cell-free conversion of HMF to FDCA using enzyme cascade system



ORGANIZATION OF THESIS

Chapter 1: Introduction and Review of literature

Chapter 2: Chemo-catalytic conversion of pineapple peel into HMF

Chapter 3: Isolation, screening and identification of microorganisms for FDCA production

Chapter 4: Media engineering and process optimization for the production of FDCA

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Chapter 1

Introduction and review of literature

Lignocellulosic biomass is a potential renewable resources, which can be utilized to synthesize green chemicals based on biological approaches (Tang et al., 2017). U.S. Department of Energy and U.S. Department of Agriculture have a vision of 30% reduction in U.S. petroleum consumption with biofuels by 2030 (Perlack et al., 2005). Biomass from agricultural resources can be used as one of the best renewable energy sources in the world instead of chemicals and fuels from non-renewable sources. As a feedstock, lignocellulosic biomass is used for the production of plastics, paper, pulp, fuels and chemicals in biorefineries, which will eventually boost up the rural economy and improve farmers` income. This will reorient the direction of chemical industries from petroleum refinery to lignocellulosic biomass refinery and lead to competitive production of bio-based products (Marques et al., 2018). The major surplus biomass are in the form of crop waste, forest residues, paper waste etc (Brodin et al., 2017) (Moscoviz et al., 2018). This biomass is mainly composed of cellulose, hemicellulose, lignin and other constituents (Kobyashi et al., 2013). Among them, cellulose is insoluble in water and made up of glucose subunits by β-1,4 glycosidic bonds (Morales et al., 2014) (Van de et al., 2011). Hemicelluloses are composed of monomers of xylose, arabinose, mannose, glucose, etc (Dhepe et al., 2010) (Peng et al., 2011). After depolymerization of the cellulose and hemicellulose, the obtained glucose can be transformed into many value added chemical products. Depolymerization will be done by acid or alkali pretreatment. Obtained glucose can be used by biorefineries for the production of biochemicals, biopolymers and biofuels. One of the main platform chemicals derived from glucose or fructose, is hydroxymethyl furfuraldehyde (HMF) (Li et al., 2017) (Zhu et al., 2015).

Conversion of fructose to HMF using chemical methods is easier compared to glucose or lignocellulosic biomass, As lignocellulosic biomass is an abundant waste resource, theoretically this can also be used for the production of HMF though either chemical or biological methods. Polysaccharides such as starch (Yang et al., 2012), cellulose (Mascal et al.,

2008), chitin and inulin (Omarl et al., 2012) have also been used as potential feed stocks for the HMF production using chemical catalysts. For all these feed stocks need a common step of depolymerization of glucose though hydrolysis followed by dehydration lead into formation of HMF (Rosatella et al., 2011) (Fig.1.1).

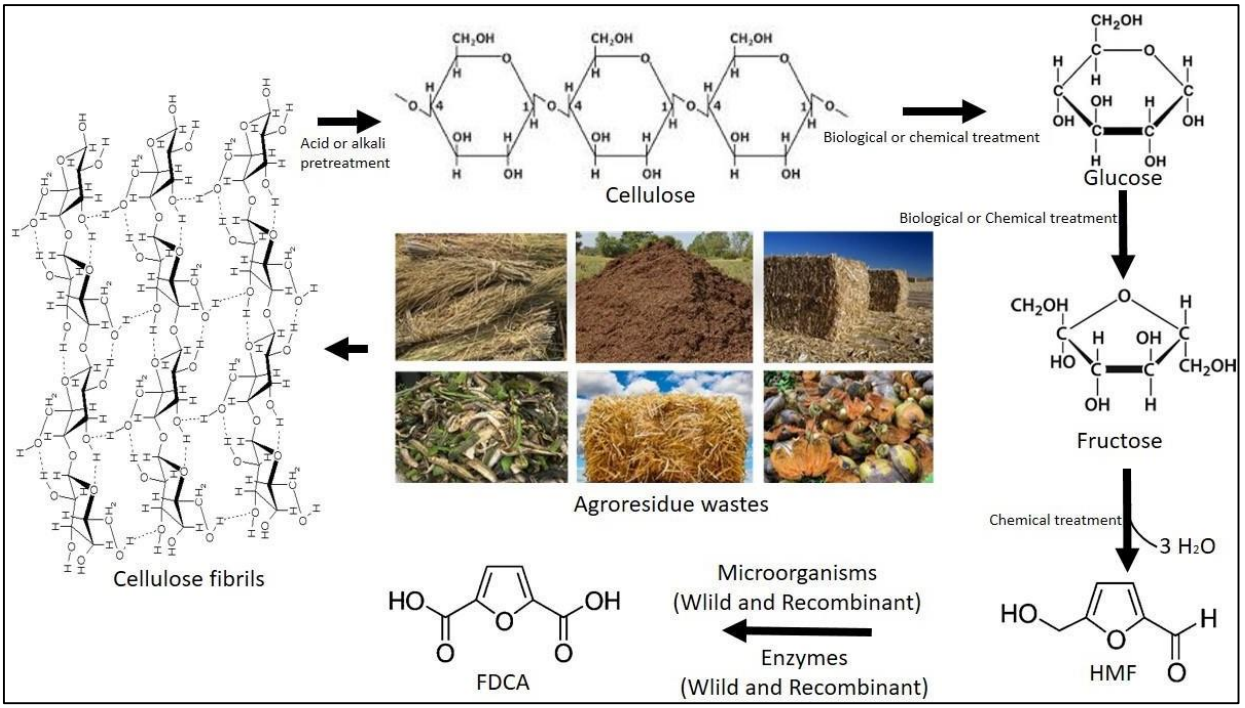


Fig.1.1. Conversion of agro-residue wastes or biomass into HMF though chemical catalysis followed by FDCA production though biological processes

The depolymerized glucose from biomass or the natural free glucose can be isomerized into fructose using chemical catalysts like titanium dioxide (TiO_2) and zirconium oxide (ZrO_2) under microwave irradiation (Qi et al., 2008). It has been reported that isomerization of glucose to fructose can also be done using immobilized glucose isomerase (Huang et al., 2010). Reports claim that HMF production from these substrates can be done using solid catalysts like aluminium sulphate and aluminium chloride, sulfate/zirconium oxide - aluminum oxide, zirconium phosphate, zirconia, aluminium chloride, ion-exchange resins and stannic chloride (Weingarten et al., 2012; Mcneff et al., 2010), lanthanide, niobium and organic-inorganic

nanocomposite based catalysts etc. (Seri et al., 2001) (Almilo et al., 2013). Due to the specialties like easiness of separation, recyclability, reaction fastness, sustainability (even at higher temperatures) and enhanced selectivity, these solid catalysts are industrially preferable (Weingarten et al., 2012) (Tong et al., 2010). HMF can also be synthesized from raw biomass, glucose and fructose using ionic liquids like1-octyl-3-methylimidazolium ([OMIM], 1-hexyl-3-methylimidazolium ([HMIM]), 1-butyl-3-methylimidazolium ([BMIM]), 1-ethyl-3-methylimidazolium ([EMIM]) etc. Ionic liquids have advantages like high stability, low vapor pressure and recyclability (Pelkovic et al., 2011) (Wang et al., 2011). The yield of HMF from monosaccharides or polysaccharides or biomass are comparatively lower than obtained from fructose. Natural processes like caramelisation (Kroh et al., 1994) and maillard reactions (Martins et al., 2005) form HMF as an intermediate.

HMF is a versatile platform chemical for the manufacture of 5-hydroxymethylfuroic acid, bishydroxymethylfuran, HMF diethers, 2,5-dimethylfuran, alkoxymethylfurfuran and 2,5-furandicarboxylic acid (FDCA). These furan derivatives have valuable fuel and/or polymer applications. Also, adipic acid, 1,6-hexanediol, levulinic acid, caprolactam and caprolactone are the non-fumaric compounds produced from HMF (Boisen et al., 2009). Among them, FDCA is the most preferred chemical by industries with broad range of applications compared to other HMF derived products. Similarity of its aromatic counterparts make it as a different structural feature from other chemicals for the polymerization applications (Gomes et al., 2011). Rudolph Fittig and Heinzelmann in 1876 reported FDCA as dehydromucic acid, who produced it by the action of concentrated hydrobromic acid and mucic acid (Fitig et al., 1876). However, the progress in research about FDCA synthesis and its application studies were discontinued and were only resumed in the last 10 years. With the emergence of biorefinary concept there are a massive move from nonrenewable fossil based processes to sustainable renewable resources based processes. According to a study conducted by USA National

Renewable Energy Limited (NREL), conversion of renewable biomass to platform chemicals has been reported. One of key green or bio-based platform chemical in this list is FDCA (Werpy et al., 2004). It has been considered and called as a 'sleeping giant' due to its applications in diverse areas in the coming future (Tong et al., 2010).

1.1. Applications of FDCA

Mainly FDCA can be used for the production of biochemicals like succinic acid (Fang et al., 2017), isodecylfuran-2,5-dicarboxylate (Del poeta et al., 1998), isononyl furan-2,5-dicarboxylate (Becker et al., 2011), dipentyl furan-2,5-dicarboxylate, diheptyl furan-2,5-dicarboxylate and poly (ethylene dodecanedioate-2,5-furandicarboxylate) (PEDF) (Jia et al., 2018). FDCA is also an important ingredient in the preparation of hexanoic acid, macrocyclic ligands (Richter et al., 1999), fungicides, corrosion inhibitors and thiolene films (Larsen et al., 2018), (Gilkey et al., 2018). FDCA derivatives like 2,5-dihydroxymethylfuran, 2,5-bis (hydroxymethyl) tetrahydrofuran can be used in the production of new polyesters as alcohol components. It is a highly demanding monomer in the production of dichloride-(FDCDCl), dimethyl- (DMFDC) and bis (hydroxyethyl)- (BHEFDC) derivatives for the production of plasticizers, polyamides, and polyesters (Sousa et al., 2015). Structurally, furan rings of FDCA are analogous to fossil derived TPA (Terepthalic Acid) (Pacheco et al., 2014) which is one of the currently used plastic material widely used today and FDCA is an eco-friendly alternative for the production of new bioplastics (Yan D et al., 2018). It can also be used as an alternative for polybutylene terephthalate (PBT) and polyethylene terephthalate (PET) (Collias et al., 2014) which are utilized in the production of film, fiber, packing materials and soft drink bottles (Tomas zewska et al., 2018; (Mattsmura et al., 2008). FDCA monomer as such cannotuse in polymer production. This has to be combined with ethylene glycol and synthesize PEF (polyethylene furonate) and PBF (polybutylene furonate). Due to its thermo-chemical, mechanical, gas barrier and recyclability properties, PEF can be used as an alternative of PET

and PBT (Eerhart et al., 2012). There were not much production of PEF from industries due to its less purity, sustainable availability and the process difficulties of FDCA production. FDCA diethyl esters have anesthetic properties similar to cocaine. It has chelating property with ions (Ca^{2+}, Cu^{2+} and Pb^{2+}) and applied as a medicine for the kidney stones removal (Lewkowski et al., 2001). Ultimately, this FDCA has been highlighted as one of the 12 platform biochemical used for the in depth research for the sustainable industrial production.

1.2. Industries involved in FDCA production

The industrial FDCA production is started by Avantium using 'YXY' process which includes carbohydrate dehydration to alkoxymethylfurfural (RMF) or methoxymethylfurfural (MMF), followed by oxidation to FDCA. This FDCA and ethylene glycol combined in the last step for the production of PEF. The pilot plant production of FDCA by Avantium, Netherlands started in 2011 aiming with a target of 40 tons of FDCA per year. After this success, Avantium has established ajoint consortium with BASF, Belgium (Synvina, Netherlands) and a pilot plant designed for FDCA production witha capacity of 50,000 tons per year with PEF production. Apart from that, another company, Corbion, Netherlands, has used a microbial process for large scale FDCA production from HMF. DuPont, United States in joint venture with Archer Daniels Midland Company (ADM), United States has developed a process to produce FDCA esters in 2016. AVA Biochem, Switzerland FDCA production capacity of approximately 30,000 tons per year has started in 2019 and it was then increased to 1,20,000 tons per year. A two-step process has been developed by Petrobras, Brazil in which first step of HMF production is from sucrose, Glucose and fructose (C6 sugars) followed by FDCA production using resin as the second step. VTT Technical Research Centre, Finland has developed a biological industrial production from hexaric acid using modified uronate dehydrogenase enzyme for the conversion of the substrate D-galacturonic acid into mesogalactaric acid (mucic acid) and yet to produce high amount of FDCA (Sajid et al., 2018). So, globally the FDCA and its derivatives all

together can give a commercial estimated production of 4 metric tons per year which may cost approximately a market value of 40 million USD from estimated FDCA production of 9 billion lb/year. This will give an approximate value of $0.85 to 2.20/lb (Werpy et al., 2004). This global market will be reached to 498.15 kilotons of FDCA production in the coming years by 2025.

Majority of the industries depend on chemical processes for the production of FDCA from different substrates. Each technology has its own drawbacks and limitations affecting its successive large scale production. Among the above industries, only BASF got 95.2% of FDCA yield from HMF in D_2O using Pt/C catalyst at 100 bar air pressure at 100 °C. All other industrial chemical technologies have used expensive catalysts, high temperature and high pressure. These processes usually carried out in D_2O or other solvents like methanol or acetic acid using HMF as the substrate with less FDCA yield. Their conditions, expensive substrates,expensive or toxic catalysts make the processes environmentally toxic or expensive technologywith less product yield. Industries like VTT and Corbion, Netherlands have used greener technologies so far. Corbion used a microbial biotransformation technology to produce FDCA from HMF. VTTmade a partial biological technology with very less FDCA yield (1.7%). VTT have produced FDCA from galataric acid with methyl trioxorhenium catalyst in methanol with less pressure (1 bar) at high temperature (100 °C). So the main problems like environment toxicity using chemical catalysts, chemical media and hazardous conditions have been overcome though biological technologies. Still, the existing biological technologies also have its own demerits like less yield and longer time.

1.3. Biotransformation of HMF into FDCA in prokaryotes and eukaryotes

HMF is an organic compound formed after the dehydration of sugars. It is formed after the long term storage of honey and thermal decomposition of sugars or lignocellulosic biomass. The sugars may be natural glucose or fructose or biomass derived origin. Fructose dehydrated

HMF molecule contains a furan ring with both aldehyde and alcohol functional group on both sides. This aldehyde or alcohol functional group makes this as a toxic molecule to living organisms. Furanic aldehydes in this respect causing ROS-associated damage to nucleic acids, proteins and cell organelles of microorganisms (Almeida et al., 2009) (Allen et al., 2010). But some microorganisms like bacteria, fungus (white rot and mycelial) and algae have natural detoxification mechanisms generally to xenobiotics, aldehydes and alcohols. Still, degradation or detoxification of HMF is naturally present for some microorganisms only. HMF detoxification is present in bacteria and fungus mainly can be exploited for industrial applications. Degradation of HMF proceeds though FDCA production (Fig.1.2) which is an industrial relevant compound with many applications. So microorganisms like bacteria and fungus are used for FDCA production from HMF. Even though, the actual mechanisms are not fully understood, there are reports for its mechanism in prokaryotes and eukaryotes are illustrated here with available information.

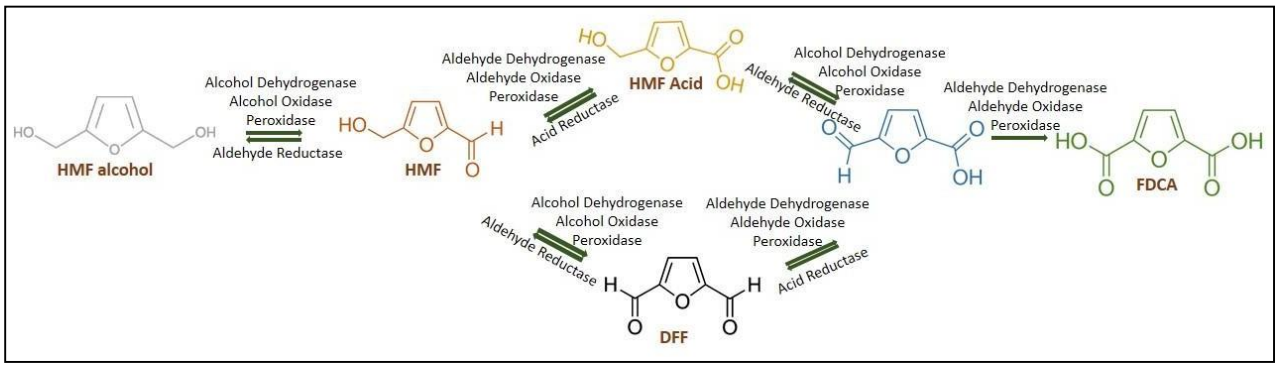


Fig.1.2. Green synthesis of HMF into FDCA though microorganisms or enzymes

1.3.1. Biotransformation of HMF in prokaryotes

HMF biotransformation or detoxification though FDCA is firstly reported in *Cupravidus basilensis* HMF 14 (Wierckx et al., 2015). In this organism, this process occurs though HMF degradation gene clusters *hmfABCDE* and *hmfFGH'H*. If the hmfABCDE gene cluster is mutated, growth of organisms is not occurred in either HMF or furfural carbon source, suggesting a shared metabolic pathway between these two aldehydes. If *hmfFGH'H* gene cluster

is mutated growthof *C. basilensis* HMF 14 is not occurred in HMF medium. It is concluded that the later gene cluster is only associated with HMF degradation pathway. Among them, HMF biotransformation of HMF to FDCA is carried out by the gene *hmfH* (oxidoreductase) in *C. basilensis*. FDCA is converted into 2-furoic acid after decarboxylation by the gene *hmfG*, which is responsible for decarboxylase (Koopman et al., 2010).

From the available reports, in bacteria, HMF can be converted into either HMF alcohol by reductase or it entered into FDCAproduction pathway in periplasm (Fig.1.3). HMF alcohol can be converted back to HMF againby dehydrogenase or oxidase or peroxidase. HMF to FDCA conversion has taken place commonly either of these classes of enzymes (Fig.1.2). HMF is getting converted into HMF acid (periplasm) or DFF (intracellular) and these two converted into FFCA. Until these all thereactions are reversible. This FFCA is getting converted into FDCA. This FDCA can be up taken by the cells for its metabolic activities after decarboxylation into 2-furoic acid. This willbe converted into 5-hydroxy-2-furoyl Co A, 2-Oxoglutaroyl Co A, 2-Oxoglutaric acid and entered into TCA cycle (Wierckx et al., 2015). The dehydrogenases, oxidases and peroxidasesare usually seen in periplasmic space of bacteria usually for the detoxification of toxic aldehydes and alcohols. Microorganisms reduce or oxidize these compounds into less or non- toxic forms for their survival and stay in lag phase. After biotransformation stage, it will be entered into metabolic pathway for its metabolic activities.

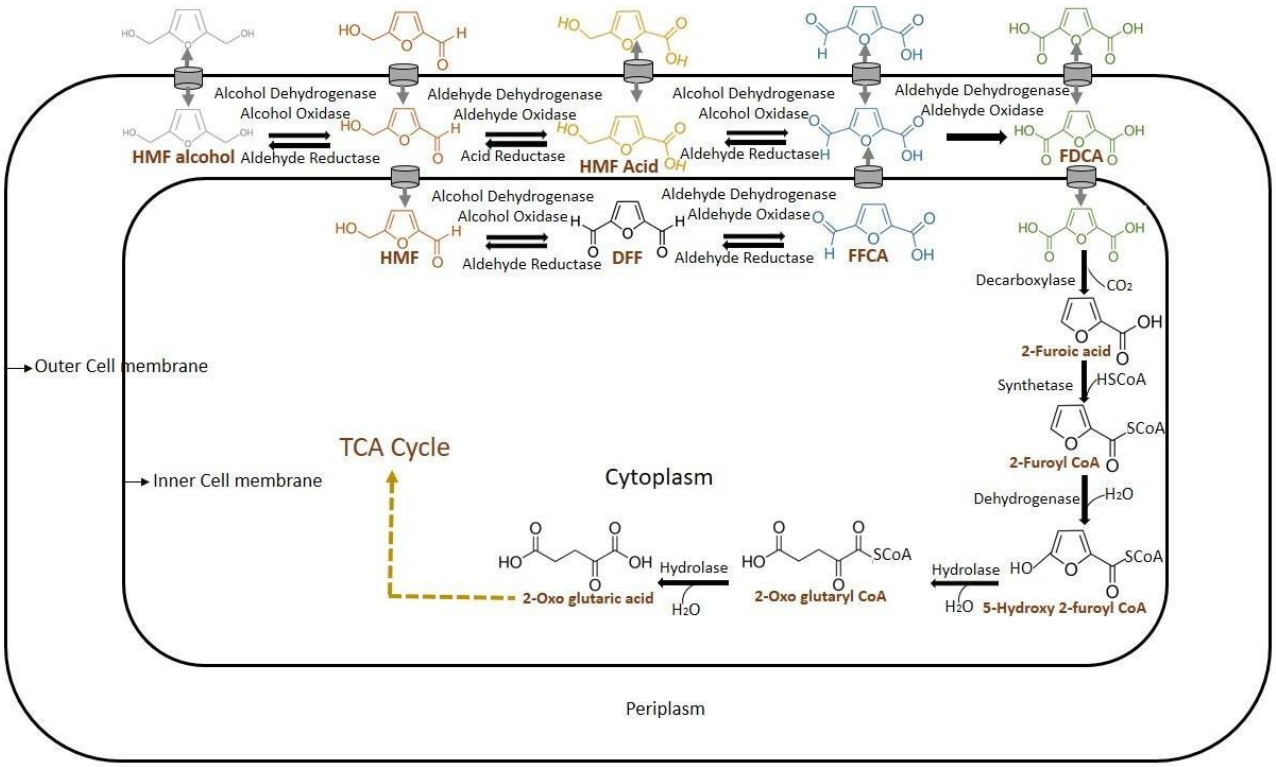


Fig.1.3. Mechanism of biotransfomation of HMF into FDCA in bacteria

1.3.2. Biotransformation of HMF in Eukaryotes

HMF biotransformation in fungus (Fig.1.4) has been reported in basidomycete *Pleurotus ostreatus* (white rot). This has the capability to detoxify HMF into HMF alcohol and FDCA production pathway. It has been reported that aryl alcohol oxidase and aryl alcohol dehydrogenase are involved in this intracellular coupled with extracellular HMF biotransformation. Elevated level of aryl alcohol oxidase and aryl alcohol dehydrogenase expression is seen after HMF induction in quantitative RT-PCR. This expression profile from RNA extract is shown that these enzymes are from the genes *aao 1-3* and *aad 1* respectively. Aryl alcohol oxidase, *aao* 4 expression is increased after 24 h of HMF induction which showed that intermediates of HMF biotransformation into FDCA is carried out by this gene. Also, this biotransformation reaction is an intracellular coupled with extracellular HMF detoxification (Feldman et al., 2015). It is reported that, HMF intermediates like HMF alcohol, HMF acid, FFCA and FDCA are produced extracellular after HMF intake in *A. flavus*. Since DFF and 2-

furoic acid is not formed in extracellular medium, and they are only observed in the intracellular mycelial fraction (Rajesh et al., 2019). So from these reports it can be concluded that dehydrogenases, oxidases and peroxidases of the fungal HMF biotransformation can be done intracellular also. Then the extracellular FDCA can be up taken to fungal cells followed by 2-furoic acid pathway like bacteria.

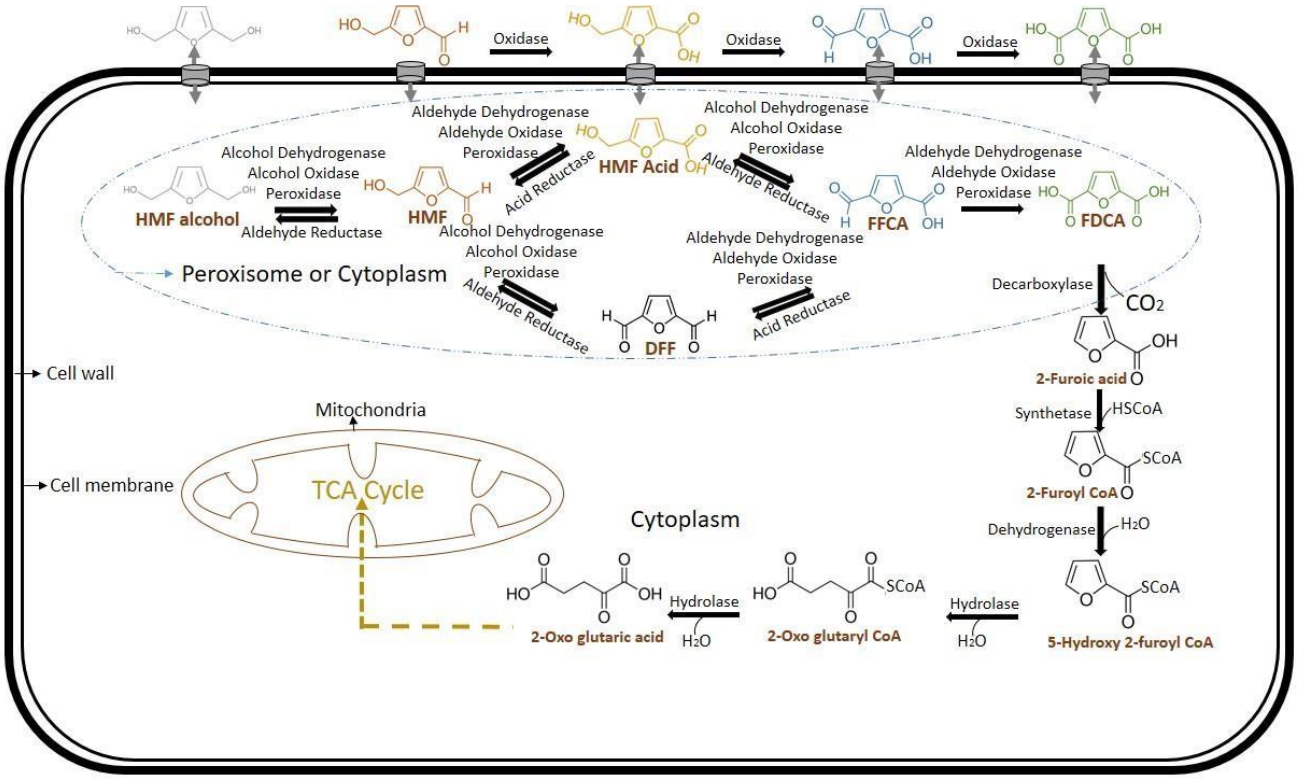


Fig.1.4. Mechanism of biotransfomation of HMF into FDCA in fungi

1.4.Technological advancements and Innovations for the green synthesis of FDCA by microorganisms

1.4.1. Engineering of media components and conditions in microbial bioprocesses for the production of FDCA

The microbial assisted biotransformation of HMF to FDCA is listed in Tab.1.1. *Burkholderia cepacia* H-2 has been isolated from the enrichment isolation technique from soil in 0.2 to 1 g/L HMF. This microorganism is capable of transforming HMF into FDCA after preliminary

analysis in 10 mL mineral salt media with 0.2 g/L HMF. After media optimizations of substrate concentration ranging from 0.5 g/L to 3 g/L and transformed 2 g/L HMF to 1.2 g/L FDCA in 125 mL batch reactor. Media pH is optimized ranging from 5.0 to 8.0 and optimized pH is noted as 7.0 and 1.2 g/L HMF produced from 2 g/L HMF after 32 h. Temperature of media is optimized ranging from 26 to 32 °C and maximum FDCA of 1.27 g/L obtained at 28 °C. These single parameter media optimizations improved the FDCA production up to 1.27 g/L from 2 g/L HMF after 24 h. Thermal algal acid hydrolysate of *Chaetomorpha linum* contains HMF, is transformed into FDCA using isolated strain *B. cepacia* H-2. Hydrolysis of 3% w/v algal biomassis done using 0.5 M HCl under 121 °C for 15 min for the HMF preparation. HMF (0.2 g/L), reducing sugars, furfural (30 mg/L) and acetic acid (104 mg/L) are analyzed in thermal algal hydrolysate and this cheap medium is used for the production of FDCA. Acid algal hydrolysateof 0, 2 and 4 times dilution is inoculated with *B. cepacia* H-2 (0.1 O.D) in batch reactor and 2g/L HMF added into it. After incubation, 1.03 g/L of FDCA obtained from two dilutions of acid algal hydrolysate after 18 h (Yang et al., 2016). This single parameter optimizations and use of cheap thermal algal hydrolysate improved the production of FDCA from HMF.

Biotransformation of HMF into FDCA is performed using the isolated strain *Methylobacterium radiotolerans* G-2. This isolated strain is enriched in 0.2 g/L HMF for the HMF utilization rates. After HPLC analysis it is confirmed that, this strain is able to produce FDCA. As part of media optimization for enhancing the production of FDCA by *M. radiotolerans* G-2 single parameter optimization strategy is applied into media. Single parameter optimization of substrate (HMF) concentration, pH and temperature is done for increasing the FDCA production. The organism is inoculated in 40 mL of mineral salt media with a cell concentration of 0.1 - 0.02 O.D in 125 mL batch reactors at 120 rpm. Media optimization of substrate concentration is done ranging from 0.5-3 g /L and best FDCA production of 1.6 g/L obtained

from 3 g/L HMF after 72 h. Optimization of media pH is done ranging from 5.0 to 8.0 and maximum of 0.5 g/L FDCA produced at pH 7.0 and 8.0 which is neutral and slightly alkaline respectively. Temperature of media is obtained ranging from 26-32 °C. The specific growth rate (0.0232-0.0274 L/h) of *M. radiotolerans* G-2 is almost similar in every media incubation temperature. Also, the FDCA concentration in media at each incubated temperature is same as 0.513 g/L. Since the FDCA degrading rate is lowest at temperature of 26 °C, this temperature used as the best one for further works. As part of FDCA production from renewable resources synthetic media like mineral salt media is avoided and algal acid hydrolysate used. This algal hydrolysate is made from acid treatment of unused or waste algae which contains reducing sugars, HMF, acetic acid and furfural. The organism is inoculated into 0, 2 and 4 fold dilutions. Maximum FDCA of 0.45 g/L is produced from 2 and 4 fold dilutions of thermal algal acid hydrolysate (Yang et al., 2018). Algal hydrolysate has avoided the use synthetic media and FDCA production has been improved after single parameter optimizations.

R. ornithinolytica BF60 is isolated from soil enrichment isolation technique showed HMF conversion and FDCA production upon preliminary analysis. Reaction conditions are optimized for the whole cell biotransformation of HMF into FDCA in phosphate buffer. First, the optimal pH of the whole-cell biocatalyst is determined ranging from 6.0 to 10.0 and better pH found as 8.0 (slightly alkaline). Optimum temperature of this biocatalyst is done ranging from 20-45 °C and its maximum activity found at 30 °C. Optimal substrate concentration (HMF) is done ranging from 25 to 150 mM. Maximum production of FDCA is reached (12.08 μmol $L^{-1}h^{-1}$) at 100 mM HMF. It is indicated that activity of microbial biocatalyst get inhibited higher than 100 mM HMF. Inoculation age of the whole cell biocatalyst is also optimized and better FDCA production observed at late exponential phase. At this stage, toxic effect of HMF is minimized and conversion of HMF into desired product FDCA is maximized. Inoculum size of the biocatalyst is determined ranging from 15 to 75 g/L and highest FDCA production resulted in 45 g/L. At this inoculum size FDCA production rate maintained at 12.08 μmol$L^{-1}h^{-}$

^{1} (9.2 g/L) from 100 mM HMF. These biochemical characters again optimized by means of a fractional factorial design and similar results obtained. After confirmations it has been confirmed that high cell concentrations might not enhance FDCA production due to the deficiency of oxygen in the media. This is because the pathway generates reducing power for the energy metabolism of the microorganisms which must be oxidized to sustain the flux (Hossain et al., 2017). This media engineering process is optimized for FDCA production by single parameter optimization and fractional factorial design, and production has been increased from preliminary results.

Enterobacter sp. has been isolated from acid pretreatment liquor drainage site after enrichment culture isolation in mineral salt media and HMF. The organism is grown for 48 h in nutrient broth and after that 20% (v/v) of its cell biomass is collected. This biomass is transferred into mineral salt media and HMF for the biotransformation. To optimize the media ingredients for maximum HMF biotransformation, 0.25% glucose and 0.25% glycerol is added to this media and production rate of FDCA got delayed. Among them media with 0.25% glucose showed much delay in the FDCA production as compared to the media with 0.25% glycerol. This might be due to the uptake of glucose into the microorganism for its metabolic activities rather than HMF biotransformation. Also cell density is higher in the glucose enriched media with HMF as compared to a media without carbon sources. So mineral salt media (MSM) with 0.5 g/L HMF converts into 0.07 g/L FDCA after 14 days (Rajesh et al., 2018). This results shows that less substrate concentration is used with less production in 14 days after media optimizations.

Acinetobacter oleivirans S27, an isolate from high altitude soil, Sikkim, India was used for the biotransformation of HMF into FDCA. It is grown in LB medium and cell biomass collected after centrifugation. This biomass is transferred into MSM with 0.5 g/L HMF. Optimization of media temperature (30 °C - 37 °C), pH (6.0 - 8.5), substrate concentration (0.5 - 3.0 g/L) and inoculum age (24 -28 h) is performed and found as 30 °C, 6.5, 1.5 g/L and after 27 h

respectively. After single parameter optimizations 0.23 g/L FDCA obtained from 1.5 g/L HMF. After making RSM design with thee parameters inoculum age, inoculum size and pH of the media is optimized for the FDCA production and optimized as 24 h, 5.0 and 5.5 respectively. So the optimized media with these conditions produce 65% yield of FDCA from 0.5 g/L HMF (Godan et al., 2019). This is mainly focused for biotransformation of FDCA and less amount of FDCA produced with less substrate detoxification.

Aspergillus flavus APLS-1 has been isolated from acid pretreatment liquor drainage of the pilot plant, India and showed HMF conversion into FDCA after preliminary analysis. Screening is done in MSM and 0.5 g/L HMF. Addition of other carbon sources (glucose and potato dextrose broth) other than HMF into MSM and HMF has showed inhibition in FDCA production due to the uptake of normal carbon sources for its metabolic activities. Upto 3.0 g/L HMF is tolerated by *A. flavus* in solid agar plates without much morphological changes. Media optimization by single parameter optimization strategy is applied to enhance FDCA production. Media is optimized with different pH (6.0 to 8.0), cell biomass size (4.0 to 8.7 g/L), biomass age (48 to 84 h) and substrate concentration (0.25 to 2.0 g/L). Pre-grown cell biomass is added into MSM containing HMF and this toxic HMF is bio-transformed into FDCA upon sequential oxidations. After this optimization maximum amount of FDCA 0.32 ± 0.06 g/L is produced from pH 6.5, biomass size 6.0 g/L, biomass age 60 h and HMF 1.0 g/L after 14 days. After, to improve the FDCA production, a Box-Behnken design is made to know the Response surface methodology of thee major parameters biomass size, biomass age and pH of the medium. Here, substrate concentration is kept as constant as 1.0 g/L. Media optimization using Box-Behnken design is improved 67% conversion efficiency of 1.0 g/L HMF (8 mM) and 0.8 g/L (6.4 mM) of FDCA produced in 14 days with biomass size of 5.7 g/L at pH 6.5 and biomass age 60 h. It is validated after validation experiments with biomass size 6.6 ± 0.1 g/L, pH 6.5, and biomass age 60 h. This condition is validated and FDCA production of 0.83 g/L (6.6 mM) obtained from 1.0 g/L (8

mM) HMF with a yield of 67% after 14 days (Rajesh et al., 2019). Even though these media optimizations have been improved biotransformation of HMF into FDCA its production occurred after 14 days.

1.4.2. Homologous and heterologous expression of genes in microorganisms by genetic engineering for the production of FDCA

1.4.2.1. Homologous expression of genes in microorganisms

The genes in the pathway of *Raoultella ornithinolytica* BF60 for biocatalytic oxidation of HMF to FDCA has been done by transcriptomic analysis. This has given the information of HMF to the undesired product 2, 5-bis (hydroxymethyl) furan (HMF alcohol) is done by the genes *adhP3* and *alkR* and the desired product FDCA by the gene *aldH*. Combinatorial deletion of two genes *adhP3* and *alkR* by λ Red recombination system resulted in 85.7% reduction in HMF alcohol and overall increase in FDCA (23.7%) production (242.0 mM). For over expression of gene vector pACYC-*hmfH-aldH* has been constructed. For this *aldH* gene amplified with 20 bp flanking homologous sequences at *Nde* I and *Xho* I sites. This *aldH* fragment has been ligated to a linearized plasmid pACYC-hmfH though recombination using the ClonExpress II Kit yielded the plasmid pACYC-*hmfH-aldH*. Biotransformation experiments have been conducted in 10 mL of pre-grown cell suspension ($O.D_{600}$ 100) and 0.5 g Calcium carbonate (Neutralizing agent) in 50 mM sodium phosphate buffer (pH 8.0) at 30°C. Fed-batch approach, where HMF concentration of 50 mM in every 12 hours until 36 hours and then 25 mM in next 36 hours were evalauted. Over-expression of*aldH* gene responsible for the oxidation of the intermediate 5-formyl-2-furan carboxylic acid (FFCA) to FDCA has resulted 96.2% yield of FDCA (264.7 mM) (Yuan et al., 2018). Even though gene expression and fed batch approach have improved FDCA production the reactionis carried out in low reaction volume which is suitable for laboratory scale work only.

1.4.2.2. Heterologous expression of genes in microorganisms

Gene (*hmfH*) of enzyme oxidoreductase from *Cupriavidus basilensis* HMF14 has been cloned into pJTmcs with a constitutive *tac* promoter as transcription control. The resultant plasmid pJThmfH is introduced into *Pseudomonas putida* S12 and this engineered whole cell biocatalyst produced FDCA from HMF in the preliminary analysis itself. HMF concentration in the media increased up to 50 mM resulted *q*FDCA from 116 ± 1.82 to 276 ± 89 µmol (g CDW)$^{-1}$ h^{-1}). In this report, HMF concentration of 75 mM or higher, an intermediate HMF acid is no longer completely converted to FDCA. This may be due to the toxicity of HMF to the host strain. Moreover, the high concentrations of acid intermediates like HMF acid and FFCA generated also resulted in lowered pH in the media. To prevent these inhibitory effects fed batch experiments have been performed. The main problems overcome by pulse feeding of HMF and adjusting the pH with NaOH or HCl in the media. In fed-batch experiments 30.1 g/L of FDCA is produced from HMF using glycerol as carbon substrate at a yield of 97%. FDCA has been recovered from its culture broth as pure dry powder (99.4%) using acid precipitation method followed by subsequent tetrahydrofuran extraction (Koopman et al., 2010). The 30.1 g/L FDCA yield is also suitable for laboratory level work which does not achieve industrial need.

The genes encoding enzymes in the conversion of HMF into FDCA is identified in *Pencillium brasilianum* C1. Actual genes involved in HMF metabolism are hmfK1, hmfP, hmfK2, hmfL3, hmf14, hmfN2, hmfQ and hmfU. In addition, a few transport or regulation genes hmfR, hmfT3, hmfT4 and hmfT5 are also identified. The gene hmfK1 has a similar function to salicylate FDCA hydroxylase FAD decarboxylation binding monooxygenase which is actually involved in FDCA decarboxylation in the fungus. *C. basilensis HmfH* and HMF or FFCA aldehyde dehydrogenase gene are genetically expressed using expression construct comprises a G418 resistance marker and URA3 homologous site for chomosomal integration into the *S. cerevisiae*

CEN.PK clone 2. *HmfH* gene construct consists of TEF1 promoter and by the CYC1 terminator for the transcription termination. HMF/FFCA aldehyde dehydrogenase gene is expressed from the TDH3 promoter and transcription is terminated by the TDH3 terminator. FDCA batch production of CEN.PK clone 2 are done in the mineral medium supplied with 1 g/L of glucose and 4 mM of HMF at 150 rpm yielded 0.22 mM FDCA after 40 h (De Bont et al., 2018) and the yield is lees as compared to other engineered microbial works.

Genes like hmfL1, hmfL2 and/or hmfN1 of *P. brasilianum* are expressed in *S. cerevisiae* CEN.PK. Resultant recombinant organisms are called as CEN.PK/PTT2, CEN.PK/PTT2-hmfN1-hmfL1-hmfL2, CEN.PK/PTT2-hmfN1-hmfL1 and CEN.PK/PTT2–hmfL1 respectively. Their activity has been checked in glucose containing mineral medium with 3 mM HMF. Among them highest FDCA production (0.93 mM) is noticed by CEN.PK/PTT2-hmfN1-hmfL1. This FDCA production is increased up to 3.02 mM from 4 mM HMF after 40 h by giving sufficient oxygen in the medium. *P. brasilianum* hmfL1 is also heterologously expressed in the yeast *Yarrowia lipolytica* Polg using pYLEX1-hmfL1 vector and conferred the ability to produce FDCA from HMF. This transformants are checked for its activity in glucose containing mineral medium supplemented with 4 mM HMF. It has showed 1.55 mM FDCA after 40 h upon HPLC analysis (De Bont et al., 2018). The work is much related to negligible FDCA yield with proof of concept.

HMF oxidase (HMFO) from *Methylovorus* sp. MP68 and HMF oxidoreductase (HmfH) from *Cupriavidus basilensis* HMF14 are inserted to *Raoultella ornithinolytica* BF60 by vector pBBR1MCS2 for improving FDCA production. After removal of *LacZα* gene, the genes HmfH and HMFO are substituted and amplified. The locus *LacZα* is substituted with the genes HmfH or HMFO though recombination. Vector pMD19-T is used for the co-expression of these genes. Restriction-enzyme-ligation method is also used for the preparation of this fusion fragment using *Hind* III, *Xba* I, and *Eco* RI restriction sites. The fusion fragment produced by

recombination is inserted into the vector pBBR1MCS2 vector by using recombination method and transformed into *Raoultella ornithinolytica* BF60. This engineered whole-cell biocatalyst has improved FDCA production from 51 mM (wild type strain) to 93.6 mM (recombinant strain). The experiments performed using pre-grown whole-cell biocatalyst and HMF in 50 mM phosphate buffer (pH 8.0) at 30 °C showed low level of intermediates like HMF alcohol and HMF (Yuan et al., 2018) . This engineered strain produces better amount of FDCA as compared to others with fast conversion.

1.4.3. Engineering of genes into the metabolic pathways of strains for the production of FDCA

HMF is getting converted into FDCA metabolically, and after that this FDCA may be up taken by the cells for its activities though TCA cycle. This is mainly done by the enzyme dicarboxylic acid decarboxylase (*dcaD*). If this is mutated, amount of FDCA in the medium will be in more amount. In a report, *R. ornithinolytica* BF60 has been mutated to block FDCA degradation to furoic acid. Mutations of genes are done by mobile group II intron based genetic engineering systems. Gene targeting is mainly done by base pairing between the intron RNA and the targeted chromosomal DNA and thus the intron fragment can be modified easily. Consecutive mutations with high efficiency can be done by Intron insertion into the chromosome and so it is a promising approach to marker less mutagenesis (Jang et al., 2012) (Li et al., 2012). The intron sequences are amplified and digested with *Bsr* G1 and *Hind* III, and ligated with the pACD4K-C-loXp TargeTron plasmid and transformed into *E. coli* JM109 cells. These plasmids are transfected into *R. ornithinolytica* BF60 by electroporation. Intron-specific primers are used for the confirmation of successful insertion by using colony PCR. It is selected using Kanamycin selectable marker by Cre-loxP-mediated recombination. After *dcaD* mutation biotransformation of 100 mM HMF into FDCA is done in 50 mM phosphate buffer (pH 8) using 45 g/L recombinant *R. ornithinolytica* BF60 at 30 °C. Also, FDCA production is

increased to 9.2 g/L. One side pathway is coming in HMF to FDCA biotransformation pathway, which reduces the production of desired product FDCA. So the HMF may biotransform into HMF alcohol instead of the desired pathway to FDCA though HMF acid or DFF. HMF to HMF alcohols are formed with the help of genes of alcohol reductase (*aldR*) in bacteria. So, if these genes are mutated, FDCA production may also be increased. So the second target alcohol reductase (*aldR*) gene is also mutated (Zong et al., 2003) in the previous *dca* mutated *R. ornithinolytica* BF60. These mutations are confirmed by probe labeling and southern blot analysis. This double mutant recombinant strain is used for the FDCA production analysis and the FDCA production is increased from 9.2 g/L to 11.3 g/L (Hossain et al., 2017) which is not up to the level of industrial standards.

In the pathway of HMF metabolism into FDCA in *R. ornithinolytica*, aldehyde dehydrogenase (*aldh1*) is responsible for the conversion FFCA to FDCA. So the over expression of *aldh1* may be increased the FDCA production. Thee aldehyde dehydrogenase genes (*aldhs*) are over expressed using the vector pBBR1MCS-2 for *R. ornithinolytica* BF60. T7 lac or promoters are added into pBBR1MCS2 with *Sal* I restriction site. T7 RNA polymerase expressed pAR1219 helper plasmid transfected into the pBBR1MCS2 vector under the control of the IPTG-inducible lac UV51. The amplified gene fragments are purified and ligated to a pMD19 simple T vector which is transfected into *E. coli*. These genes are re-amplified with restriction enzyme digestion sites using primers. It is ligated to pET 28a and pBBR1MCS-2 vectors after digestion and transfected into double mutant *R. ornithinolytica* BF60 (RTFB60-2). These *aldh1* recombinants are confirmed by restriction analysis and DNA sequencing. Expression of these thee genes from *R. ornithinolytica* and enzymatic activities have been done by aldehyde reductase, decarboxylase and aldehyde dehydrogenase activity assays. After over expression of *aldh1* into *R. ornithinolytica* BF60 (RTFB60-2) FDCA titer increased to 13.9 g/L which is almost 1.7 times greater than wild-type strain with the molar conversion of 89% from 51%

(Hossain et al., 2017).When higher concentration of HMF (>100 mM) is used for the conversion of HMF into FDCA an inhibition is observed without its complete oxidation.

Fourteen gene expression cassettes are constructed to improve the FDCA production in *R. ornithinolytica* BF60. Genes, *hmfH* (HMF oxidoreductase) and *hmfo* (HMF oxidase) expression cassettes are constructed for fine-tuning of FDCA synthesis from HMF. The genes HMFO from *Methylovorus* sp. MP688 and HmfH from *Cupriavidus basilensis* HMF14 are synthesized and codon-optimized. It is expressed in *R. ornithinolytica* BF60. Plasmids pRSF, pACYC and pCDF, are formulated after replacement of T7 promoter of the respected plasmids with promoters trc and/or tac based on pRSFDuet-1, pACYCDuet-1 and pCDFDuet-1 skeleton. They are electroporated into *R. ornithinolytica* BF60 and λ Red recombination system is used for gene deletion (Datsenko et al., 2000). The recombinant strains are harvested after 24 h of incubation (OD_{600} of 100) and suspended (10 mL) in 50 mM phosphate buffer (pH 8.0) in 100 mL conical flasks. This strain is improved the FDCA production of 108.9 mM with yield of 73%. It is almost 16% higher yield than the non-recombinant strains. Ribosomal binding site sequences of *hmfH* are computationally designed using the RBS calculator and these sequences are assembled into *HmfH* expression cassettes. Ribosomal binding site sequences are inserted into plasmids by using a whole-plasmid PCR with suitable primers for the mutation sites. This expression cassette in *R. ornithinolytica* BF60 improved FDCA yield (93%) of 139.6 mM. Based on RNA-sequencing-based transcriptomics, it has been confirmed that genes dkgA, aldR, akR, adhP1, and adhP2 are responsible for the reduction of HMF into HMF alcohol in *R. ornithinolytica* BF60. These five genes are deleted by a method combinatorial deletion which led to less production of HMF alcohol from HMF. This led to enhancement (12%) in FDCA production of 175.6 mM. This FDCA synthesis is again improved by fed batch strategy (50, 25, 25, 25, and 25 mM HMF in every 12 h from 24-72 h) and 221.5 mM FDCA produced with 88.6% yield. (Yuan et al., 2018). Even though, the FDCA amount is approximate to 34.5 g/L

(221.5 mM) its conversion rate (HMF to FDCA) is less as compared to other metabolically engineered strains.

Sl. No.	Name of the organisms	Media	HMF (g/L)	FDCA (g/L)	Yield (%)	Time (h)	Reference
1	*B. cepacia* H 2	MSM	2	1.27	51.21	24	Yang & Huang., 2016
2	*B. cepacia* H 2	Algal acid hydrolysate	2	1.03	41.53	18	Yang & Huang., 2016
3	*M. radiotolerans* G-2	MSM	3	1.6	43.01	72	Yang & Huang., 2018
4	*M. radiotolerans* G-2	Algal acid hydrolysate	3	0.45	12.1	-	Yang & Huang., 2018
5	*R. ornitholytica* BF 60	Phosphate buffer	12.6	9.2	58.88	14	Hossain et al., 2017
6	*Enterobacter* sps	MSM	0.5	0.07	11.29	336	Rajesh et al., 2018
7	*A. oleiovirans* S 27	MSM	0.5	0.4	64.52	24	Godan et al., 2019
8	*A .flavus* APLS-1	MSM	1	0.83	66.93	336	Rajesh et al., 2019
9	*R .ornitholytica* BF 60	Sodium phosphate buffer	35	41.29	95.14	72	Yuan et al., 2018
10	*P. putida* S 12	Glycerol + HMF	23	30.1	97	144	Koopman et al., 2010
11	*P. brasilanam* CEN.PK Clone 2	Glucose + HMF	0.5	0.03	4.84	40	deBont et al., 2018
12	*P. brasilanam* CEN.PK/PTT2-hmfN1-hmfL1	Glucose + HMF	0.5	0.47	75.81	40	deBont et al., 2018
13	*Y. lipolytica* pol g PVLEX1-hmfL1	Glucose + HMF	0.5	0.24	38.71	40	deBont et al., 2018
14	*R. ornitholytica* BF 60	Phosphate buffer	12.6	14.6	93.45	144	Yuan et al., 2018
15	*R. ornitholytica* BF 60	Phosphate buffer	12.6	11.3	72.32	144	Hossain et al., 2017
16	*R. ornitholytica* BF 60	Phosphate buffer	12.6	13.9	88.96	144	Hossain et al., 2017
17	*R. ornitholytica* BF 60	Phosphate buffer	32	34.5	88.6	144	Yuan et al., 2018

Tab.1.1. Microbial technology for the biotransformation of HMF into FDCA

1.5. Bioprocesses using recombinant and wild enzymes for the synthesis of FDCA

Enzymatic technologies for the biotransformation of HMF into FDCA are summarized in the Tab.1.2. HMF oxidase is a FAD-dependent oxidase from the family of the glucose-methanol-

choline oxidoreductase, which has an oxidase activity on HMF to produce FDCA with molecular oxygen as a cofactor. HMF oxidase gene from *C. basilensis* HMF 14 is expressed in *E. coli* BL21. Affinity chomatography is used for the purification of this expressed gene. This expressed oxidase has the potential of oxidizing HMF alcohol to FDCA by four consecutive oxidations. Each oxidation step is a two-electron oxidation in which one molecule of O_2 is utilized and one molecule of H_2O_2 is liberated by the enzyme. Highest yield (95%) of FDCA is obtained in reaction with 20 µM HMF oxidase and 20 µM FAD from 4 mM HMF after 15 hin 100 mM phosphate buffer (pH 7.0). The reaction is carried at ambient pressure (0.1 MPa) and temperature (25 °C) and above 95% of yield is also obtained if the reaction is kept for morethan 24 h. HMF oxidase has wide substrate specificity for its sequential oxidations and product formation. Normally, a proton is transferred from the alcohol group of any active site followed by a hydride transfer (from 'C' atom of alcohol) to the FAD cofactor by FAD dependent oxidases after alcohol oxidation (Gadda et al., 2008). Since this transfer is not possible in the case of aldehydes most FAD-oxidases are not capable of aldehyde oxidations (Dijkman et al., 2013). It has been confirmed that the full oxidation of HMF (which has aldehyde and alcohol moiety) to FDCA by this HMF oxidase. Even it has higher range of substrate specificity, and aldehyde and alcohol oxidation at ambient temperature and pressure, it needs longer reaction time of more than 24 h for the 100% product yield (Dijkman et al., 2014).

Thee fungal aryl alcohol oxidase enzymes (*Pery*AAO, *Post*AAO and *Badu*AAO), recombinant galactose oxidase (GAO) (expressed in *Aspergillus oryzae*) and peroxygenase from *Agrocybe aegerita* (AaeUPO) are tested for their capability to oxidize HMF into FDCA by multienzyme cascade reaction processes. Thee fungal aryl alcohol oxidase (AAO) enzymes *Pery*AAO, *Post*AAO and *Badu*AAO are produced and purified from wild fungi *Pleurotus eryngii*, *Pleurotus ostreatus* and *Bjerkandera adusta* respectively. Aryl alcohol oxidase enzymes are purified by ion exchange chomatography steps using Q-sepharose and Mono Q columns

followed a size exclusion chromatography using Sephadex 75 column. All these enzymes are analyzed for its conversion efficiency into suitable products for selecting the cascade system for the conversion to HMF to FDCA. Among the three fungal enzymes *Pery*AAO which efficiently converts HMF to DFF is selected for the multi enzyme cascade reactions. AaeUPO is selected because of its efficiency in the conversion of FFCA to FDCA which also utilizes the H_2O_2 produced by the oxidase in the sequential oxidation steps. Galactose oxidase (belongs to the copper radical oxidases family) is selected due to its capacity to oxidize HMF acid to FFCA. So, after all the preliminary analysis of these thee enzymes a multi enzyme reaction set up is converted 9.7 mM HMF into 7.9 mM of FDCA after 24 h with 80% yield in 50 mM phosphate buffer (pH 7.25) (Karich et al., 2018). Here, recombination and purification of thee enzymes for the conversion of HMF into FDCA will not be economically viable for the large scale processes. Also, 24 h is needed for 80% conversion into desired product FDCA.

Oxidative conversion of HMF into FDCA is taken place by an enzymatic cascade system using the enzymes Aryl alcohol oxidase (AAO) from *P. eryngii* and Peroxygenase (UPO) from *Agrocybe aegerita*. AAO is able to transform HMF and DFF by oxidation in presence of O_2 and FFCA and H_2O_2 is formed. HMF to FFCA conversion is taken place after four successive electron oxidations by AAO. Here UPO mainly catalyzes the conversion of HMF into HMF acid and FFCA to FDCA with the expense of H_2O_2, produced from the AAO reactions. After enzyme cascade reaction simultaneous action of AAO and UPO on HMF have not improved FDCA yield. It might be because of UPO oxidization of HMF to HMFCA taken place with the expense of H_2O_2, which is produced by the AAO after HMF to FFCA conversion. So there may be limited amount of H_2O_2 for the action of UPO for the conversion of FFCA to FDCA. In the enzyme cascade system using *P. eryngii* AAO (5 µM) and *A. aegerita* UPO(0.65 µM), 91% FDCA is obtained from 3 mM HMF after 120 h in 5 mL phosphate buffer (pH 6.0 – 7.0)

at 25 °C (Carro et al., 2015). It is a long term reaction for the production of FDCA with low substrate concentration.

The enzymatic cascade is involved with thee fungal oxidoreductases for the production of FDCA from a new substrate 5-methoxymethylfurfural (MMF). MMF is converted into MMFA (5-methoxymethylfurancarboxylic acid) followed by HMF acid, FFCA and FDCA after sequential oxidations. Thee fungal enzymes are involved in this cascade system Aryl-alcohol oxidase (AAO), unspecific peroxygenase (UPO) and Methanol oxidase (MOX). The gene (CDNA) coded for AAO from *P. eryngii* is expressed in *E. coli* W3110 harboring the pFLAG1 vector. *A. aegerita* UPO gene is inserted into the vector pPICZ-B-PaDa-I and heterologously expressed in *P. pastoris*. The enzyme is purified using Sepharose FF and Q-source chomatography columns. MOX is available commercially made from *Pichia pastoris*. MOX is taken methanol released for in situ producing H_2O_2 which is produced by aryl-alcohol oxidase acts as fuel for the peroxygenase reactions. Conversion of MMF is taken place after three or four sequential oxidation steps. It is mainly dependent on whether breakdown of ether leaves an alcohol or a carbonyl function in the furfuraldehyde. Enzyme cascade reactions of AAO (5 μM), UPO (5 μM) along with MOX (1μM) is converted 1.5 mM MMF into 98% FDCA after 120 h of incubation. This reaction is carried out in 100 mM phosphate buffer (pH 7.0) in presence of H_2O_2 (1 mM) and methanol (1 mM) at temperature 28 °C (Carro et al., 2018). This reaction also led with expensive thee enzymes, low substrate concentration and long reaction time. The substrate MMF has not much advantage than the commonly used substrate HMF for the production of FDCA.

An enzyme cascade reaction using this periplasmic aldehyde oxidase, galactose oxidase M3–5 and catalase is performed for the conversion of HMF into FDCA. It has been reported that *E. coli* periplasmic aldehyde oxidase (PaoABC) is a 135 KDa heterotrimeric enzyme which uses oxygen as the electron acceptor has the capability to convert toxic aldehydes into nontoxic

intermediates. It has a large molybdenum cofactor containing PaoC subunit (78.1 KDa), a medium sized FAD containing PaoB subunit (33.9 KDa), and a small [2Fe–2S] PaoA subunit (21.0 KDa) (Neuman et al., 2009) (Leimkuhler et al., 2013). His6 tag fused (N terminal) PaoABC genes heterologously expressed into *E. coli*, pMN100 is transformed into *E. coli* TP1000 cells (deleted mobAB genes). This His-tagged PaoABC is eluted in column chomatography with 100 mM imidazole in 50 mM sodium phosphate (pH 8.0) and 300 mM NaCl. Galactose oxidase variant M3-5 gene (GOase M3-5) is transformed into *E. coli* BL21. The enzyme is purified with 5-mL-Strep-Tag-II column (GE Healthcare) pre-equilibrated with buffer 50 mM sodium phosphate and 300 mM NaCl. It is followed by dialysis in 30 KDa cut-off dialysis membrane and eluted. Along with these purified PaoABC, GOase M3-5 enzymes, catalase is also used for the experiments which purchased from Sigma. In a one pot sequential reaction GOase M3-5 (3.3 mg/mL), catalase (3.3 mg/mL) and 50 mM HMF is added into 400 mM potassium phosphate buffer (pH 7.0) and incubated at 37 °C in a shaking incubator. After all HMF is oxidized, as determined by HPLC, PaoABC (13.3 mg/mL) is added into this reaction mixture and placed back into the shaking incubator for the analysis of intermediates. After 8 h, 90% FDCA is produced with 100% HMF conversion (Mckenna et al., 2015). Use of thee enzymes and low substrate concentration will not make the process economically viable. The multiple substrate specificity of PaoABC oxidation led into conversion of toxic substrates into desired products which make advantage in future.

Tandem oxidations of galactose oxidase (GO) and lipase is designed for the conversion of HMF to FDCA. GO, (8 U) is incubated in deionized water and sodium acetate buffer (50 mM) to replace phosphate buffer. The DFF yield (92%) is increased after 96 h from 30 mM HMF. After this preliminary analysis for the tandem oxidations HMF to FDCA, HMF is transformedinto DFF by GO after 48 h with 75% conversion efficiency. This DFF (22.5 mM) is oxidized using Immobilized lipase B (9.6 mg) from *Candida antartica* (CAL-B, Novozyme) and H_2O_2

(30% v/v) and complete uptake of DFF is taken place within 7 h in 2 mL butanol-Ethanol (1:1, v/v). This DFF is converted into FFCA as the intermediate which is completely transformed into 88% of FDCA after 24 h at 40 °C (Qin et al., 2015).

Four enzymes, periplasmic aldehyde oxidase (PaoABC), galactose oxidase M3-5, horse radish peroxidase and catalase are used for the biotransformation of HMF into FDCA as Continuous one-pot reaction set up. It is known that Horse radish peroxidase (HP) can activate GOase, and is used in this reaction. Here, PaoABC uses atmospheric oxygen from air as an electron acceptor. The reaction is carried out in 200 mM phosphate buffer (pH 7.0) using enzymes PaoABC (28.9 mg/mL), GOase M3-5 (3 mg/mL), catalase (3.30 mg/mL) and HP (1 mg/mL) at 37 °C. This enzyme cascade system is converted 50 mM HMF into FDCA (100% yield) after 6 h (Mckenna et al., 2017). It is noted that unwanted formation of HMF acid during FDCA synthesis can be avoided by stepwise addition of the enzymes. Use of atmospheric oxygen is resulted in higher rate of HMF transformation and therefore higher substrate concentration is also used in this study (>100 mM HMF).

Combined-cross linked enzyme aggregates (combi-CLEAS) are multi-functional biocatalyst used for the formulation of immobilization of two or more enzymes. Also combi-CLEAS are applied as versatile carrier free immobilized systems. So these can be used for merging multi-step enzyme cascade and non-enzyme cascade biotransformations of HMF into one pot reaction (Ning et al., 2014) (Dalal et al., 2007). PaoABC is immobilized into PaoABC-Gel and catalase is entrapped into CLEA and both CAT-CLEA and PaoABC-Gel are joined to form a complex. This PaoABC-Gel/CAT-CLEA complex is capable of tolerating higher substrate concentration (DFF) up to 200 mM with recyclable capacity of 14 times without loss in product (FDCA) yield (Mckenna et al., 2017).

Sl. No	Name of the enzymes	Media	HMF (g/L)	FDCA (g/L)	Yield (%)	Time (h)	Reference
1	1) HMF oxidase	Phosphate buffer	0.5	0.59	95	15	Dijkman et al., 2013
2	1) Pery AAO 2) Aae UPO 3) GAO	Phosphate buffer	1.2	1.21	80	24	Karich et al., 2018
3	1) *P. eryngii* AAO 2) *A. aegerita* UPO	Phosphate buffer	0.39	0.43	91	120	Carro et al., 2015
4	1) AAO 2) UPO 3) MOX	Phosphate buffer	0.19	0.23	98	120	Carro et al., 2018
5	1) PaoABC 2) GAO M3-5 3) Catalase	Phosphate buffer	6.3	7	90	8	Mckenna et al., 2015
6	1) GAO 2) Lipase	Butanol-Ethanol & Sodium acetate buffer	3.78	4.12	88	48	Qin et al., 2015
7	1) PaoABC 2) GAO M3-5 3) HRP 4) Catalase	Phosphate buffer	6.3	7.81	100	6	Mckenna et al., 2017

Tab.1.2. Enzymatic technologies for the biotransformation of HMF into FDCA

1.6. Challenges on the green synthesis of FDCA

The reports suggest that the green synthesis of FDCA is possible by using microorganisms and enzymes. Still none of the industries have established a lab for FDCA by complete green technologies. So far these industries are trying to produce FDCA from lignocellulosic biomass waste and cheap sources at least by partial chemical treatments with mild or toxic chemicals. Apart from that biological synthesis of FDCA favors non-toxic media ingredients. In the case of microbial assisted FDCA production, the reported microorganisms show highest yield of FDCA production is showed after media optimizations, genetic engineering and metabolic engineering approaches. But the highest production of FDCA by these microorganisms happen after 2-3 days only, which is a drawback for the large scale production by Industries. Hence, microbial conversion of HMF into FDCA is a time consuming process. Even the conversion of HMF to FDCA is a complex biotransformation, intermediates like DFF and FFCA get transformed into FDCA quickly as compared to HMF acid which interferes or less convert into FDCA. HMF acid is seen in extracellular medium of both bacteriaand fungus along with HMF alcohol, FFCA and FDCA during biotransformation process. Uptake of HMF is faster but the extracellular HMF acid uptake is a slower process for all microorganisms whichis a rate limiting step and time consuming for the biotransformation of HMF into FDCA. It might be due to the less toxicity of HMF acid to bacteria or fungus and the non-availability of enzymes for its alcohol group to aldehyde group conversion. Produced FDCA might be taken up by microrganisms,if there is no carbon source is present in the medium and it will be progressing though TCA cycle forthe metabolism. So engineering of media has to play with microbial growth conditions along with HMF biotransformation focusing on FDCA synthesis.

Also, microorganisms with higher substrate tolerance can be created though media engineering, genetic engineering, transport protein engineering and metabolic engineering approaches. Here the toxic aldehyde, HMF, can be bio-transformed, only if the cells have the capacity to uptake it more

without substrate toxicity. So, these approaches made HMF tolerant microorganisms up to 150 mM and with higher FDCA yield. This HMF toxicity can be overcome by fed batch or continuous fermentation modes for large scale FDCA production. Among them, the reports are concentrating on batch and fed batches only, which have many limitations. Still, this substrate tolerant concentration and FDCA production is not enough for the large scale production of industries. The high HMF tolerant and FDCA produced microorganisms have to be exploited and explored by engineering or natural approaches from the laboratory scale to industrial scale. Another concern is that most of the microbial biotransformation works have been carried out in flask level only. These works have not yetdone in fermenters. So higher scale of these works and its pilot plant works should be established in future. And the techno economic analysis (TEA) and Life cycle assessment (Dijkman et al., 2014) of FDCA production though microbial transformations are also needed to be carried out with itsenvironmental effects.

Enzyme biotransformations are fast and time consuming. But most of the works have been successively carried out with recombinant and wild enzymes though one pot or enzyme cascade system which make enzyme assisted biotransformations as not economical. Also, the inhibitors like H_2O_2 hinder its activity and reduce substrate binding and product formation. So, this should be eliminated or used by another enzyme using H_2O_2. Alcohol to aldehyde group and aldehyde to acid group of HMF and its intermediates have to be transformed only using multiple enzymes which make the sequential reaction as a complex one. Enzymes have higher substrate tolerance and high yield but all the reactions are in laboratory scale only. Also, these experiments or reactions are carried out in milliliters only. So this will be another challenge for the biotransformation of HMF into FDCA using enzymes from infant stage to pilot scale with TEA and LCA analysis. Then only industries will come forward for its large scale production.

Chapter 2

Chemo-catalytic conversion of pineapple peel into HMF

2.1. Introduction

Industrial production of wide range of chemicals heavily relies on fossil resources and this create huge environmental issues. There is an urgent need to find alternative green and sustainable methods for the production of industrial chemicals. Agricultural residues offers a potential source of sustainable raw materials for the production of wide range of products. Among this, pineapple waste is a renewable material that could be a good source of sugars and can be used for the synthesis of valuable products. Pineapple is one of the most popular tropical fruits consumed worldwide with a global production of 25.8 million tonnes (t) per year. Also, India is the fifth largest producer of pineapple in the world. Kerala has an average production of 85,500 t production per year with a productivity of 8.4t/ha. Pineapple parts (Fig. 2.1) mainly consist of peel, core, stem and crown. During pineapple production, processing and consumption several tons of by-products like pineapple peel, leaves, crown and juice waste are produced which are usually discarded in soil (Sznida., 2018) and it causes environmental problems (Campos et al., 2020).

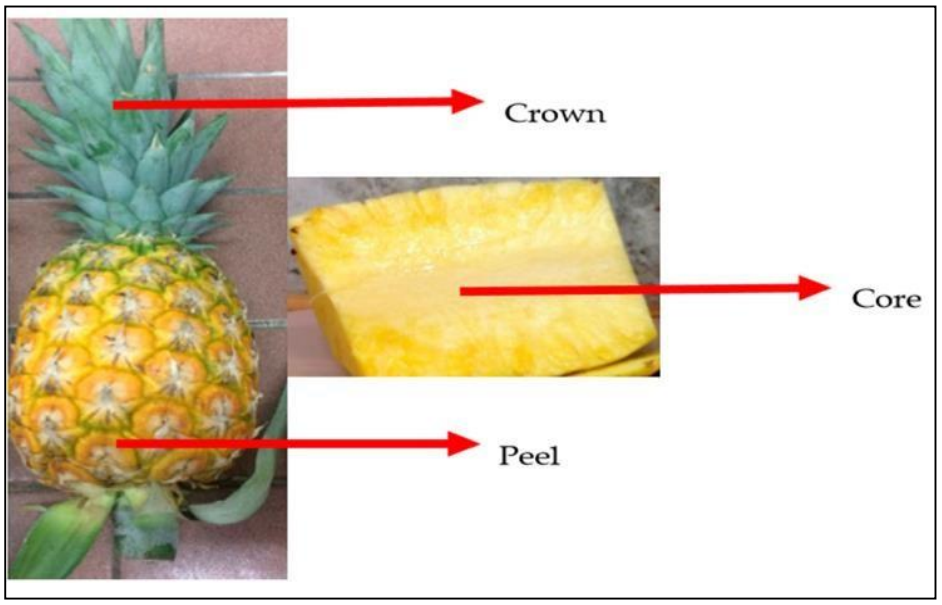


Fig. 2.1. Pineapple parts

These waste are mostly generated from poor handling of fresh fruit, storage, or lack of good and reliable transportation system (Praveena and Estherlydia 2014). Also, disposal of this waste requires

a huge capital investment, majority of which is the transportation cost. Improper management of these wastes would result in the deterioration of environmental quality which can be attributed mainly to the degradation of the sugar-rich contents. So, creation of high value-added product like HMF from pineapple waste will have a positive effect not only in economic point and environmental cleanliness, but also maintain pineapple plantations, help farmers and associated industries. In view of this, pine apple waste was treated with catalysts like chromium (III) chloride ($CrCl_3$) and Seralite SRC (solid acid catalyst) in ionic liquid 1-butyl-3-methyl immidazolium chloride (BMIM.Cl) to produce HMF.

2.2. Materials and methods

2.2.1. Chemicals

Monosaccharide standards such as glucose, xylose, galactose, arabinose, mannose, cellobiose, sucrose and fructose were purchased from Sigma-Aldrich, India. $CrCl_3$, Seralite SRC and BMIM.Cl were purchased from SRL, India.

2.2.2. Composition analysis of pineapple waste

2.2.2.1. Preparation of pineapple waste

Pineapple waste was collected from CSIR-NIIST canteen. It was separated as peel, core and juice waste. Pineapple peel and core waste were cut into small pieces of size 1 cm^3 and kept in hot air oven for drying at 60 ^oC for 60 h. It was milled in mixer grinder to produce fine powder.

2.2.2.2. Extraction of free sugars

Free sugar was extracted using B-811 Soxhlet extraction system (Fig. 2.2). Pineapple peel, core and juice waste (5 g each) were weighed and wrapped inside the Whatman filter paper and kept in the soxhlet extraction system to remove non-volatile compounds. Soxhlet extraction was carried out in two different stages, water extraction followed by ethanol extraction. Then the extracted liquid was

analysed in HPLC and sugar amount was estimated. The wet biomass was kept in the hot air oven for 12 h at 50 ˚C and the dried pineapple peel, core and juice waste was used for further studies. .

Fig.2.2. Buchi B-811 soxhlet extraction system

2.2.2.3. Composition analysis after acid hydrolysis

Moisture of the dried of pineapple peel, core and juice waste were determined and approximately 300 mg of dry biomass was transferred to pressure tubes. 72% (w/v) of sulphuric acid (H_2SO_4) (3 mL) was added to each tubes and the content were mixed well using a glass rod and placed in a shaking water bath at 40 ˚C at 100 rpm for 2 h. After 2 h, these tubes were transferred to hydrolysis flasks and 84 mL of distilled water were added into them. The flasks were autoclaved for 1 h at 121 ^oC at 15 psi. The content were allowed to cool after autoclaving and then filtered using vacuum filtration apparatus through dried filter paper (weighed before). The collected solid cake was kept for 12 h for drying at 50 ^oC. The obtained liquid filtrate (15 mL) was then neutralised using calcium carbonate ($CaCO_3$) followed by centrifugation twice at 10,000 rpm for 5 min at 4 ^oC. The clear supernatant was taken for HPLC analysis to determine the amount of sugars in it. Monosaccharides were analysed by HPLC (Shimadzu, Japan) using RezexTM RPM Monosaccharide PB^{+2} column (Phenomenex, India) with

pore size 5 µm and size 300*7.8 mm with 0.05 M H_2SO_4 (0.6 ml/min) as mobile phase at 80 ºC for 30 min. They were detected by RI detector.

The acid soluble lignin in the non-neutralized filtrate samples were determined by diluting 20 times and absorbance noted at 205 nm using spectrophotometer. The weight of the filter cakes were determined after drying overnight at 50 ºC andthen samples transferred into crucibles. It was kept in a muffle furnace at 575 ºC for 4 h. It was then allowed to cool and the weight of the ash was calculated. Cellulose, hemicellulose, lignin and ash content of pineapple peel, core and juice waste were determined.

2.2.3. free sugar analysis in pineapple peel powder

Pineapple peel powder (500 mg) was dissolved in 10 ml of water and incubated for 1h. The free sugars like glucose, fructose and sucrose were analyzed by HPLC.

2.2.4. Production of HMF from pineapple peel

Dried, fine powder of pineapple peel was treated with catalysts $CrCl_3$ and Seralite SRC in BMIM.Cl as a solvent to produce HMF and samples were analysed by HPLC. The reaction mixture was optimized at different temperature, amount of catalyst and amount of substrate at different time intervals to maximize the HMF production.

2.2.4.1. Optimization of temperature

Pineapple peel powder of 50 mg was treated with 2 different catalysts $CrCl_3$ (40 mg) and Seralite SRC (50 mg) in 1g of BMIM.Cl and the reactions were kept at various temperature such as 70, 80, 90, 100, 110, and 120 °C. Samples were taken at different time intervals such as 15 min, 30 min, 1h, 2h and 4h. Reaction results were compared and optimum temperature was selected for further studies.

2.2.4.2. Optimization of catalyst amount

Pineapple peel powder of 50 mg was treated with different amount of catalysts $CrCl_3$ and Seralite SRC

in 1 g of BMIM.Cl and the reactions were kept at 100 °C. Samples were analysed at different time intervals.

2.2.4.3. Optimization of substrate amount

Different amount of pineapple peel powder (50-90 mg) was treated with $CrCl_3$ of 20 mg in 1g of BMIM.Cl and the reactions were kept at 100 °C. Samples were analysed at different time intervals.

2.2.5. Production of HMF from commercial sugars

Glucose and fructose (50 mg each) were treated with the similar optimized conditions used for pineapple peel powder. Reaction mixtures were treated with 20 mg of $CrCl_3$ in 1 g BMIM.Cl and incubated at 100 °C for 4 h. Samples were analysed at different time intervals.

2.2.6. Purification of HMF

The collected reaction mixture was mixed with double the volume of chloroform in a separating funnel and kept for 1 h. This was repeated two times. Two phases were obtained, in which lower phase containing the HMF and chloroform, and upper phase with catalyst and reacted peel powder waste. The lower phase which contains HMF was separated and chloroform was evaporated using rotary evaporator and analyzed by HPLC.

2.3. Results and Discussion

2.3.1. Composition analysis of pineapple waste

Free sugars present in the pineapple peel, core and juice waste after acid hydrolysis were analysed. Cellulose, hemicellulose, lignin and ash content in the pineapple peel, core and juice waste were determined (Tab.2.1). Cellulose and hemicellulose content in pineapple peel were observed as 41.6±2% and 22.76±1.14% respectively. In pineapple core, cellulose and hemicellulose were observed as 49.34±2.47% and 18.71±0.93% respectively. The amount of lignin and ash content in pineapple peel waste powder were estimated as 26.86±1.34% and 0.67±0.03% respectively and its total sugar

content as 91.89±4.59%. It has been reported that pineapple peel powder contain 36.21±5.31% cellulose, 21.37±4.15% hemicellulose and 4.56±0.03% ash content (Dai et al., 2019). Free sugars like sucrose, glucose and fructose content in 50 mg of pineapple peel (Fig. 2.3) were found to be 6.09±0.37, 5.0±0.3and 6.5±0.39 mg respectively. For one gram of pineapple peel sucrose, glucose and fructose were calculated as 0.12±0.01, 0.10±0.01 and 0.13±0.01 g/g respectively.

	Composition (%)				
	Cellulose	Hemicellulose	Lignin	Ash	Total (%)
Pineapple peel powder	41.6 ± 2	22.76 ± 1.14	26.86 ± 1.34	0.67 ± 0.03	91.89 ± 4.59
Pineapple core	49.34 ± 2.47	18.71 ± 0.93	19.23 ± 0.96	0	87.29 ± 4.36
Pineapple juice waste	46.06 ± 2.3	17.3 ± 0.86	17.22 ± 0.86	0.1 ± 0.005	81.93 ± 4.09

Tab. 2.1. Composition of pineapple peel, core and juice waste powder

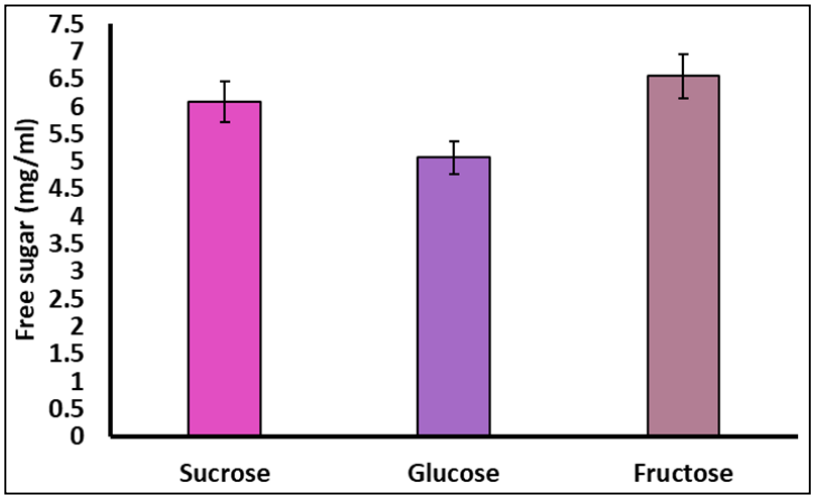


Fig. 2.3. Free sugars present in the pineapple peel powder (50 mg)

2.3.2. Production of HMF from pineapple peel

2.3.2.1. Temperature optimization

The two different catalysts, $CrCl_3$ (40 mg) and seralite SRC (50 mg) were treated at different temperatures. $CrCl_3$ was treated at temperature ranging from 70 - 120 °C (Fig.2.4) and seralite SRC at

90 – 110 °C (Fig.2.5) with 50 mg of pineapple peel powder in 1 gm of BMIM.Cl and optimum temperature was found to be 100 °C for both catalysts. After 30 min of the reaction, 12.27±0.61 mg/ml of HMF (0.245±0.01 g/g) was produced from 50 mg of pineapple peel using $CrCl_3$ catalyst. It is better than the solid acid catalyst seralite SRC which produced only 8 mg/ml (0.16±0.01 g/g) of HMF.

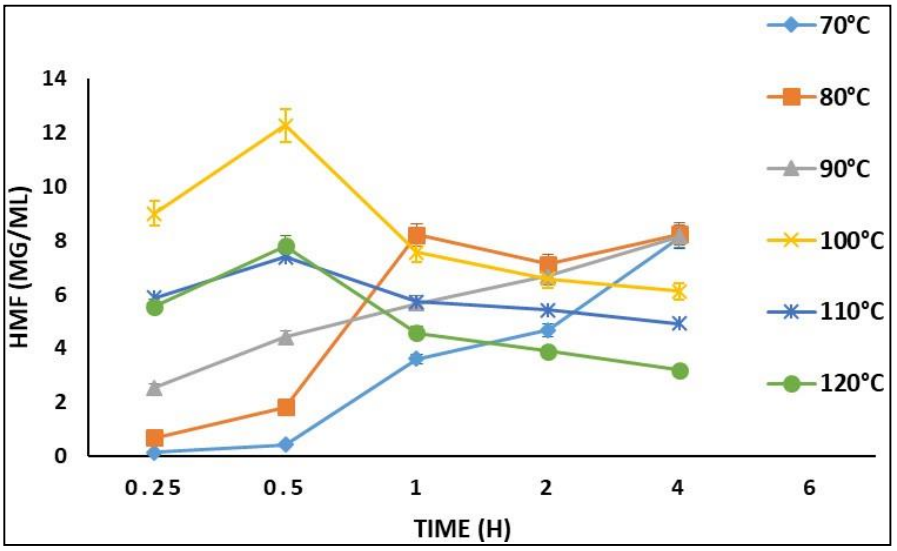


Fig.2.4. Production of HMF with $CrCl_3$ at different temperatures

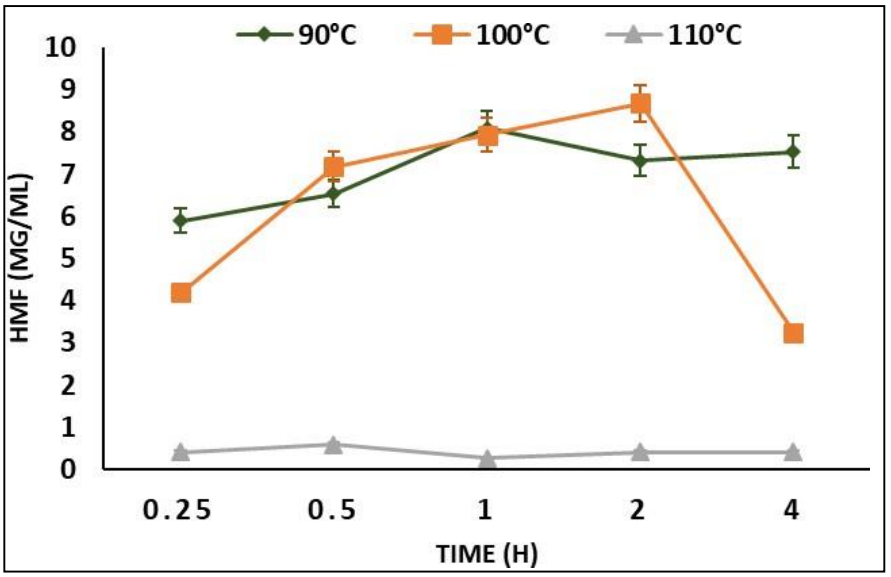


Fig.2.5. Production of HMF with seralite SRC at different temperatures

2.3.2.2. Optimization of catalyst concentration

Pineapple peel (50 mg) was treated with different concentration of catalysts such as $CrCl_3$ and Seralite SRC in 1 g of BMIM.Cl and the reactions were kept at 100 °C. Among them, 20 mg of $CrCl_3$ (Fig.2.6) showed best production with HMF yield of 14.83±0.74 mg/ml (0.3 g/g) after 1 h. In the case of seralite SRC (Fig.2.7), 100 mg of the catalyst converted 11.26±0.56 mg/ml (0.23 g/g) of HMF after 2 h. After this, $CrCl_3$ was selected for further studies.

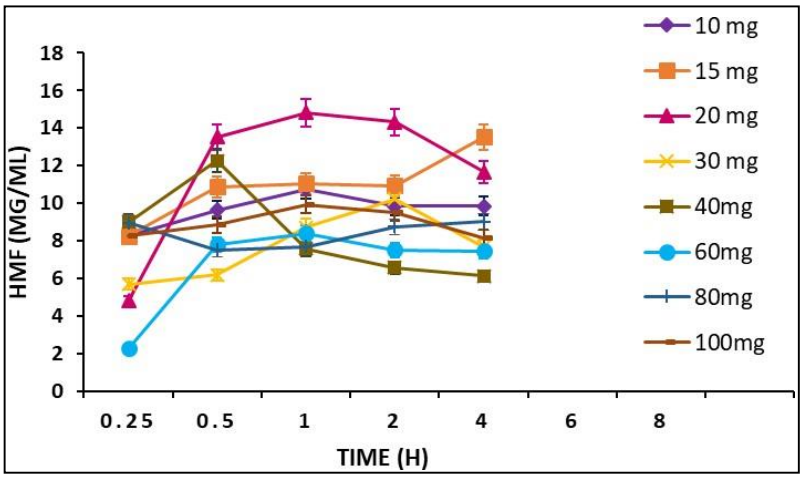


Fig.2.6. Optimization of amount of $CrCl_3$ on HMF production

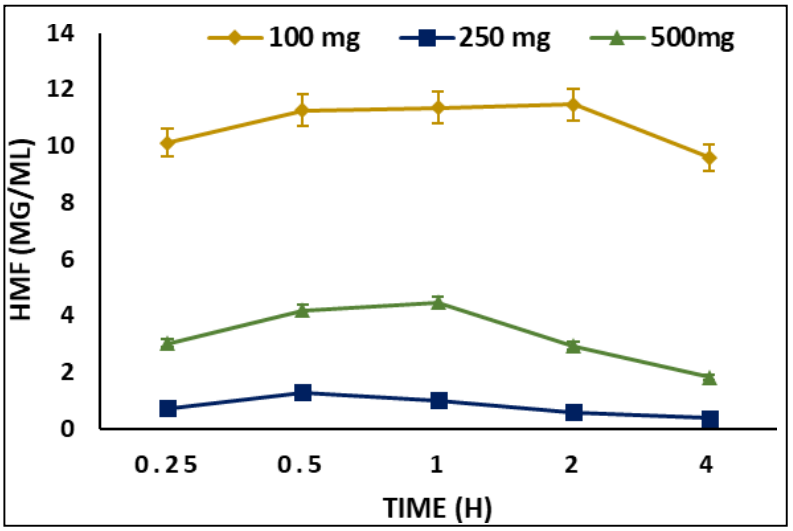


Fig.2.7. Optimization of amount of seralite SRC on HMF production

2.3.2.3. Optimization of substrate amount

Different amount of pineapple peel powder (50-90 mg) was treated with 20 mg of $CrCl_3$ in 1 g of BMIM.Cl and the reactions were kept at 100 °C. After 1 h of the reaction, 22.67±1.36 mg/ml of HMF (Fig.2.8) was produced from 80 mg of pineapple peel powder which was approximately 0.29±0.02 g/g.

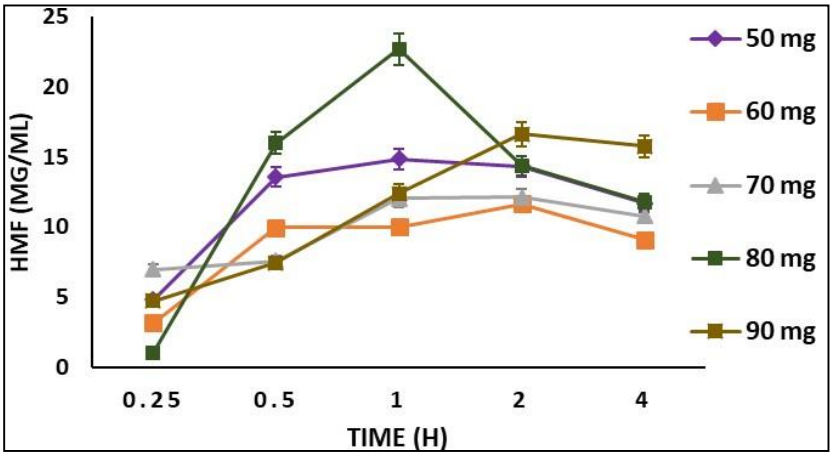


Fig.2.8. Optimization of amount of pineapple peel on HMF production

2.3.3. Production of HMF from commercial sugars

Glucose and fructose (50 mg each) were treated with 20 mg of $CrCl_3$ in 1 g of BMIM.Cl and incubated at 100 °C to know the action of catalyst on these monosaccharides (Fig.2.9). Fructose converted into 34.4±2.4 mg/ml (0.69±0.05 g/g) of HMF in 15 min and glucose converted into 18.6±1.3 mg/ml (0.37±0.03 g/g) of HMF in 30 min respectively. In percentage yield, (Fig.2.10) HMF from fructose and glucose were 98.28±1.49 and 53.16±3.19% respectively.

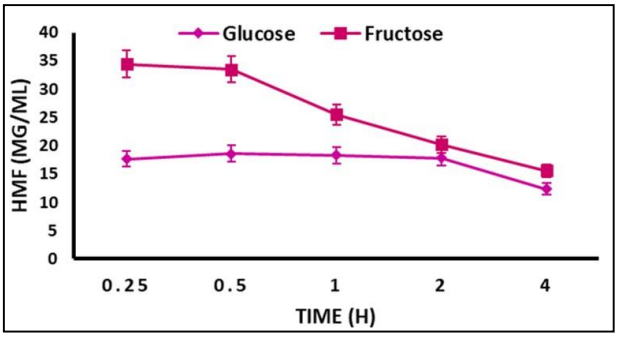


Fig.2.9. Conversion of commercial sugars into HMF

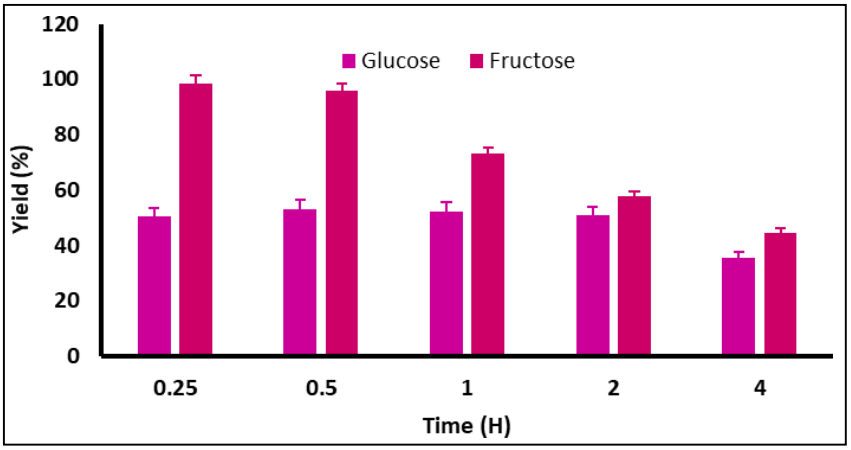


Fig.2.10. Percentage yield of commercial sugars into HMF

2.3.4. Purification of HMF

Pineapple peel was converted into HMF and it was purified using chloroform extraction method. It was tested using HPLC and showed 98% of purity (Fig.2.11). According to the reports, after dehydration of fructose to HMF some amount of levulinic acid and humins are obtained (Jung et al., 2021).

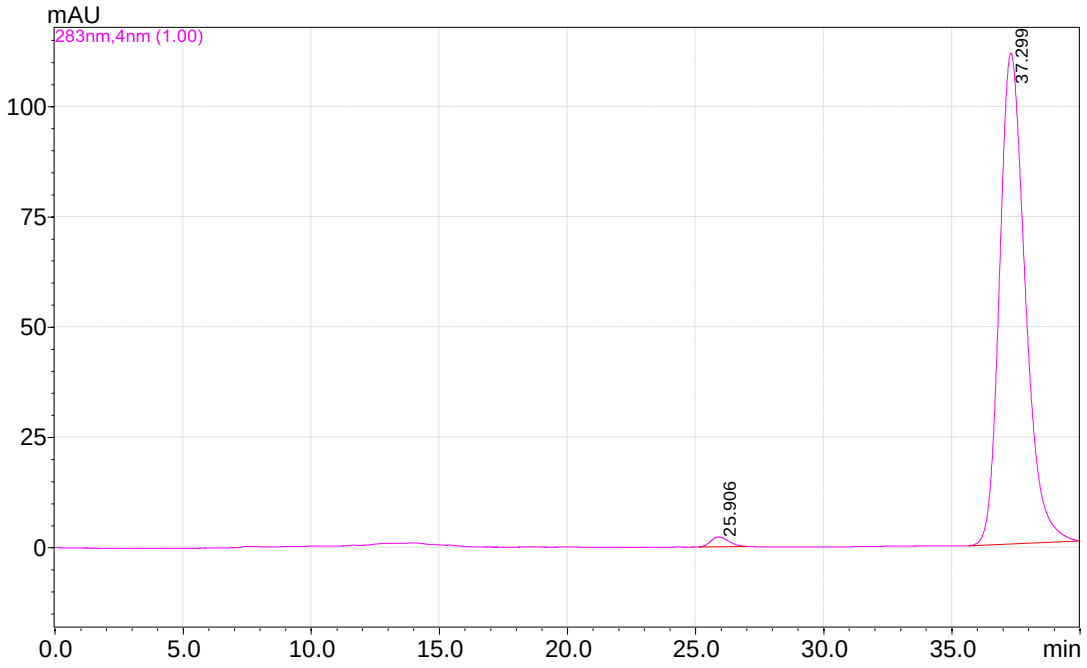


Fig.2.11. HPLC chromatogram of sample with HMF

2.4. Summary

Composition analysis of pineapple peel and core powder showed 49.34 and 41.6% cellulose, 22.76 and 18.71% hemicellulose respectively. Maximum production of 0.29±0.02 g/g HMF was obtained from pine apple peel powder at 100 °C with an incubation time of 1 h. In these conditions, fructose converted into 0.69±0.05 g/g of HMF in 15 min and glucose converted into 0.37±0.03 g/g of HMF in30 min respectively. In percentage yield, HMF from fructose and glucose were 98.28±1.49 and 53.16±3.19% respectively. HMF synthesized from pineapple peel was purified by chloroform extraction followed by rotary evaporation.

Chapter 3

Isolation, screening and identification of microorganisms for FDCA production

3.1. Introduction

Even though large scale FDCA synthesis through chemical catalysts are reported (Zhang & Deng, 2015), these production methods involve metal salts and organic solvents using high temperature and high pressure which is non-eco-friendly and expensive. As an alternative to chemical processes (Han et al., 2017; Xu et al., 2017), greener and environmental friendly approaches are more attractive in order to solve the environmental and global warming issues. In this context, the biological conversion of HMF to FDCA through microbial routes is highly important and acceptable as this method is green and solves most of the environmental related issues. Biocatalysts offer a potential candidates for many chemical reactions as it works in mild reaction conditions and environmentally benign. Usually, HMF is a toxic chemical for the microorganism due to the presence of furan aldehyde group which inhibits metabolism. Also, these aldehydes form reactive oxygen species which damage proteins, nucleic acid and all other cell organelles. But, there are certain microorganisms which can survive in media containing HMF and transform more toxic HMF to comparatively less toxic FDCA. In our study, whole cell biocatalytic approach was used for the biotransformation of HMF to FDCA. Microorganisms from different sources were isolated at different concentration of HMF and screened for FDCA production. After screening, potent microorganisms were identified and selected for further studies.

3.2. Materials and methods

3.2.1 Chemicals

HMF and FDCA were procured from Sigma Aldrich, India. 5-(Hydroxymethyl)furfuryl alcohol (HMF alcohol), 5 – Hydroxymethyl-2-furancarboxylic acid (HMF acid), Diformyl furan (DFF), Formyl furancarboxylic acid (FDCA) and 2-furoic acid were procured from Carbosynth Chemicals, UK.

3.2.2. Isolation of microorganisms by enrichment culture technique

The soil sample was collected from Acid Pre-treatment Liquor (APL) drainage site (CSIR-National Institute Interdisciplinary Science and Technology, Trivandrum) at Kerala, India. For the enrichment culture technique, five gram of soil sample was suspended in 95 mL of Mineral Salt Media (MSM) of pH 7.2 containing HMF ranging from 0.25 g/L to 3 g/L in 250 ml Erlenmeyer flasks and kept in incubated shaker at 200 rpm for 72 hrs at 30 °C. One ml of the above suspension culture was diluted in 99 mL sterile distilled water and a series of dilutions ($10^{-2} – 10^{-10}$) were performed. Single colonies of different morphologies were isolated on HMF agar plates (Nutrient and Potato dextrose agar) ranging from same HMF concentrations (0.25 g/L to 3 g/L) (Yang & Huang., 2016). The plates were incubated for two to five days at 30 ºC and 37 ºC for fungal and bacterial cultures respectively. Isolated single colonies were stored in a refrigerator at 4 ºC.

3.2.3. Screening of microorganisms for FDCA production

Isolated microorganisms were pre-cultured before inoculating into appropriate media. Single colony of pure culture bacteria was inoculated into 50 ml nutrient broth and kept for 14 hrs at 37 °C at 200 rpm. From this, one percentage inoculum was transferred into 100 ml nutrient broth in 250 ml conical flasks and kept for 24 hrs in an incubated shaker at 37 °C at 200 rpm. From this, 10 percentage inoculum was collected and centrifuged at 8000 rpm for 10 min. The lower phase contained biomass was collected and washed three times in phosphate buffer saline (PBS) solution and transferred into 50 ml of MSM (pH 7.2) containing 0.5 g/L HMF in 250 ml conical flasks and kept for 14 days in an incubator shaker at 200 rpm at 37 °C (Yang & Huang, 2016). The samples were withdrawn in frequent intervals and analysed by HPLC.

In the case of non-sporulating fungus, 3 mm potato dextrose agar disc containing pre-grown fungus was inoculated into 100 ml potato dextrose broth in 250 ml conical flasks and kept in

an incubator shaker at 200 rpm at 30 °C for 72 hrs (Rajesh et al., 2018). For sporulating fungus, $1x10^6$ spores were inoculated into 100 ml potato dextrose broth in 250 ml conical flasks and kept in an incubator shaker at 200 rpm at 30 °C for 72 hrs. In both cases, after 72 hrs, media with organism (20 ml) was collected followed by filtration using a sieve (2 mm pore size) and funnel. Collectedbiomass was washed three times in PBS. It was transferred into 50 ml of MSM (pH 7.2) containing 0.5 g/L HMF in 250 ml conical flasks and kept for 14 days in an incubator shaker at 200 rpm at 30 °C. The samples were withdrawn in frequent intervals and analysed by HPLC.

3.2.4. Biocatalytic activity of AS-3 for FDCA production in presence of HMF and different carbon sources

FDCA production of isolated bacterial strain AS-3, was carried out in presence of HMF and different carbon sources. The carbon sources used were 0.25% glucose, and 0.25% glycerol apart from 0.5 g/L HMF in 50 ml MSM. Production media contained MSM with 0.5 g/L HMF and the pre-grown organism AS-3, along with carbon sources. This reaction mixture was incubated in a shaker at 200 rpm at 30 °C for 20 days and the samples were withdrawn in specific intervals for analysis. Pure single colony of AS-3 was inoculated in MSM containing 0.5g/L HMF and growth was observed for five days. For understanding its intolerance, the same experiment was performed in presence of 0.25% and 0.50% glucose.

3.2.5. Biocatalytic activity of fungus (CO-2 and APLS-1) for FDCA production in presence of HMF and different carbon sources

FDCA production of CO-2 and APLS-1, were carried out in presence of HMF and different carbon sources as explained earlier for AS-3. Production media contained MSM with 0.5 g/L HMF and the pregrown organisms (as described earlier) for fungus along with other carbon sources. This reaction mixture was incubated in a shaker at 200 rpm at 30 °C for 20 days and the samples were analysed.

3.2.6. Identification of the AS-3 and APLS-1

Bacterial DNA was isolated according to the manufacturer's protocol (Gene Elute™ Bacterial Genomic DNA Kit, Sigma). The 16S rRNA gene was amplified in a Biorad- Thermal cycler using universal primers 27F: 5' AGAGTTTGATCMTGGCTCAG 3' and 1492R: 5' TACGGYTACCTTGTTACGACTT 3'. Conditions of the PCR cycle was initial denaturation at 94 °C for 2 min, followed by 30 cycles of 94 °C for 1 min, 54 °C for 1 min, and 72 °C for 2 min. PCR products were purified using a QIA quick gel extraction kit (Qiagen) followed by sequencing on an ABI model 3130 automatic DNA sequencer using a Big Dye terminator cycle sequencing kit (Applied Biosystems).

Fungal spores ($1x10^6$ spores) were inoculated in 100 ml potato dextrose broth and kept for 48 hrs in an incubator shaker at 200 rpm at 30 °C. The biomass was filtered using Whatman filter paper and fungal DNA was isolated according to the universal phenol–chloroform protocol. The Internal Transcribed Spacer region was amplified in a thermal cycler (Biorad) using universal primers ITS 1F: 5' CTTGGTCATTTAGAGGAAGTAA 3' and ITS 4R: 5' CAGACTTGTATATGGTCCAG 3'. Conditions of the PCR cycle were initial denaturation at 95 °C for 5 min followed by 30 cycles of 95 °C for 1min 15 sec., 65 °C for 1 min and 72°C for 2 min. and the final step extension at 72°C for 10 min. PCR products were purified using a QIA quick gel extraction kit (Qiagen) and sequenced using above mentioned instrument.

3.2.7. Analysis of HMF alcohol, HMF, HMF acid, DFF, FFCA, FDCA and 2-furoic acid

The amount of HMF alcohol, HMF, HMF acid, DFF, FFCA, FDCA and 2-furoic acid in the reaction mixture were determined by using gradient RP-HPLC (Shimadzu, Japan). The column used for the analysis of these compounds was C–18 (Phenomenex) with pore size 5 µm and size 150 x 4.6 mm kept at 40 °C. Mobile phases used were 0.06N H_2SO_4 (mobile phase A) and

Acetonitrile (mobile phase B). Acetonitrile was run at 6-2% for 1-2 min and 2-6% next 2 to 15 min at flow rate of 1 ml/min for 20 min.

3.3. Results and discussion

3.3.1. Isolation and screening of microorganisms for FDCA production

Total 49 microorganisms were isolated from different sources (Fig. 3.1) (Tab.3.1). Based on the morphological differences, isolates were segregated into bacteria (46 Nos.) and fungus (3 Nos.). Also, bacteria, *Gluconobacter oxydans* MTCC 904 and three fungus, *Pleuroteus* sps, *Calocybe* sps MM 6/7, and *Calocybe* CO-2 were purchased for our study. So, total 53 strains were selected for screening of FDCA production. Among them, a bacteria AS-3 and, two fungus CO-2 and APLS-1 showed better FDCA production as compared to other isolates and it was selected for further studies. It has been reported that, after screening, five strains are isolated and grown in the media containing HMF showed FDCA production upon analysis (Yang and Huang., 2016).

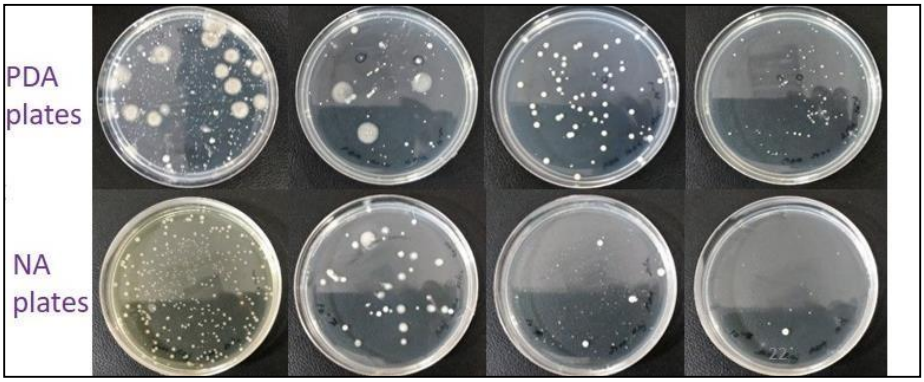


Fig. 3.1. Isolated microorganisms

Sl.No	Source	Number of microorganisms	
		Bacteria	Fungus
1.	APL - drainage soil	4	3
2.	Long term stored APL	4	
3.	NIIST Soil	7	
4.	Leaf litter soil	3	
5.	Drainage soil	3	
6.	North Eastern ghat	20	
7.	Western ghat	5	
	Total (Isolated strains)	**49**	
	Purchased strains:		
9.	*Gluconobacter oxydans* MTCC 904	1	
10.	Mushrooms from Koonpura (*Pleuroteus* sps, *Calocybe* sps MM 6/7, CO-2 16/6)		3
	Total	**53**	

Tab. 3.1. Total isolated and purchased microorganisms

3.3.2. Biocatalytic activity of AS-3 for FDCA production in presence of HMF and different carbon sources

The organism AS-3 was shown better FDCA production in the MSM containing 0.5 g/L HMF only. Maximum production was obtained after 14 days of about 0.07 g/L, without any carbon sources other than HMF (Fig.3.2). When carbon sources like 0.25% glucose and 0.25% glycerol was added, FDCA production decreased (Fig.3.3). It is meant that, in presence of carbon sources and HMF, AS-3 utilized glucose and glycerol rather than HMF for its metabolic activities. Also, other carbon sources might have supported the growth of this microorganism, instead of the FDCA production from HMF. Hence, in presence of these carbon sources FDCA production decreased by two times as compared to MSM only with HMF.

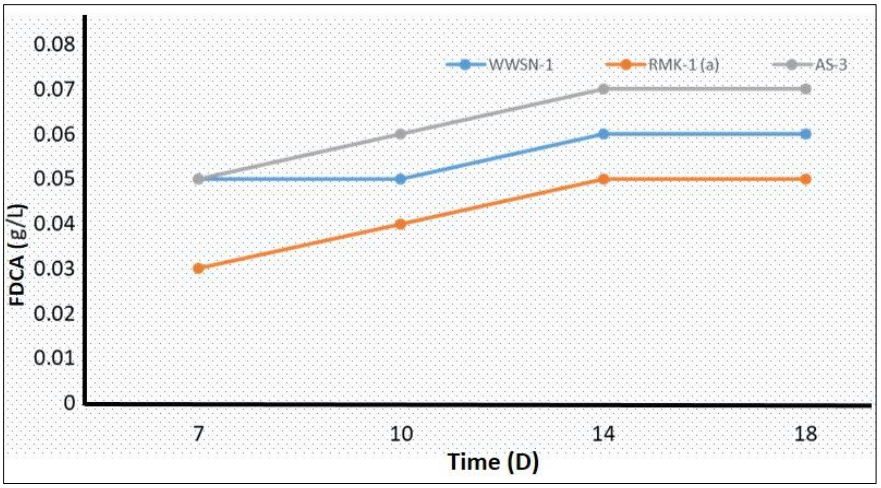


Fig. 3.2. Bacterial strains showing FDCA production

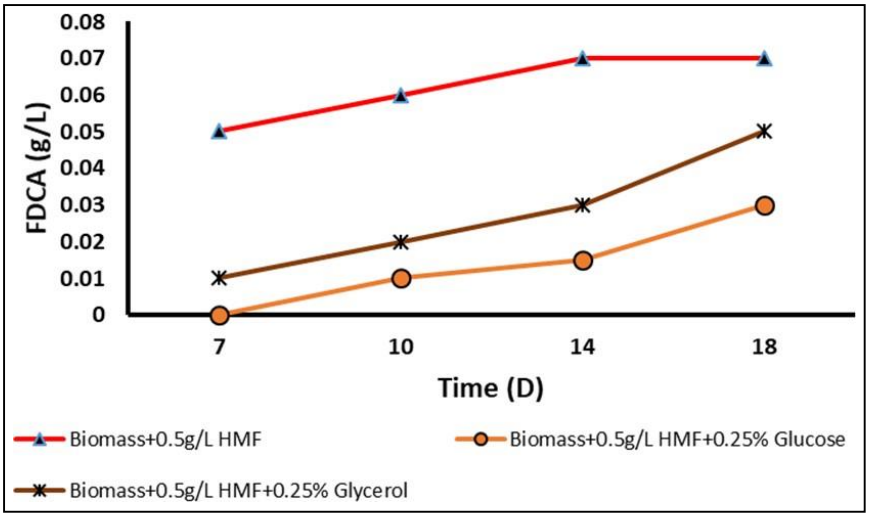


Fig.3.3. FDCA production of AS-3 in MSM with different carbon sources

3.3.3. Biocatalytic activity of fungus (CO-2 and APLS-1) for FDCA production in presence of HMF and different carbon sources

The isolated and purchased strains were selected for screening of FDCA production. Among them, fungus CO-2 and APLS-1 (Fig.3.4) showed better FDCA production as compared to other isolates and selected for further studies. In presence of 0.25% glucose (Fig.3.4.A) and 0.25% glycerol (Fig.3.4.B) both fungus showed reduction in FDCA production as compared to

MSM with HMF (Fig.3.4.C). It was similar to the result of bacteria. Among these two, APLS-1 showed better FDCA production (0.25 g/L) as compared to CO-2 (0.05 g/L) and selected for further studies. APLS-1 of 3.98 g/L Cell Dry Weight (CDW) (Fig.3.4.D) biomass was transferred into MSM and HMF, showed 0.14 g/L of FDCA production.Further optimizations were needed to increase the FDCA production in APLS-1. In a work 51.0% (mol/mol) conversion ratio of HMF is noticed in engineered *Raoultella ornithinolytica*BF60 at 30 °C in 50 mM phosphate buffer (pH 8.0) as a production media (Hossain et al.,2016).

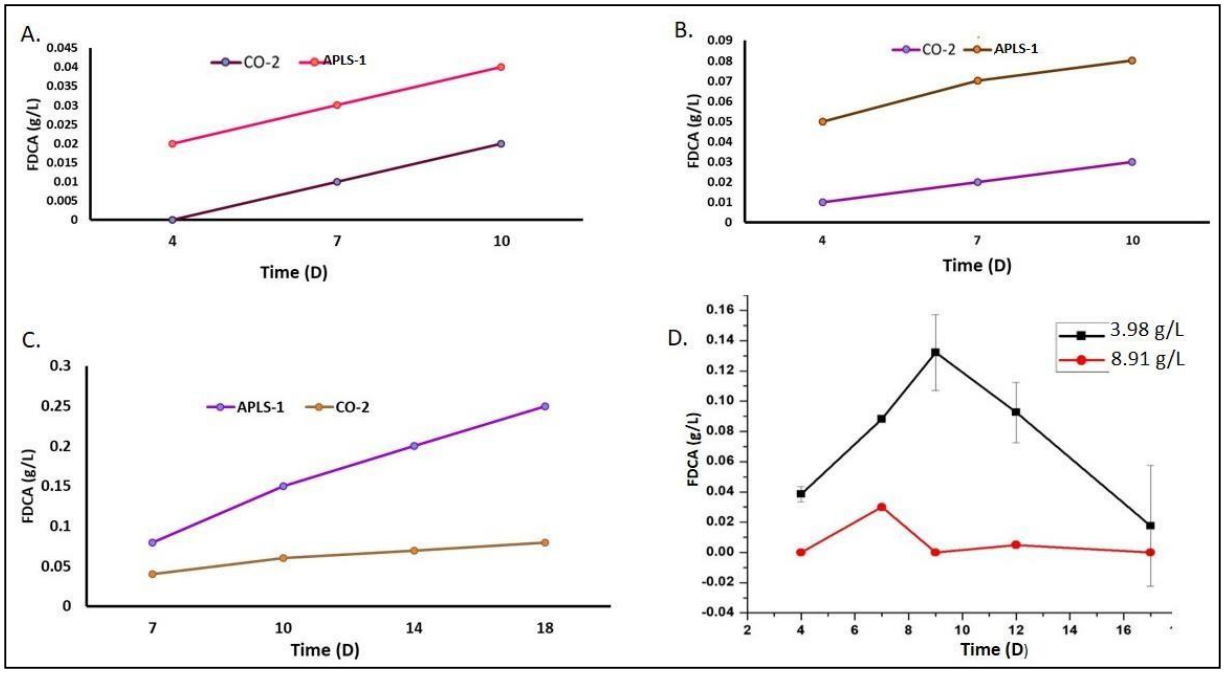


Fig.3.4. FDCA production of CO2 & APLS-1. A. FDCA production in MSM+HMF+glucose, B. FDCA production in MSM+HMF+glycerol, C. FDCA production in MSM+HMF, D. FDCA production using APLS-1 (diff. inoculum size)

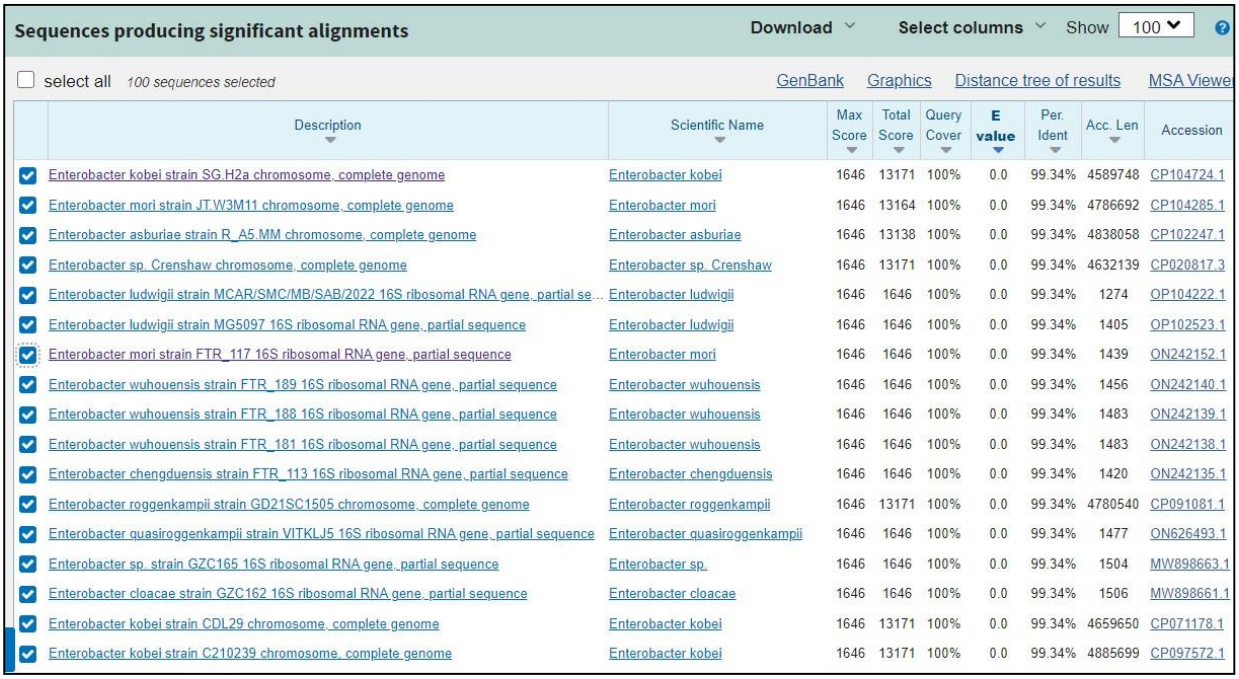
3.3.4. Identification of the AS-3 and APLS-1

3.3.4.1. Identification of AS-3

Amplified 16S rRNA region of bacteria AS-3 was sequenced and compared with NCBI-BLAST. The BLAST analysis showed 99% of sequence similarity with *Enterobacter* sps (Fig.3.5).

Sequence of AS-3:

5’-GCCCTCCCGAAGGTTAAGCTACCTACTTCTTTTGCAACCCACTCCCATGGTGTGA
CGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGTAGCATTCTGATCTACG
ATTACTAGCGATTCCGACTTCATGGAGTCGAGTTGCAGACTCCAATCCGGACTAC
GACGCACTTTATGAGGTCCGCTTGCTCTCGCGAGGTCGCTTCTCTTTGTATGCGCC
ATTGTAGCACGTGTGTAGCCCTACTCGTAAGGGCCATGATGACTTGACGTCATCC
CCACCTTCCTCCAGTTTATCACTGGCAGTCTCCTTTGAGTTCCCGGCCKRACCGCT
GGCAACAAAGGATAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATTTCACA
ACACGAGCTGACGACAGCCATGCAGCACCTGTCTCAGAGTTCCCGAAGGCACCA
AWSCATCTCTGSWAAGTTCTCTGGATGTCAAGAGTAGGTAAGGTTCTTCGCGTTG
CATCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCATTTG
AGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGTCGACTTAACGCGTTAGCTCCG
GAAGCCACGCCTCAAGGGCACAACCTCCAAGTCGACATCGTTTACGGCGTGGAC
TACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTGAGCGTCAGTC
TTTGTCCAGGGGGCCGCCTTCGCCACCGGTATTCCTCCAGATCTCTACGCATTTC
ACCGCTACACCTGGAATTCTACCCCCCTCTACAAGACTCTAGCCTGCCAGTTTCG
AATGCAGTTCCCAGGTTGAGCCCGGGGATTTCACATCCGACTTGACAGACCGCCT
GCGTGCGCTTTACGCCCAGTAATTC -3’



Sequences producing significant alignments Download Select columns Show 100

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Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Enterobacter kobei strain SG.H2a chromosome, complete genome	Enterobacter kobei	1646	13171	100%	0.0	99.34%	4589748	CP104724.1
Enterobacter mori strain JT.W3M11 chromosome, complete genome	Enterobacter mori	1646	13164	100%	0.0	99.34%	4786692	CP104285.1
Enterobacter asburiae strain R_A5.MM chromosome, complete genome	Enterobacter asburiae	1646	13138	100%	0.0	99.34%	4838058	CP102247.1
Enterobacter sp. Crenshaw chromosome, complete genome	Enterobacter sp. Crenshaw	1646	13171	100%	0.0	99.34%	4632139	CP020817.3
Enterobacter ludwigii strain MCAR/SMC/MB/SAB/2022 16S ribosomal RNA gene, partial se...	Enterobacter ludwigii	1646	1646	100%	0.0	99.34%	1274	OP104222.1
Enterobacter ludwigii strain MG5097 16S ribosomal RNA gene, partial sequence	Enterobacter ludwigii	1646	1646	100%	0.0	99.34%	1405	OP102523.1
Enterobacter mori strain FTR_117 16S ribosomal RNA gene, partial sequence	Enterobacter mori	1646	1646	100%	0.0	99.34%	1439	ON242152.1
Enterobacter wuhouensis strain FTR_189 16S ribosomal RNA gene, partial sequence	Enterobacter wuhouensis	1646	1646	100%	0.0	99.34%	1456	ON242140.1
Enterobacter wuhouensis strain FTR_188 16S ribosomal RNA gene, partial sequence	Enterobacter wuhouensis	1646	1646	100%	0.0	99.34%	1483	ON242139.1
Enterobacter wuhouensis strain FTR_181 16S ribosomal RNA gene, partial sequence	Enterobacter wuhouensis	1646	1646	100%	0.0	99.34%	1483	ON242138.1
Enterobacter chengduensis strain FTR_113 16S ribosomal RNA gene, partial sequence	Enterobacter chengduensis	1646	1646	100%	0.0	99.34%	1420	ON242135.1
Enterobacter roggenkampii strain GD21SC1505 chromosome, complete genome	Enterobacter roggenkampii	1646	13171	100%	0.0	99.34%	4780540	CP091081.1
Enterobacter quasiroggenkampii strain VITKLJ5 16S ribosomal RNA gene, partial sequence	Enterobacter quasiroggenkampii	1646	1646	100%	0.0	99.34%	1477	ON626493.1
Enterobacter sp. strain GZC165 16S ribosomal RNA gene, partial sequence	Enterobacter sp.	1646	1646	100%	0.0	99.34%	1504	MW898663.1
Enterobacter cloacae strain GZC162 16S ribosomal RNA gene, partial sequence	Enterobacter cloacae	1646	1646	100%	0.0	99.34%	1506	MW898661.1
Enterobacter kobei strain CDL29 chromosome, complete genome	Enterobacter kobei	1646	13171	100%	0.0	99.34%	4659650	CP071178.1
Enterobacter kobei strain C210239 chromosome, complete genome	Enterobacter kobei	1646	13171	100%	0.0	99.34%	4885699	CP097572.1

Fig.3.5. BLAST result of sequence of AS-3

3.3.4.2. Identification of APLS-1

For fungus, Internal Transcribed Spacer (ITS) region of APLS-1 was amplified and sequenced. Obtained sequence was compared with NCBI – BLAST and it showed 100% similarity to *Aspergillus flavus* (Fig.3.6). The sequence was submitted in GenBank under the Accession number MK434161.

ITS Sequence of APLS-1:

5’-CTCCCACCC TGTTTACTGA ACCTTAGTTG CTTCGGCGG CCCGCATAC

TGGCCGCCGG GGGCTCTCAG CCCCGGGCCC GCGCCCGCC GAGACACCA

GAACTCTGTC TGATCTAGTG AAGTCTGAGT TGATTGTAT GCAATCAGT

AAAACTTTCA ACAATGGATC TCTTGGTTCG GCATCGATG AGAACGCAG

GAAATGCGAT AACTAGTGTG AATTGCAGAA TTCCGTGAA CATCGAGTC

TTGAACGCAC ATTGCGCCCC CTGGTATTCC GGGGGGCAT CCTGTCCGA

CGTCATTGCT GCCCATCAAG CACGGCTTGT GTGTTGGGT GTCGTCCCC

CTGCGGGGGG GACGGGCCCC AAAGGCAGCG GCGGCACCGC GTCCGATCCT

CGAGCGTATG GGGCTTTGTC ACCCGCTCTG TAGGCCCGGC CGGCGCTTGC

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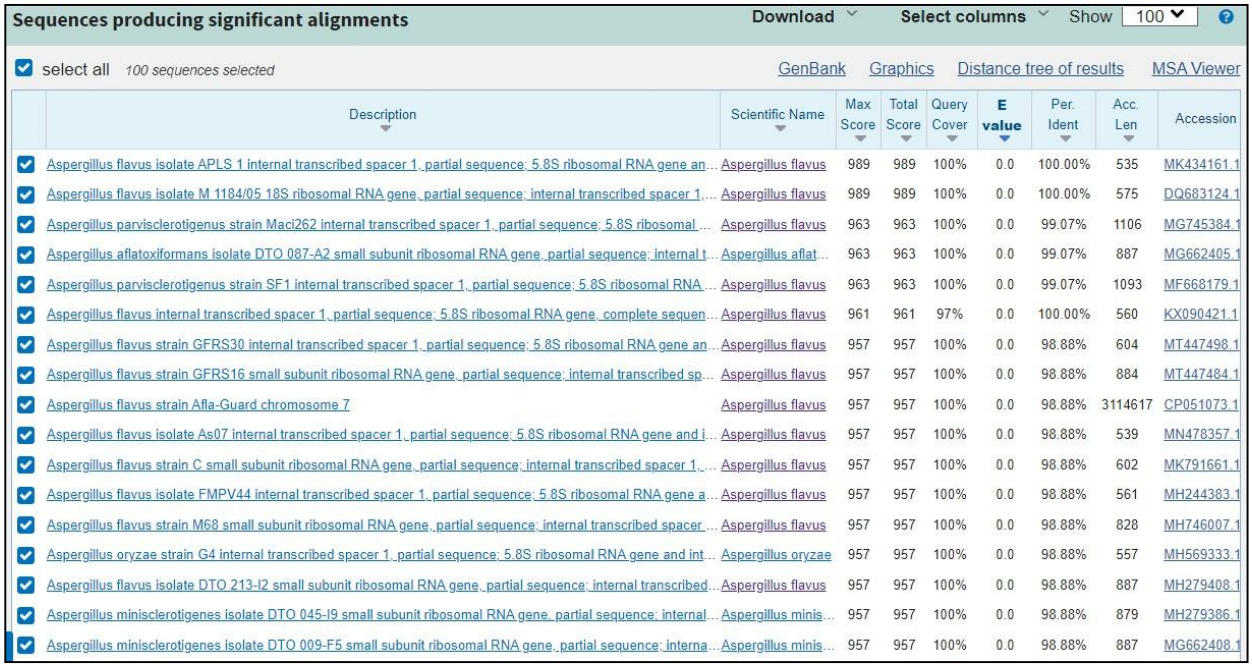
CCGCTGAACT TAAGCATATC AATAAGCGGA GGAAA -3’



Sequences producing significant alignments Download Select columns Show 100

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Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Aspergillus flavus isolate APLS 1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene an...	Aspergillus flavus	989	989	100%	0.0	100.00%	535	MK434161.1
Aspergillus flavus isolate M 1184/05 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1,...	Aspergillus flavus	989	989	100%	0.0	100.00%	575	DQ683124.1
Aspergillus parvisclerotigenus strain Maci262 internal transcribed spacer 1, partial sequence; 5.8S ribosomal ...	Aspergillus flavus	963	963	100%	0.0	99.07%	1106	MG745384.1
Aspergillus aflatoxiformans isolate DTO 087-A2 small subunit ribosomal RNA gene, partial sequence; internal t...	Aspergillus aflat...	963	963	100%	0.0	99.07%	887	MG662405.1
Aspergillus parvisclerotigenus strain SF1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA ...	Aspergillus flavus	963	963	100%	0.0	99.07%	1093	MF668179.1
Aspergillus flavus internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequen...	Aspergillus flavus	961	961	97%	0.0	100.00%	560	KX090421.1
Aspergillus flavus strain GFRS30 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene an...	Aspergillus flavus	957	957	100%	0.0	98.88%	604	MT447498.1
Aspergillus flavus strain GFRS16 small subunit ribosomal RNA gene, partial sequence; internal transcribed sp...	Aspergillus flavus	957	957	100%	0.0	98.88%	884	MT447484.1
Aspergillus flavus strain Afla-Guard chromosome 7	Aspergillus flavus	957	957	100%	0.0	98.88%	3114617	CP051073.1
Aspergillus flavus isolate As07 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and i...	Aspergillus flavus	957	957	100%	0.0	98.88%	539	MN478357.1
Aspergillus flavus strain C small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1,...	Aspergillus flavus	957	957	100%	0.0	98.88%	602	MK791661.1
Aspergillus flavus isolate FMPV44 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene a...	Aspergillus flavus	957	957	100%	0.0	98.88%	561	MH244383.1
Aspergillus flavus strain M68 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer ...	Aspergillus flavus	957	957	100%	0.0	98.88%	828	MH746007.1
Aspergillus oryzae strain G4 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and int...	Aspergillus oryzae	957	957	100%	0.0	98.88%	557	MH569333.1
Aspergillus flavus isolate DTO 213-I2 small subunit ribosomal RNA gene, partial sequence; internal transcribed...	Aspergillus flavus	957	957	100%	0.0	98.88%	887	MH279408.1
Aspergillus minisclerotigenes isolate DTO 045-I9 small subunit ribosomal RNA gene, partial sequence; internal...	Aspergillus minis...	957	957	100%	0.0	98.88%	879	MH279386.1
Aspergillus minisclerotigenes isolate DTO 009-F5 small subunit ribosomal RNA gene, partial sequence; interna...	Aspergillus minis...	957	957	100%	0.0	98.88%	887	MG662408.1

Fig.3.6. BLAST result of sequence of APLS-1

3.3.5. Analysis of HMF, FDCA and other intermediates

The HMF alcohol, HMF, HMF acid, DFF, FFCA, FDCA and 2-furoic acid in the reaction mixture (Fig.3.7) were determined by using gradient RP-HPLC with retention time 6.5, 8.1, 8.9, 10.3, 12.7, 14.8, 16.5 min respectively.

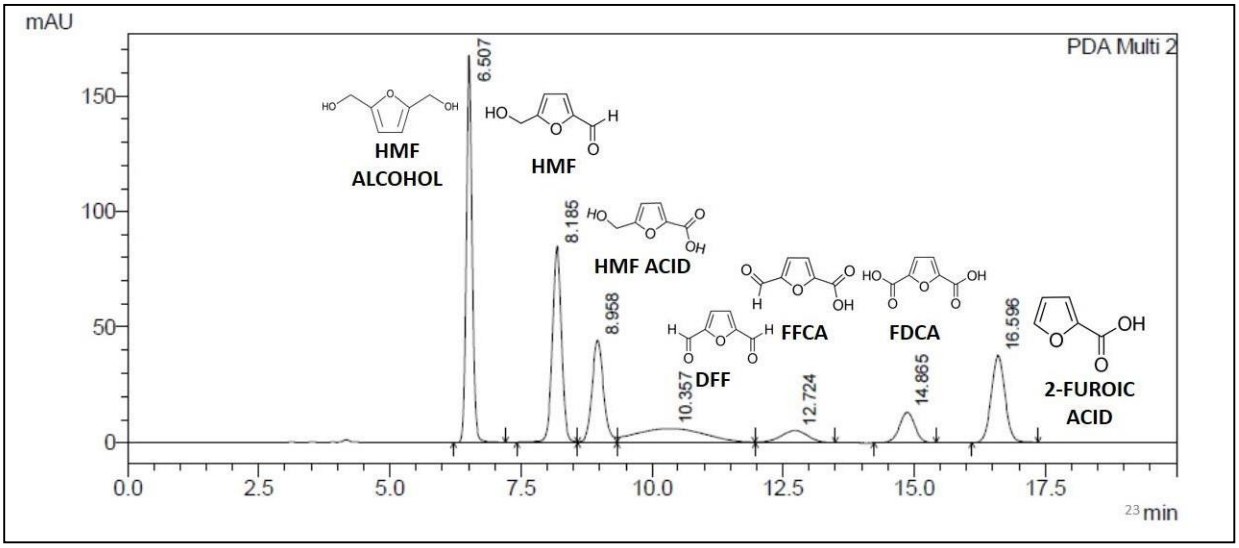


Fig.3.7. HPLC analysis of HMF, FDCA and other intermediates

3.4. Summary

Total 53 microrganisms were evaluated for its ability to convert HMF to FDCA. Among the isolates, *Enterobacter* sps (AS-3) and *Aspergillus flavus* APLS-1 showed stable FDCA production and selected for further studies. *Enterobacter* sps (AS-3) and *A. flavus* APLS-1 was found to be potent organism for the conversion of HMF to FDCA. The FDCA production efficiency of *Enterobacter* sps was 10% and that of Aspergillus flavus APLS-1 was 40%. Addition of carbon sources like glycerol (0.25%) and glucose (0.25%) in the production media delayed the FDCA production. Based on the FDCA yield, *A. flavus* APLS-1 was selected for further studies.

Chapter 4

Media engineering and process optimization for the production of FDCA

4.1. Introduction

A. flavus is an imperfect, filamentous, mesophilic, spore-forming fungi, mostly growing as a saprophyte in the soil. *A. flavus* colonies appear as powdery masses of branched mycelia with yellowish-green spores. Process optimization involves the simultaneous optimization of several parameters (e.g. suitable carbon and energy sources, nitrogen sources, other key macro-nutrients, micro-nutrients etc.) to improve the product yield which is necessary for further scale-up of the fermentation process. Current study is based on fungal assisted conversion of HMF to FDCA and its derivatives. In view of this, single parameter optimization for the production of FDCA by *A. flavus* was done. Process parameters like spore count, pH, inoculum size, inoculum age and substrate concentration for FDCA production have been optimized. The factors affecting fermentation yield was finally optimized by statistical Box-Behnken Design (BBD).

4.2. Materials and methods

4.2.1. Selection of production media

Various media were evaluated in order to select the best production media *A. flavus* APLS-1 was grown in Potato Dextrose Broth (PDB) for 72 h and this biomass was transferred to different media followed by incubation at 30°C and analysed the FDCA production. Media ingredients in the MSM (pH 7.2) were performed with 0.5 g/L HMF, 1 g/L HMF and 0.03 g/L NAD^{+}. It was essential to know which media is best for the reaction. For this, different media like MSM, K_2HPO_4 Buffer (50 mM) (pH 8), KH_2PO_4 Buffer(50 mM) (pH 7) and distilled water were used with HMF (0.5 g/L) for checking the productionof FDCA. For all experiments, 72 h aged inoculum size of 3.98 g/L CDW *A. flavus* APLS-1 was used.

4.2.2. Optimization of production media

To optimize the pH, the reaction was carried in 50 ml MSM inoculated with 72 h grown *A. flavus* APLS-1 biomass of 3.98 g/L cell dry weight (CDW) at 30 °C. The range of pH used were from 6 to 8. For the optimization of biomass size, the reactions were carried out with 72 h grown biomass with an optimized pH of 6.5 at 30 °C. The range of inoculum size (biomass size) selected were 4 to 8.7 g/L CDW. To optimize inoculum age (biomass age), reaction media was inoculated with optimized 6 g/L biomass, pH 6.5 at 30 °C. The range of inoculum age used was from 48 to 72 h. Optimization of pre-inoculum spore count was performed ranging from 10^5-10^7 spores/ml in PDB and this inoculum transferred into previous optimized media. Using this optimized condition of spores, APLS-1 was grown in PDB and this inoculum was used for substrate optimization. For the optimization of substrate concentration, the reaction was done in MSM inoculated with optimized 6 g/L CDW at an age 60 h and pH 6.5 at 30 °C. Substrate (HMF) concentration used was ranging from 0.25-2 g/L. All the experiments were done in 50 ml MSM in 250 ml flasks and kept in an incubator shaker at 200 rpm for 14 days.

4.2.3. Optimization of FDCA production using Box-Behnken design (BBD)

A BBD was used to evaluate the influence of three major parameters like pH, biomass size and biomass age on FDCA production in 1 g/L HMF (Tab.4.1). The pH range used in the BBD was from 6-8 with inoculum size ranging from 3.98-8.91 g/L CDW. Inoculum age used in this design was ranging from 48-72 h. Total 15 experiments were conducted. Linear regression and analysis of variance were estimated to know the significant effect of variables on the response ($p < 0.05$). Regression relation between all the process variables and the response variable were plotted. All these statistical analysis were done in Minitab 17 software (Ver. 15).

Sl.No.	pH	Inoculum Size (CDW-g/L)	Inoculum age (h)
1	6	3.98	60
2	6	5.70	48
3	8	3.98	60
4	6	5.70	72
5	7	3.98	72
6	7	5.70	60
7	7	8.91	48
8	7	5.70	60
9	8	5.70	48
10	8	8.91	60
11	8	5.70	72
12	7	5.70	60
13	7	8.91	72
14	6	8.91	60
15	7	3.98	48

Tab.4.1. BBD design used for the optimization

4.2.4. Validation of FDCA production

The optimum conditions obtained from the BBD was validated based on predicted and experimental values using software. The software predicted conditions like pH (6.3), inoculum size (6.5 g/L CDW) and age (58 h) with FDCA production of 0.99 g/L. Based on these conditions an experimental design (Tab.4.2) was created manually and compared the result with predicted result. Its yield and selectivity percentage were calculated.

Sl.No	pH	Biomass size (CDW-g/L)	Biomass age (h)
1	6	6.6	58
2	6	6.6	60
3	6	8.9	62
4	6.5	6.6	58

Tab.4.2. Experimental design for validation

4.3. Results and discussion

4.3.1. Selection of production media

In the first set of experiment, already grown biomass was transferred into 50 ml MSM with different ingredients like HMF and NAD^+ (Fig.4.1.A). The hypothesis here was to know whether NAD^+ can increase the oxidation rate of HMF or not. Among them, MSM with HMF as sole carbon source showed better FDCA production rather than adding NAD^+ as media supplement. For selection of production media (Fig.4.1.B) MSM with 0.5 g/L HMF showed increased FDCA production as compared to other media. This media showed complete detoxification and oxidation reaction of HMF into HMF acid, FFCA and FDCA. So, MSM with HMF was the best production media selected for further studies.

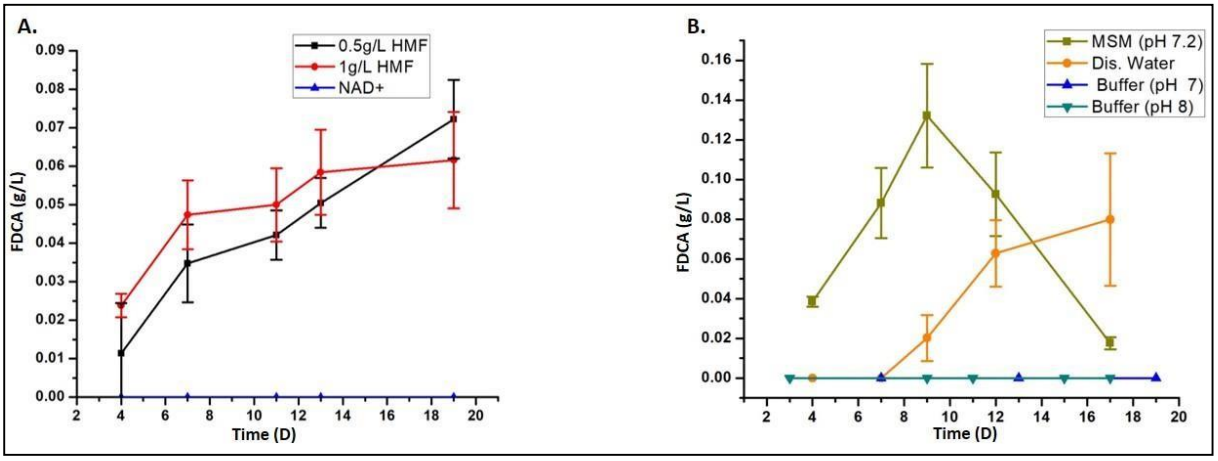


Fig.4.1. Selection of production media. A. MSM with different ingredients, B. Media selection

4.3.2. Single parameter optimization of production media

Single parameters like pH, inoculum size, inoculum age and substrate concentration were optimized for the FDCA production (Fig.4.2). Results revealed that optimum pH of the reaction was found to be 6.5 in which 0.5 g/L of HMF was converted into 0.04±0.01 g/L (Fig.4.2.A). At pH 8, a drastic declining in the FDCA production was noticed. Optimum inoculum size was found as 6 g/L CDW which converted 0.5 g/L HMF into 0.06±0.01 g/L of FDCA (Fig.4.2.B). As the inoculum size increases the production rate was reduced. It might be because of fast metabolic reaction in which the produced FDCA might be utilized into its metabolic pathway. It has been reported that higher cell concentration cannot transform HMFinto FDCA in *R. ornithinolytica* (Hossain et al., 2017). The maximum activity of inoculum agewas 60 h which gave 0.12±0.01 g/L of FDCA from 0.5 g/L HMF (Fig.4.2.C). Harvesting the cells at late exponential phase has been shown better FDCA production in *R. ornithinolytica* (Hossain et al., 2017). Optimum spore count (Fig.4.2.D) of *A. flavus* for the pre-inoculum sizein PDB was optimized as 10^6 spores/ml. From this PDB, inoculum was transferred into mediacontaining MSM with HMF and 0.15±0.01 g/L of FDCA produced. The HMF concentration ranging from 0.25-2 g/L (Fig.4.3.A) were evaluated for the biotransformation and maximum production of

FDCA was observed as 0.32±0.05 g/L (Fig.4.3.B) in the medium supplementedwith 1 g/L HMF after 14 days of incubation. It has been reported that, after single parameter optimizations 0.23 g/L FDCA obtained from 1.5 g/L HMF (Godan et al., 2019). Another studyreported that after single parameter media optimizations, 1.28 g/L FDCA is produced from 2 g/L HMF by the organism *Burkholderia cepacia* H-2 (Yang and Huang., 2016).

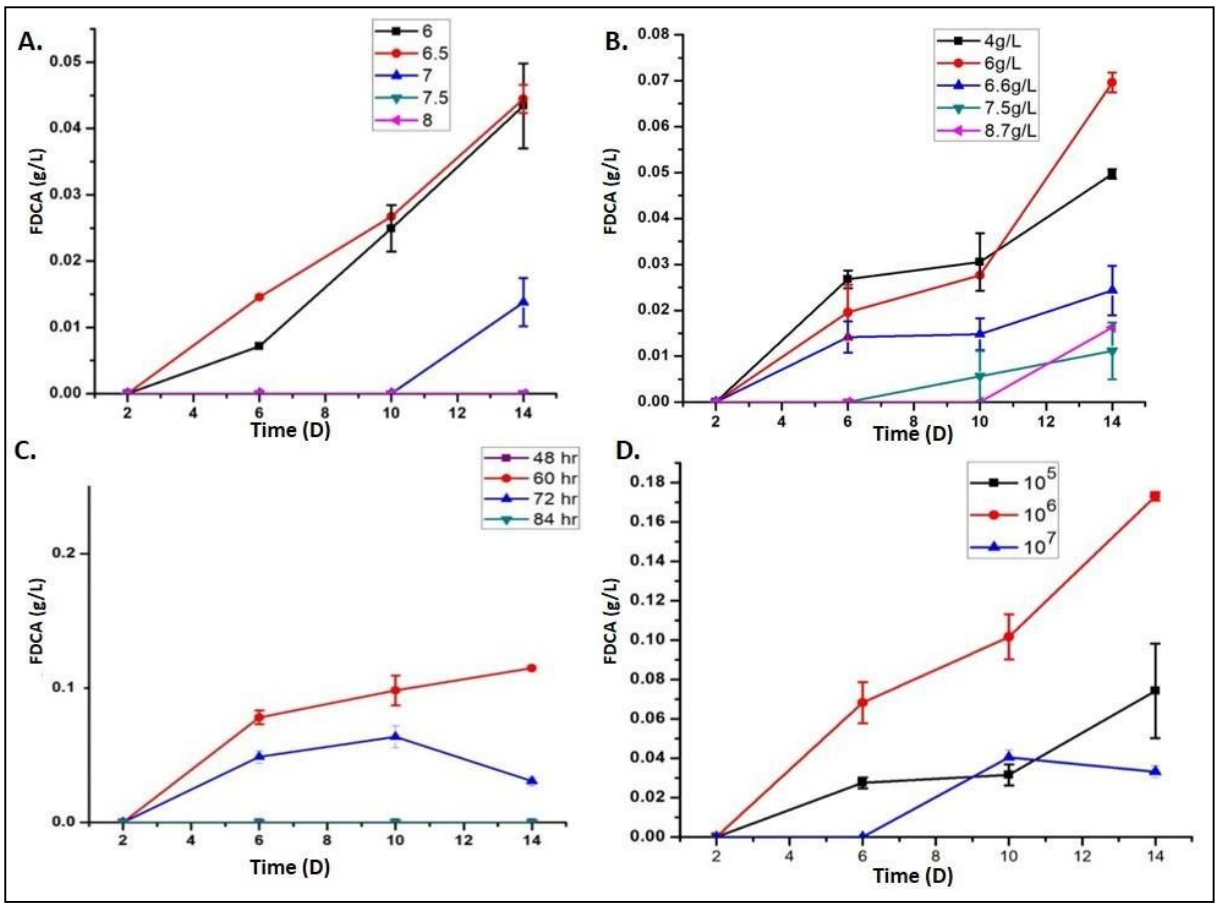


Fig.4.2. Single parameter optimization. A. Optimization of pH, B. Inoculum size, C. Inoculum age, D. Spore count for the production of FDCA

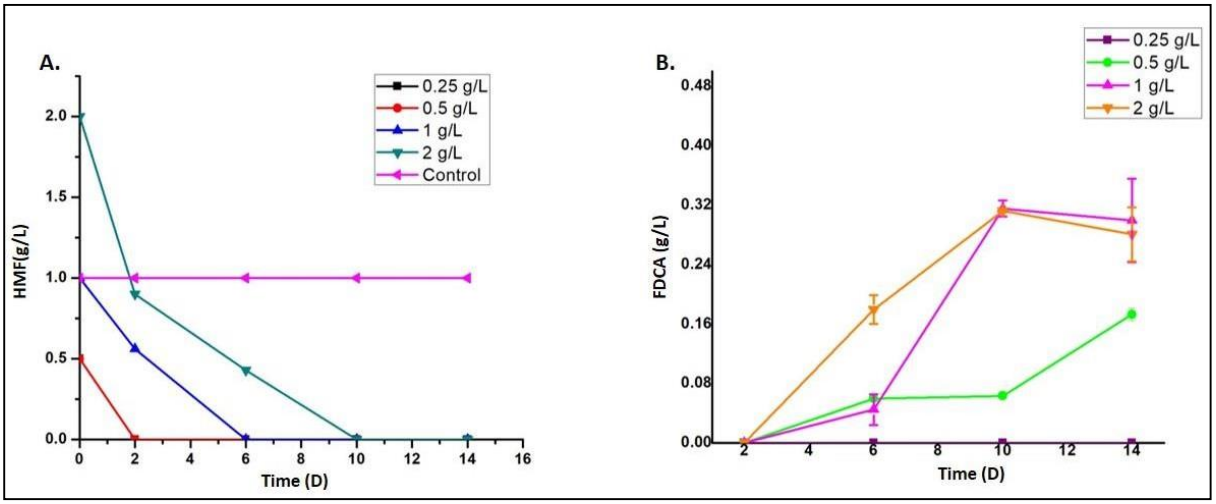


Fig.4.3. Single parameter optimization. A. Utilization of HMF, B. FDCA production

4.3.3. Optimization of FDCA production using Box-Behnken design (BBD)

Three major parameters like pH, inoculum size and inoculum age were selected to know the multi-parameter influence on HMF biotransformation and FDCA production. According to the Box-Behnken design, 15 set of experiments were done in which 6th, 8th, 12th, and 14th set showed better FDCA production from 1 g/L HMF after 14 days (Tab.4.3). Contour plot (Fig.4.3) was drawn for the FDCA production after 14 days in terms of inoculum size and inoculum age (Fig.4.3.A), pH and inoculum size (Fig.4.3.B), pH and inoculum age (Fig.4.3.C). Results revealed that, biomass size and biomass age and pH influenced highly in the production of FDCA. Among them 6th set showed 0.8 g/L (6.4 mM) FDCA from 1 g/L HMF (8 mM) after 14 days (Fig.4.5) where the conditions were pH 7, biomass size 5.7 g/L and biomass age 60 h with yield of 64.51%. It has been observed that after making RSM design with three parameters inoculum age, inoculum size and pH of the media optimized to produce 65% yield of FDCA from 0.5 g/L HMF by *A. oleivirans* S27 (Godan et al. 2019). Recent report suggest that higher cell concentrations might make oxygen as a limiting factor by generating reducing power. This reducing power may be oxidized again to sustain the flux and do not enhance the FDCA production (Hossain et al., 2017). The HPLC chromatogram of HMF, FDCA and other

intermediate compounds are shown in Fig 4.5. No DFF peaks were observed in the HPLC profile after biotransformation by *A. flavus* APLS-1 which means this may be intracellular and other HMF intermediates will be extracellular.

FDCA (mM) = -15.33 + 1.26 A + 0.22 B + 0.40 C- 0.12 A*A - 0.01 B*B -0.004 C*C -0.03 A*B + 0.008 A*C + 0.002 B*C (Where, A – pH, B - CDW (g/l), C - Inoculum age)

Sl.No	**pH**	**Inoculum Size (CDW-g/L)**	**Inoculum age (h)**	**FDCA production (14^{th} Day)**
1	6	3.98	60	0.74
2	6	5.70	48	0.53
3	8	3.98	60	0.54
4	6	5.70	72	0.09
5	7	3.98	72	0.05
6	**7**	**5.70**	**60**	**0.80**
7	7	8.91	48	0.41
8	**7**	**5.70**	**60**	**0.79**
9	8	5.70	48	0.13
10	8	8.91	60	0.51
11	8	5.70	72	0.05
12	**7**	**5.70**	**60**	**0.79**
13	7	8.91	72	0.69
14	6	8.91	60	0.79
15	7	3.98	48	0.59

Tab.4.3. BBD result for the optimization

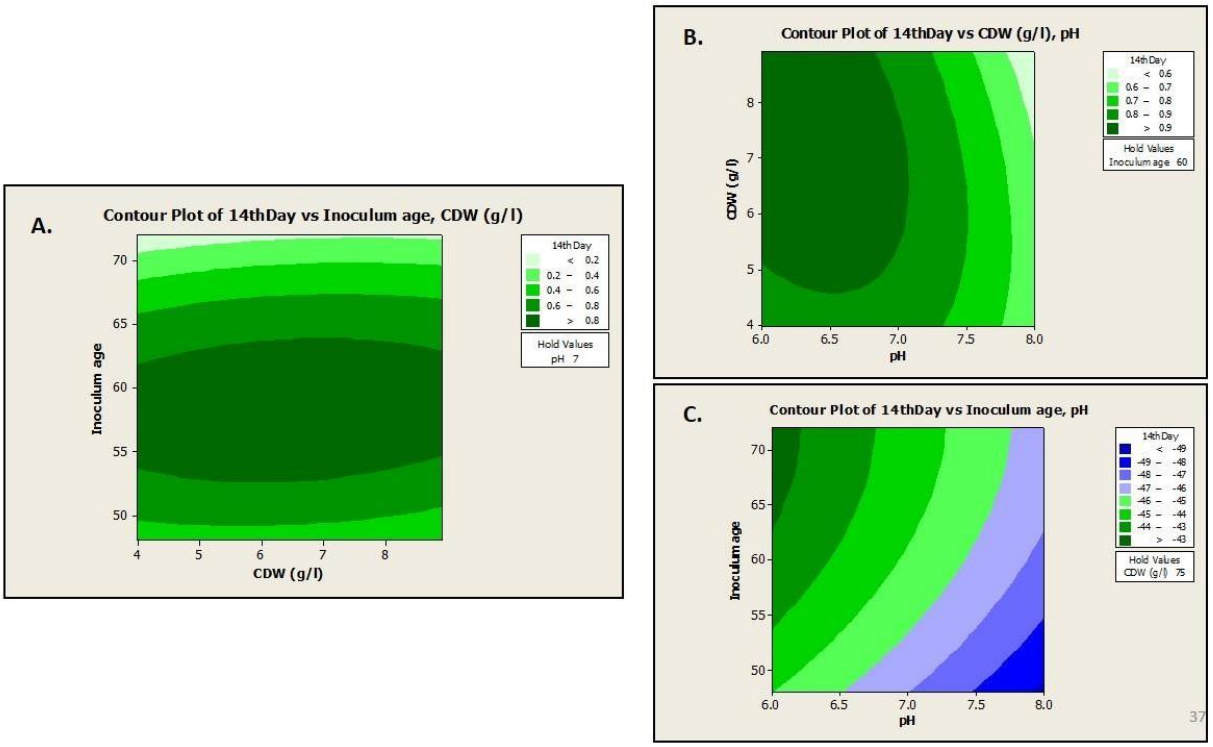


Fig.4.4. Contour plot on FDCA production

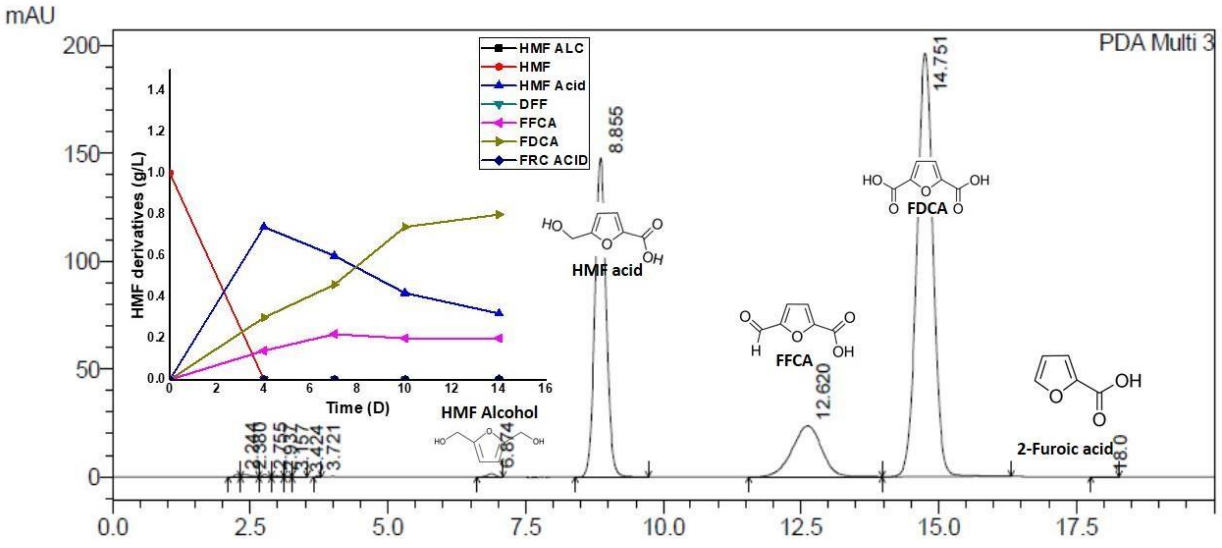


Fig.4.5. FDCA production of 6th set with HPLC chromatogram

4.3.4. Validation of FDCA production

According to the validation design results (Tab.4.4) the 4th set with pH 6.5, biomass size 6.66 g/L and biomass age 60 h showed FDCA production of 0.83 g/L (6.6 mM) from 1 g/L (8 mM) HMF with yield of 67% after 14 days (Fig.4.6). It showed a selectivity percentage of 62.4% with 67% yield. Theoretical and experimental yields of FDCA were compared (Tab. 4.4) and the results were comparable with correlation coefficient 0.92. According to the intermediate compound analysis these samples showed a rapid conversion of HMF to HMF acid followed by slower conversion of HMF acid to FFCA. It might be due to the absence of respective oxidative enzymes or channel proteins or by the limitation of oxygen uptake for the sequential oxidation for the conversion of HMF acid to FFCA.

Sl.No	**pH**	**Biomass size (CDW-g/L)**	**Biomass age (h)**	**Experimental FDCA yield (g/L)**	**Theoretical FDCA yield (g/L)**
1	6	6.6	58	0.783	0.96
2	6	6.6	60	0.792	0.93
3	6	8.9	62	0.69	0.89
4	**6.5**	**6.6**	**58**	**0.83**	**0.97**

Tab.4.4. Validation result for the optimum production of FDCA

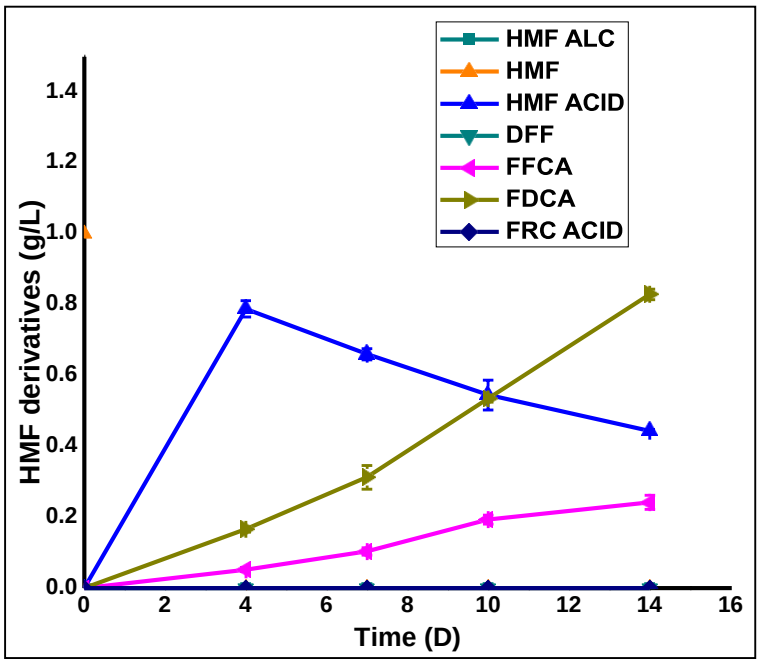


Fig.4.6. Optimized condition for FDCA production

4.4. Summary

Upon single parameter optimization 0.32±0.05 g/L FDCA was obtained from 1 g/L HMF at inoculum age 60 h, inoculum size 6 g/L and pH 6.5 after 14 days. Statistical optimization was performed based on most effective parameters like inoculum age, inoculum size and pH and 0.8 g/L FDCA was obtained from 1 g/L HMF at inoculum age 60 h, inoculum size 8.91 g/L and pH 7 after 14 days. There were around 2.5 times increase in production of FDCA after optimization. HMF was utilized completely (100%) within 2 days and yield of FDCA was 64.5%. Validation design was created using Minitab software and optimum production of 0.83 g/L FDCA obtained (58 h, 6.66 g/L and pH 6.5) with a selectivity percentage of 62.4% with 67% yield. It was approximately with yield of 0.83 g/g.

Chapter 5

Various fermentation strategies for FDCA production

5.1. Introduction

Process scale-up is an important step in bioprocess development to increase the amount of desired product. For this, flask-scale process has to be mimicked in bioreactor and further operational parameters need to be optimized. In this study, batch production of FDCA was performed in parallel and 2L fermenter. Also, FDCA production was performed in immobilized packed bed bioreactor for continuous use of whole cell catalyst. The major limitation of batch process in FDCA production is the toxicity of HMF to the organisms. In order to convert higher substrate concentrations, fed-batch fermentation approaches were evaluated.

5.2. Materials and methods

5.2.1. Production of FDCA in parallel fermenter

Pre-cultured fungal inoculum was inoculated in PDB with $1x10^6$ spores/ml for 58 h in 250 ml conical flasks. From this, 6.66 g/L CDW was filtered, washed (in PBS) and transferred into MSM in parallel fermenter (0.6 L) with 1 g/L HMF. Working volume of this bioreactor was 300 ml and it was kept at 30 °C with 160 rpm and 0.285 VVM for 12 days.

5.2.2. Production of FDCA in 2 Liter fermenter

Pre-grown fungal biomass (6.66 g/L CDW) was transferred into 2 L fermenter containing 1 g/L HMF in 500 mL MSM. The bioreactor was operated at 30 °C with 160 rpm with aeration rate of 1 VVM, and incubated for 12 days.

5.2.3. Immobilized packed bed bioreactor with media recycle

Fungal inoculum *A. flavus* APLS-1 was inoculated in PDB with $1x10^6$ spores/ml in 250 ml conical flasks along with polyurethane foam cubes (PUF) ($1cm^3$) (1 g) in potato dextrose media for 58 h. This immobilized fungal biomass was transferred into a packed bed bioreactor with a size of 12x3 inches (l*b). MSM (600 ml) was recycled into it from downward to upward direction and incubated in room temperature. This media was circulated using a peristaltic pump with a flow rate of 1 ml/min and kept for 8 days.

5.2.4. FDCA production by fed batch approach

Different strategies of HMF feeding (Tab.5.1) were applied under fed batch approach in 50ml MSM. Among five strategies, first one was with initial 0.75 g/L and fed at every 12 h intervals with 0.25 g/L HMF for 48 h. Second strategy was with initial 1 g/L HMF and fed at every 12 h intervals with 0.25 g/L HMF for 48 h. Third strategy was with initial 0.5 g/L fed at every 24 h intervals with 0.25 g/L HMF for 4 days. Fourth one was with initial 0.75 g/L followed by 0.5 g/L HMF in every 24 h for 4 days. Strategy fifth was with initial 1 g/L HMF and fed at every 24 h with 0.5 g/L HMF for 4 days. These were inoculated with pre-grown fungal biomass (6.66 g/L CDW) and kept at 30 °C for 14 days.

Strategy 1 – 0.75+0.25+0.25+0.25+0.25	12 hrs interval
Strategy 2 – 1+0.25+0.25+0.25+0.25	
Strategy 3 – 0.50+0.25+0.25+0.25+0.25	24 hrs interval
Strategy 4 – 0.75+0.50+0.50+0.50+0.50	
Strategy 5 – 1+0.50+0.50+0.50+0.50	

Tab.5.1. Different strategies of fed batch

5.2.5. Conversion of pineapple peel derived HMF into FDCA

HMF was synthesized from pineapple peel as described earlier in the chapter 2. From this, 1 g/L HMF was transferred into 50 ml MSM in 250 ml conical flasks. It was inoculated with pre-grown fungal biomass (6.66 g/L CDW) and kept at 30 °C for 14 days.

5.3. Results and discussion

5.3.1. Production of FDCA in parallel fermenter

After 14 days of incubation in parallel fermenter (5.1.A), pH of the media (5.1.B) declined from 6.5 to 3.5 and dissolved oxygen (DO) (5.1.C) was declined from 65 to 50% followed by an increase. In the case of FDCA production, (5.1.D) low level of FDCA (0.04 g/L) was produced. There was an accumulation of HMF alcohol (0.26 g/L) in the media which means HMF reduction occurred more than the oxidation reactions. Also, high amount of HMF acid was

observed in the media even after 12 days. But, after sequential oxidations from HMF to HMF acid, there were a delayin the conversion of HMF acid to FFCA followed by FDCA production. Fungal inoculum gotattached to the impeller of fermentor after 12 days. This might be the reason that organism could not actively participate in sequential oxidation reactions after HMF acid production.

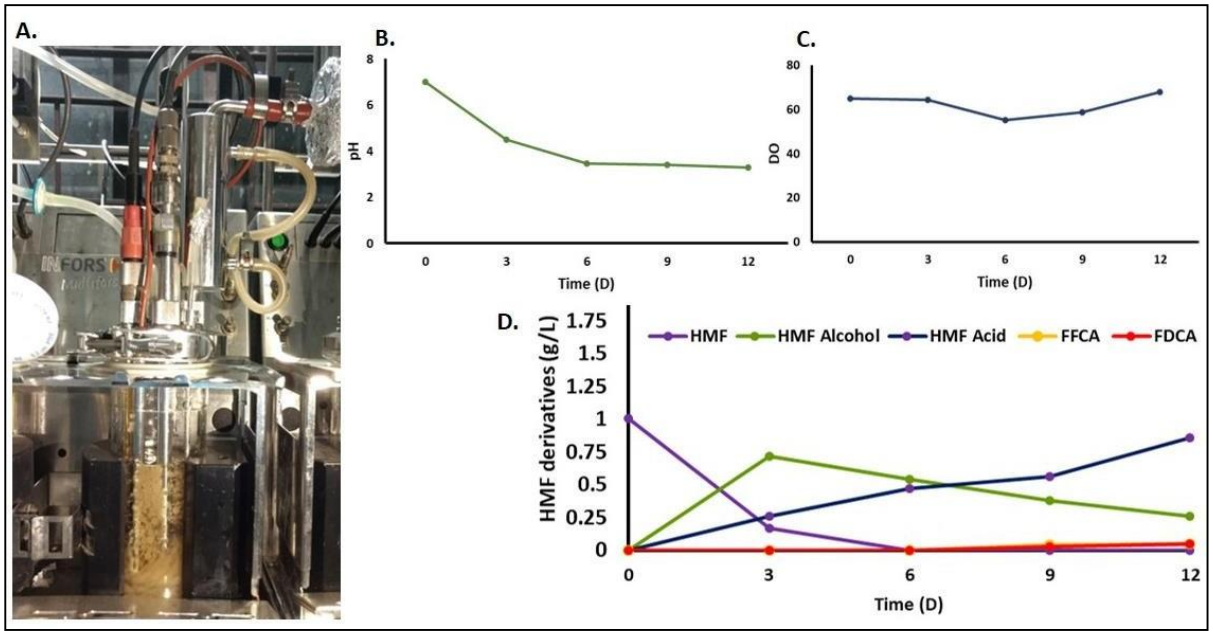


Fig.5.1. FDCA production in parallel fermenter A. Parallel fermenter B. pH of media, C. DO in the media, D. FDCA production and HMF derivatives

5.3.2. Production of FDCA in 2 L fermenter

In this, complete HMF uptake was happened within 2 days by the organism and less amount of HMF alcohol was produced. After sequential oxidations HMF acid was converted into FFCA followed by FDCA production. It was noted that, the organism was highly active in conversion of HMF to FDCA and pH of the media was declined to 4.5. The change in pH to acidic is an indication of the formation of acid intermediates in which the presence of FDCA will be highly acidic in nature. DO was less than 30 after 8 days from 55 which showed that organism used very high amount of oxygen for its growth and oxidation reactions. After 12 days, 0.5 g/L FDCA was produced from 2 L fermenter. A similar study has been reported by

Koopman et al. (2010) where FDCA production was performed in 1 L fermenter using an engineered *Pseudomonas putida* S12.

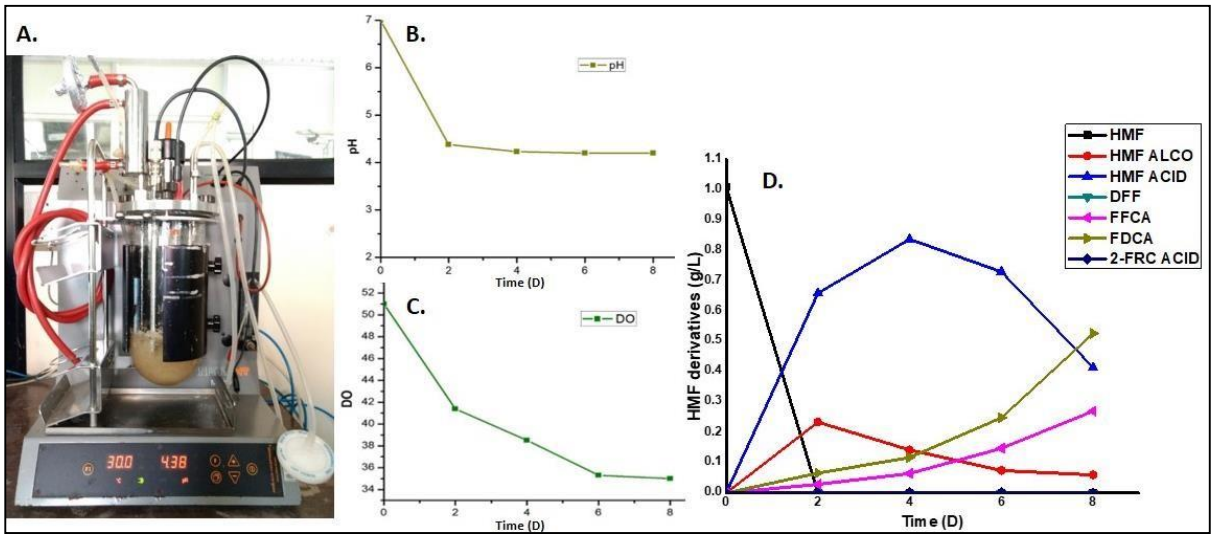


Fig.5.2. FDCA production in 2 L fermenter. A. 2 L fermenter, B. pH of the media, C. DO in the media, D. FDCA production and other HMF derivatives

5.3.3. Immobilized packed bed bioreactor with media recycle

A. flavus was immobilized on PUF and MSM was recycled with 1 g/L HMF and it converted 100% HMF within two days. Even though it showed increased amount of HMF biotransformation to HMF acid followed by FFCA production, very low level of FDCA was obtained. The immbolized fungus was detached from the PUF after four days and float on the media and traveled through the tubes. This blocked the lines of the bioreactor followed by leakage of the media to outside which enhanced the chance of contamination. To avoid this, suitable scaffold is needed to develop for the better immobilization of the APLS-1 in future FDCA production studies. Further optimization of inoculum age of APLS-1 and media flow in packed bed bioreactor might also increase the FDCA production in future.

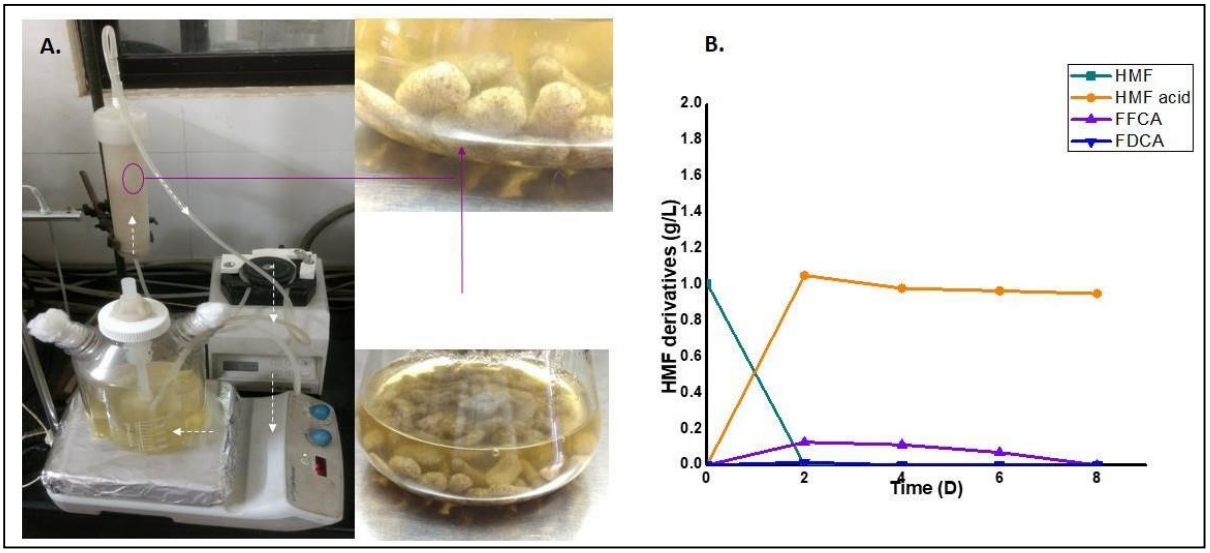


Fig.5.3.A. FDCA production in packed bed bioreactor. B. HMF derivatives in the packed bed bioreactor

5.3.4. FDCA production by fed batch approach

Among different strategies (Fig.5.4.A & B) were applied, strategy 2 (Fig.5.4.C) with 1 g/L HMF followed by 0.25 g/L HMF in every 12 h for 48 h showed increased amount of FDCA production after incubation period. In this, complete HMF was utilized within 48 h and 1.18±0.09 g/L FDCA produced after 14 days with 51% yield. It was observed that the time intervals of fed batch addition of HMF reduced, APLS-1 utilized HMF immediately. But, after a certain limit, this rate of utilization was slowed which affected FDCA production. It might be due to the substrate toxicity of HMF in a short period of time. Hence, a balanced addition of HMF is required in an optimized time period for higher production of FDCA. Total yield of FDCA was 0.59±0.05 g/g. It has been reported that, 1.19±0.03 g/g of FDCA was obtained after fed batch approach of HMF using genetically engineered *Pseudomonas putida* S12 strain (Koopman et al., 2010).

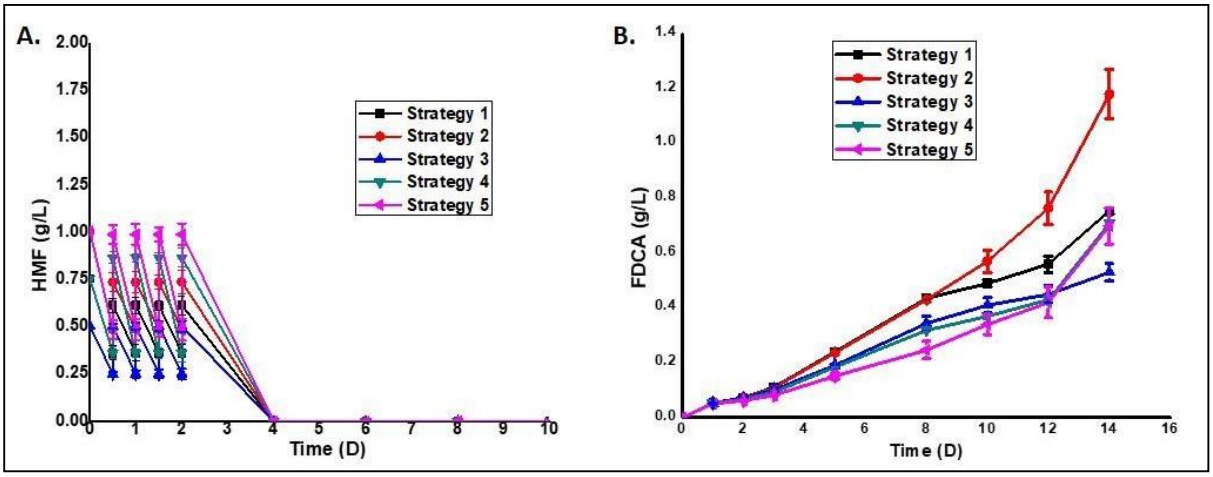


Fig.5.4.A. Utilization of HMF during fed batch approach. B. FDCA production after different strategies of fed batch

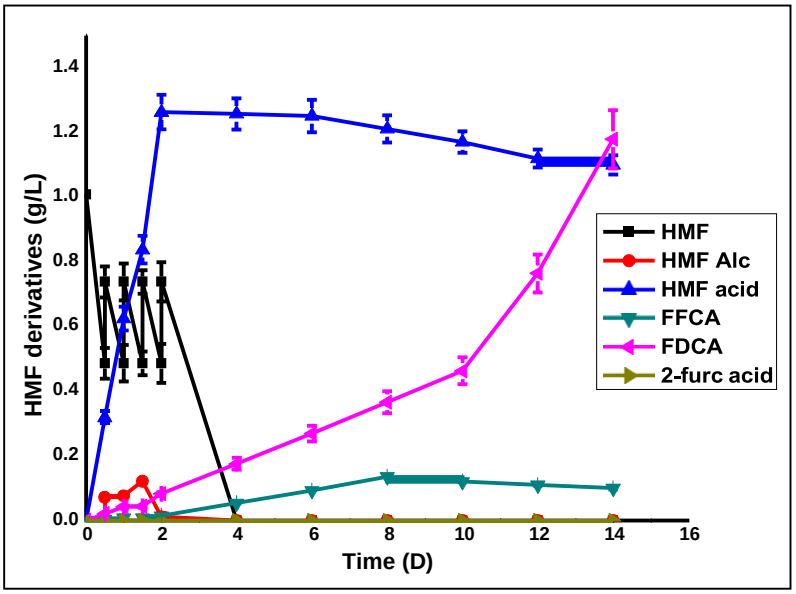


Fig.5.4.C. FDCA production of second strategy

5.3.5. Conversion of pineapple peel derived HMF into FDCA

Pineapple derived HMF was utilized for the production of FDCA. In this, 1 g/L HMF was completely utilized within 2 days. There was HMF alcohol observed until 6th day. Also, HMF acid is seen in the reaction in higher amount until 14th day. This rate limiting step shows oxidation of reaction was slow and 0.16±0.01 g/L FDCA was obtained after 14 days. It may be due to impurities like levulinic acid and other humins in the media. These will divert the oxidation reactions and enter into other metabolic pathways and hinder the FDCA production.

In a similar work, acid algal hydrolysate biomass is used which contain low amount of HMF and added 2 g/L HMF into it additionally produced 0.98 g/L FDCA by *B. cepacia* H-2 (Yang & Huang., 2016). In another work, diluted acid algal hydrolysate which contain HMF is used as the renewable media for the growth of *M. radiotolerans* G-2 and 0.45 g/L of FDCA produced (Yang et al., 2018).

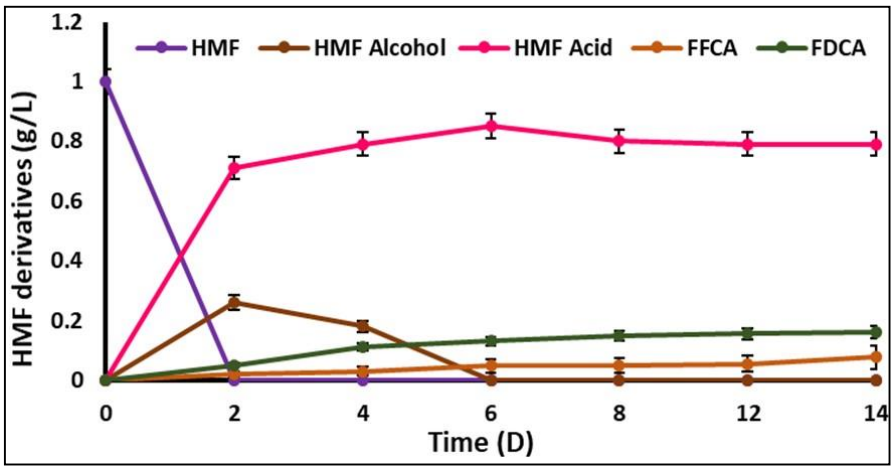


Fig.5.5. Conversion of pineapple derived HMF into FDCA

5.4. Summary

FDCA production in 2 L fermenter showed 0.5 g/L FDCA (40% yield) from 1 g/L HMF after 12 days with yield of 0.5 g/g. The FDCA yield in immobilized (*A. flavus* APLS-1) packed bed bioreactor was very low. In Fed batch approach, in a strategy of initial 1 g/L HMF followed by 0.25 g/L HMF in every 12 h up to 48 h produced 1.18±0.09 g/L FDCA (51% yield) in 14 days. The yield of FDCA was 0.59±0.05 g/g. In the case of pineapple derived HMF 0.16±0.01 g/L FDCA was obtained with yield of 0.16±0.01 g/g.

Chapter 6

Enzymatic and gene expression analysis in *A. flavus* APLS-1 for FDCA production

6.1. Introduction

Only a few microorganisms have been reported that have the ability to convert HMF into FDCA. Analysis of enzymes responsible for the conversion of HMF into FDCA will be essential to improve further research works in *A. flavus* APLS-1. Also, identification of mechanism of conversion of HMF into FDCA in *A. flavus* APLS-1 would be very much crucial for the future improved bioprocesses. Hence, enzyme assay for dehydrogenase, reductase and decarboxylase were performed for the conversion of HMF into FDCA. Expressed specific enzymes and other proteins were identified by the liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis by comparing treated (HMF –fed batch) with un-treated at different time intervals. Up-regulated proteins and respective pathways were identified and compared with the previous reports. Further gene expression profiles of control and HMF stressed cultures were identified by transcriptomic analysis at different time intervals in order to understand the possible mechanism for the conversion of HMF into FDCA.

6.2. Materials and methods

6.2.1. Assay of dehydrogenase, reductase, and decarboxylase

6.2.1.1. Assay of dehydrogenase

The *A. flavus* APLS-1 was grown in PDB for 60 h and filtered and transferred to MSM flasks. There were treated and un-treated MSM flasks in which treated was added with 1 g/L HMF and un-treated without HMF. After 24 h, the fungal biomass were homogenized using mortar and pestle in liquid nitrogen and it was suspended in K_2HPO_4 buffer. The solution was then centrifuged at 20,000g for 20 min. The supernatant which contains proteins were separated from cell debris. For the assay, reaction mixtures were prepared in 200 mM K_2HPO_4 buffer (pH 7), which included 10 mM HMF, 2.5 mM NAD^+ and crude protein extract (treated and un-

treated). The mixtures were incubated for 30 min. The enzyme activity was analyzed using spectrophotometer at 340 nm.

6.2.1.2. Assay of reductase

Reaction mixtures were prepared according to above procedure in K_2HPO_4 buffer (200 mM) (pH 7), with HMF (10 mM), NADPH (1 mM), and crude protein extract (treated and un-treated). The samples were incubated for 30 min. The enzyme activity was analyzed by spectrophotometer at 340 nm.

6.2.1.3. Assay of decarboxylase

According to the reports, FDCA is converted into 2-furoic acid by decarboxylase. Hence, the fungal biomass was pre-grown in PDB and transferred to MSM flasks. The treated flasks were with MSM and FDCA and un-treated was with MSM only. Crude proteins were isolated from these samples and used as enzyme extract for the experiments. For the assay, reaction mixtures were prepared in 200 mM K_2HPO_4 buffer with 10 mM FDCA, 1 mM pyridoxal phosphate (PLP) (pH-7) and crude protein extract and incubated for 30 min. After the specified time of incubation the presence of 2-furoic acid was analyzed by HPLC.

6.2.2. Proteomic analysis of intracellular crude protein in *A. flavus* APLS-1

The *A. flavus* APLS-1 was grown in PDB and filtered and transferred to MSM flasks with HMF (treated) (initial 1 g/L HMF and fed at every 12 h intervals with 0.25 g/L HMF for 48 h) and without HMF (un-treated). Treated flasks were labeled as tests and un-treated as controls. For protein isolation, separate flasks were taken at frequent intervals (1^{st}, 3^{rd}, 6^{th}, 9^{th} and 12^{th} days) and this fungal biomass was homogenized using liquid nitrogen in 100 mM ammonium bicarbonate (NH_4CO_3) buffer. It was centrifuged at 20,000 g for 20 min. The pellet with cell debris was removed and supernatant containing protein was taken for further analysis. Protein estimation of samples were carried out by Bradford assay and sent for protein profiling. From

these, 100 µg of protein samples (1 mg/ml) were subjected to trypsin digestion followed by centrifugation at 20,817 g at 4 °C for 12 min. The supernatant was removed and stored at -20 °C until analysis by LC-MS/MS.

The digested peptides were passed and separated by reverse phase chromatography using nanoACQUITY UPLC system (Waters, United Kingdom) followed by data processing by MassLynx4.1 SCN781 software. The mass spectrometry analysis of peptides were performed in positive electrospray ionization mode with NanoLockSpray^{TM} source using Waters SYNAPT G2 High-Definition MS^{TM} system. The obtained mass spectra was analysed by Progenesis QI for Proteomics V3.0 (Non-Linear Dynamics, Waters) for label free protein quantification. The data processing parameters were performed according to the protocol reported by Vineetha et al., 2020. One peptide match from the desired protein was taken for identification and the quantification of proteins were performed by (average intensity of the most abundant unique peptides) Hi-N algorithm. Obtained proteins were searched using *A. flavus* sps (strains ATCC MYA-384/AF70) as reference organisms from UniProt database (UniProt consortium, 2021). The mean triplicates of proteomic data and one way ANOVA ($P>0.05$) of all the samples (treated and un-treated) were calculated using SPSS/PC+version 16 (SPSS Inc. Chicago, USA).

6.2.3. Transcriptomic analysis of differentially expressed genes in *A. flavus* APLS-1

The fungal biomass was grown in PDB and filtered and transferred to MSM flasks (test flasks) with initial 1 g/L HMF followed by 0.25 g/L HMF fed at every 12 h intervals for 48 h and control MSM flasks without HMF. For RNA isolation, separate flasks were taken at frequent intervals (1st, 3rd and 6th days or 24, 72, and 144 h) and 200 mg of the samples were sent for RNA isolation, sequencing and analysis.

6.2.3.1. Extraction of RNA

This fungal biomass was homogenized using liquid nitrogen, and total RNA isolation was done using RNeasy mini kit (Qiagen, Germany) by following manufacturer's protocol. This was purified using Direct-zol RNA Miniprep Kit (Zymo Research, USA). RNA quality assessment was examined using RNA ScreenTape System (Agilent, USA) in a 4150 TapeStation System (Agilent, USA). The integrity of RNA was determined by RNA integrity number (RINe) and measured by the software. RNA concentration in each samples were determined by Qubit® 3.0 Fluorometer (ThermoFisher Scientific, USA) using the Qubit™ RNA BR Assay Kit (ThermoFisher Scientific, USA).

6.2.3.2. Preparation of cDNA from mRNA

Total RNA (250 ng) was used to enrich the mRNA using NEBNext Poly (A) mRNA magnetic isolation module (New England Biolabs, USA) and enriched mRNAs were further taken for the library preparation using the NEBNext® Ultra™ II RNA Library Prep Kit for Illumina (New England Biolabs, USA). In brief, the enriched mRNAs were primed with NEBNext Random Primers and chemically fragmented in a magnesium-based buffer at 94 °C for 10 min in order to get an inserts of ~200 nucleotides. The fragmented mRNAs were reverse transcribed to form cDNA according to the manufacture's protocol (NEBProtoScript® First Strand cDNA Synthesis Kit). The purified RNA of 1- 6 µl was mixed with 2 µl of primer $d(T)_{23}VN$ and made up to 8 µl with nuclease free water followed by denaturation at 70 °C for 5 min. This mixture was transferred to M-MuLV reaction mix (6 µl) and M-MuLV enzyme mix (2 µl). From this, 20 µl of reaction mixture was incubated at 42 °C for 1 hr followed by inactivation at 80 °C for 5 min. The double stranded cDNA fragments obtained were cleaned up by using 1.8X of AMPure XP beads (Beckman Coulter, USA).

6.2.3.3. Library preparation

For obtaining cDNA blunt ends, 3’ to 5’ exonuclease end repair was performed by enzyme mix which removed the 3’ overhangs and filled the 5’ overhangs. The adenylated 3’ end was ligated with loop adapters and cleaved with uracil specific excision reagent (USER) enzyme. Library size of 400-600 bp were created with the addition of AMPure XP beads (Beckman Coulter, USA). Furthermore, the cDNA was amplified by 12 cycles of PCR with the addition of NEBNext Ultra II Q5 master mix. Based on the manufacturer's instructions, the libraries with different indices were multiplexed with NEBNext® Multiplex Oligos and loaded on the Illumina HiSeq instrument (Illumina, USA). The amplified products were then purified using 0.9X AMPure XP beads (Beckman Coulter, USA) and the final DNA library was eluted in 0.1X TE buffer.

6.2.3.4. Library quantification and validation:

The library concentration was determined in a Qubit.3 Fluorometer (Life technologies, USA) using the Qubit dsDNA H (High Sensitivity) assay Kit (ThermoFisher Scientific, USA). Dye and the buffers in the kit were diluted at 1:200 ratio and 1µl of the library was mixed with the dye mix. It was incubated at RT for 2 min and were analysed by Qubit 3 Fluorometer (Life technologies, USA). The library quality assessment was carried out using Agilent D1000 ScreenTape System (Agilent, USA) in a 4150 TapeStation System (Agilent, USA). The purified library (1 µl) was mixed with D1000 (3 µl) sample buffer and vortexed at 2000 rpm for 1 min and spun down to collect the sample to the bottom of the strip. The strip was then loaded on the Agilent 4150 TapeStation System.

6.2.3.5. Sequencing and identification of differentially expressed genes

The sequence data was generated using Illumina HiSeq and its quality was checked using FastQC (Andrews., 2010) and MultiQC (Ewels etal., 2016) software. The data was checked for

quality of base distribution, % bases (Q20 & Q30), %GC, and sequencing adapter contamination. QC theshold of the samples were Q20 >95% and selected raw reads were processed to remove adapter sequences and low quality bases using fastp (Chen et al., 2018). The obtained sequences were compared with the annotated whole genome of *A. flavus* NRRL3357 (NCBI: txid332952) (Skerker etal., 2021; Hatmaker et al., 2020; Nierman et al., 2015; Arnaud et al., 2012) as a reference genome (NCBI) and gene ids were identified. The gene ids (gene names) were converted into UniProt ids by UniProt database and its respective accession numbers (AC) with protein names obtained. Transcriptome at different time points was analyzed by comparing the samples of treated (test) and un-treated (control) based on the differential expression of logarithmic gene fold change ($Log_2FC \geq 1$ and ≤ -1, and $FDR \leq 0.05$).

6.2.4. Pathway analysis in *A. flavus* APLS-1

The pathways attributed to differentially expressed proteins and genes in *A. flavus* APLS-1 were identified using Kyoto Encyclopedia of Genes and Genomes (KEGG) mapper (Kanehisa et al., 2010) by comparing with *A. flavus* NRRL3357 (NCBI: txid332952). Results obtained from this were compared with previous published reports in bacteria and fungi for the conversion of HMF into FDCA followed by TCA cycle and possible pathway was elucidated. Heat map was generated for the essential genes using Genesis v.1.8.1 (Sturn et al., 2002) for different time intervals (24, 72, and 144 h).

6.3. Results and discussion

6.3.1. Assay of dehydrogenase, reductase and decarboxylase

After spectrophotometry analysis crude intracellular enzyme extract from the treated HMF organism showed higher O.D. rather than the un-treated samples due to the conversion of NAD^+ to NADH (Fig. 6.1.A). Since, the O.D. of treated are increased as compared to the un-treated samples presence of dehydrogenase was confirmed. In reductase assay, depletion of

NADPH was noted and crude intracellular enzyme extract from treated organism showed reduction in O.D as compared to the un-treated samples (Fig. 6.1.B). Reduction of O.D in treated samples were due to higher conversion rate of NADPH to $NADP^+$ and thus O.D. got reduced. Hence, presence of reductase in treated samples were confirmed. The presence of decarboxylase in crude enzyme extract was confirmed by the production of 2-furoic acid after HPLC analysis. The HPLC chromatogram showed a peak of 2-furoic acid at 17.9 min.

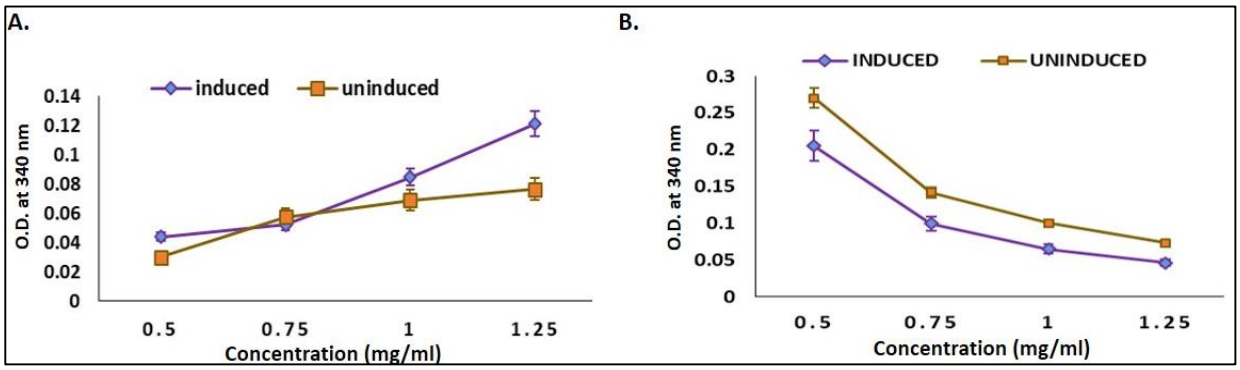


Fig. 6.1.A. Assay of dehydrogenase, B. Assay of reductase

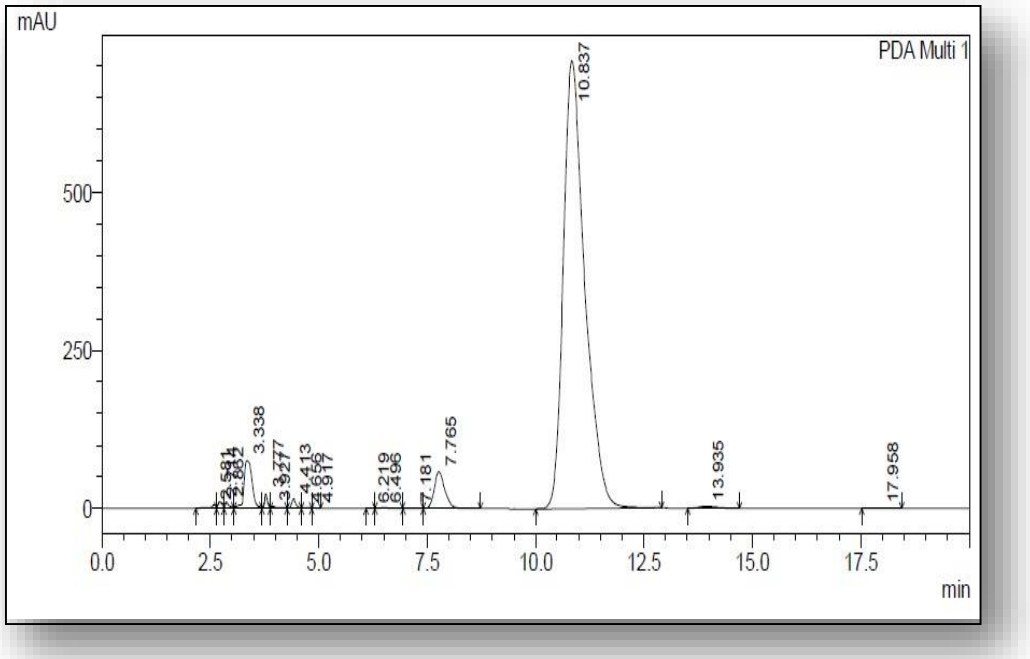


Fig. 6.2. Assay of decarboxylase

6.3.2. Proteomic analysis of intracellular crude protein in *A. flavus* APLS-1

6.3.2.1. Analysis of differentially expressed proteins

Identification and analysis of expressed proteins from HMF treated and un-treated *A. flavus* APLS-1 were carried out using UniProt database. Predicted UniProtKB accession number (AC) and its respective proteins were compared against the *A. flavus* strains ATCC MYA- 384/AF70 using UniProt database. According to the proteomic data, a total of 489 differentially expressed proteins were identified after comparison of the crude proteins of treated and un- treated samples of APLS-1. It was observed that, as the number of days increase, number of up-regulated proteins are decreased and down-regulated proteins increased. Almost 249 proteins were up-regulated (>2fold change) after first day in treated APLS-1 and this number reduced to 20 after 12 days (Fig. 6.3). Also, 80 proteins were down-regulated (>2-fold change)after first day and its number increased to 393 after 12 days.

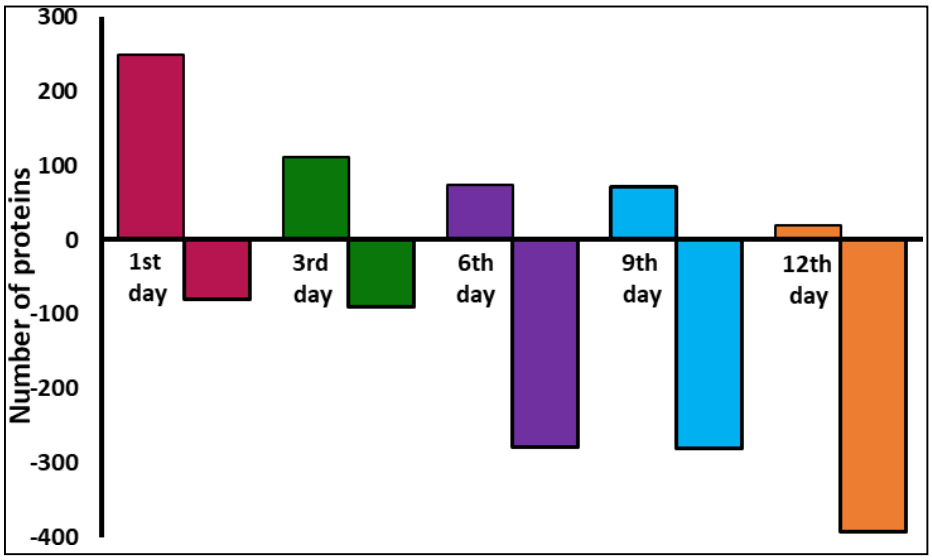


Fig. 6.3. Up-regulated and down-regulated proteins (>2-fold change)

6.3.2.2. KEGG pathway analysis of expressed proteins

KEGG pathway analysis of differentially expressed proteins (>2 fold change) were carried out from HMF treated and un-treated APLS-1 for different time intervals (1-12 days). After KEGG analysis of up-regulated proteins in HMF Treated APLS-1, respected proteins under different

metabolisms were represented in a percentage distribution (Fig. 6.4). Majority of the up-regulated proteins were distributed under carbohydrate, genetic information processing and amino acid metabolisms (each >10%) for 1 to 12 days. Also, less than one percentage of proteins were only distributed for metabolisms of xenobiotics and biodegradation, glycan biosynthesis and cofactors and vitamins.

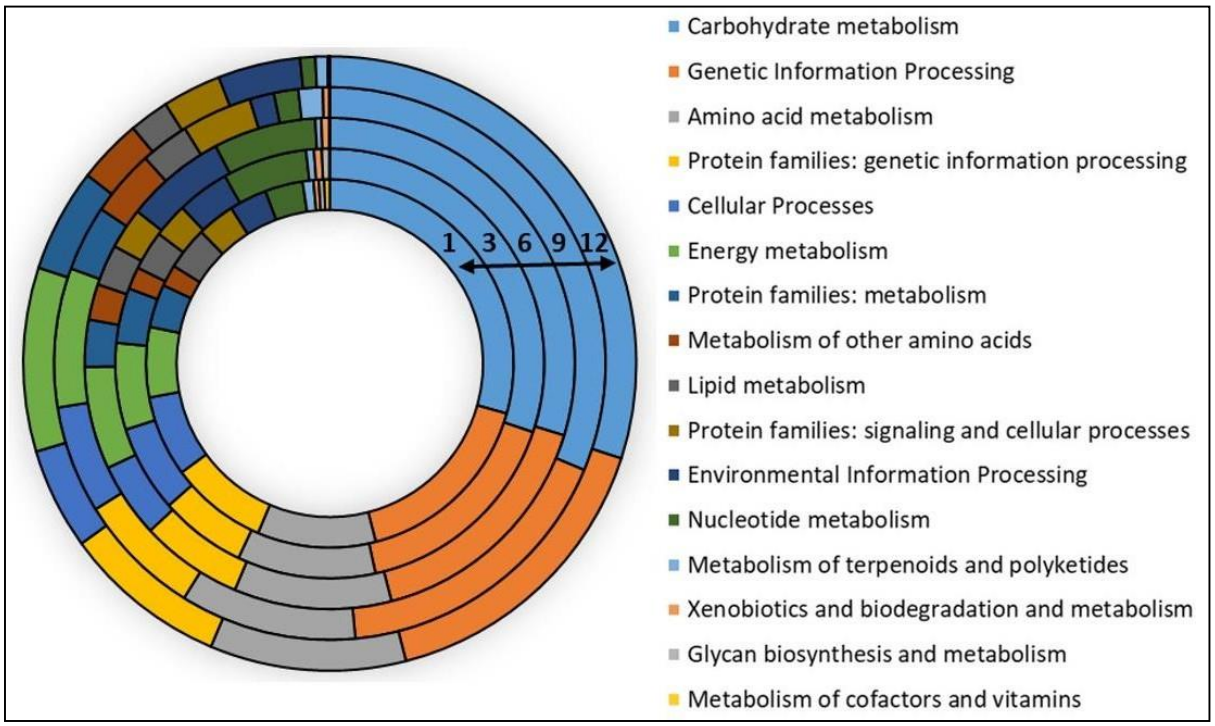


Fig. 6.4. Percentage distribution of KEGG pathway analyzed up-regulated proteins from HMF treated APLS-1 at different time intervals (1-12 days)

6.3.2.2.1. Effect of energy metabolism in response to HMF

After the KEGG pathway analysis some proteins were categorized into energy metabolism (6.5). The highest up-regulated protein from the energy metabolism classification was F-type H^+ transporting ATPase subunit alpha (AC: A0A2P2HK17), which showed 88.15, 14.82, 8.08 and 32.72 times in treated HMF samples after 1^{st}, 3^{rd}, 6^{th} and 12^{th} days respectively. Also, F-type H^+ transporting ATPase subunit alpha, is one of the subunits of the F_1 complex of F-ATP synthase (complex V) which lead the formation of ATP from ADP in the electron transport

chain (Amzel et al., 2003). Another protein, NADH dehydrogenase (ubiquinone) 1 alpha/beta subcomplex 1 (Complex I) (AC: A0A2P2H7Z1), expressed more than 5.12 and 1.65 fold in HMF treated samples after 24 and 72 h respectively. It is one of the largest five complexes of the electron transport chain, which transfer electrons from NADH to coenzyme Q10 and transfer protons across inner mitochondrial membrane for ATP synthesis (Voet et al., 2013). Succinate dehydrogenase flavoprotein subunit (AC: A0A2P2H8T5) was another protein participated in the energy synthesis which up-regulated 1.78, 1.51 and 1.56 times for different time intervals. It is a subunit complex II (succinate dehydrogenase) of the electron transport chain, which transfer electrons from succinate to ubiquinone (coenzyme Q10) by converting succinate into fumarate and aid in the synthesis of $FADH_2$ from FAD (Kenney., 1975).

Other important proteins expressed related to energy metabolism were V-type H^+-transporting ATPase subunit E (AC: A0A2P2HJD5) and H^+ transporting ATPase (AC: A0A2P2HA29) which showed highest fold change of 32.52 and 54.29 times after 24 h of HMF induction. These are the major subunits of ATPase in oxidative phosphorylation pathway which degrades the ATP and do proton transport across intracellular and plasma membranes and keep the cells acidic (Johnson et al., 1982). The presence of proteins related to energy metabolism conclude that APLS-1 used the HMF for the energy metabolism especially for ATP synthesis and its degradation.

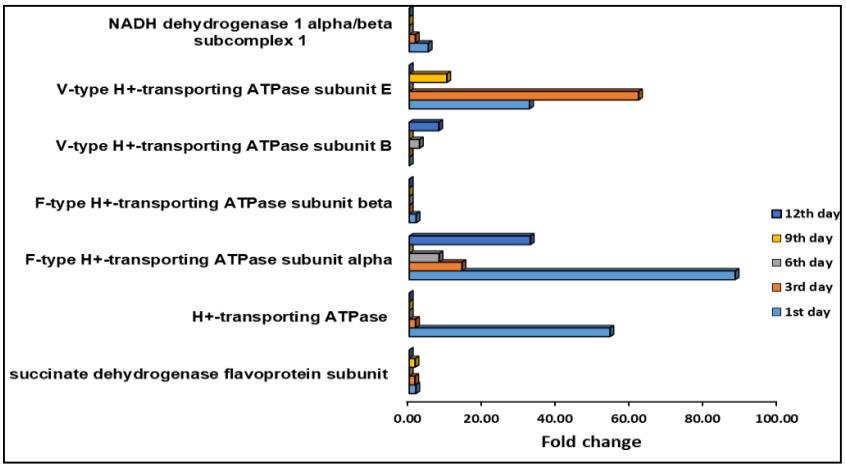


Fig.6.5. Relative protein expression in the energy metabolism (oxidative phosphorylation)

6.3.2.2.2. Effect of metabolism of cofactors in response to HMF

Purine-nucleoside phosphorylase [EC: 2.4.2.1] (AC: A0A3M7JR08) was the other important enzyme which showed a fold change of 3.86 and 4.54 after 24 and 72 h respectively after exposure of HMF (Fig.6.6.A). It is one of the major enzymes fall under nicotinate (Vit. B3) and nicotinamide metabolism. Nicotinate and nicotinamide are the precursors of NAD^+/NADH which are the cofactors of dehydrogenases.

6.3.2.2.3. Expression of proteins involved in xenobiotics biodegradation and metabolism

Some of the up-regulated proteins were involved in xenobiotics biodegradation and metabolism (Fig. 6.6.B). Among them, glutathione S-transferase [EC: 2.5.1.18] (AC: A0A2P2GZK5) showed highest of 57.79 fold after 24 h of exposure to HMF. Also, alcohol dehydrogenase [EC: 1.1.1.1] (AC: A0A364LPV9) and aldehyde dehydrogenase [EC: 1.2.1.3] (AC: A0A2P2H1W6) are the other enzymes which showed significant fold change. According to the KEGG data, HMF degradation or biotransformation is a part of the furfural degradation pathway (map00365) which fall under xenobiotics biodegradation and metabolism (map09111). This furfural and 5-(hydroxymethyl) furfural degradation pathways from *C. basilensis* HMF14 have been submitted by the authors Koopman et al., 2009 in KEGG database.

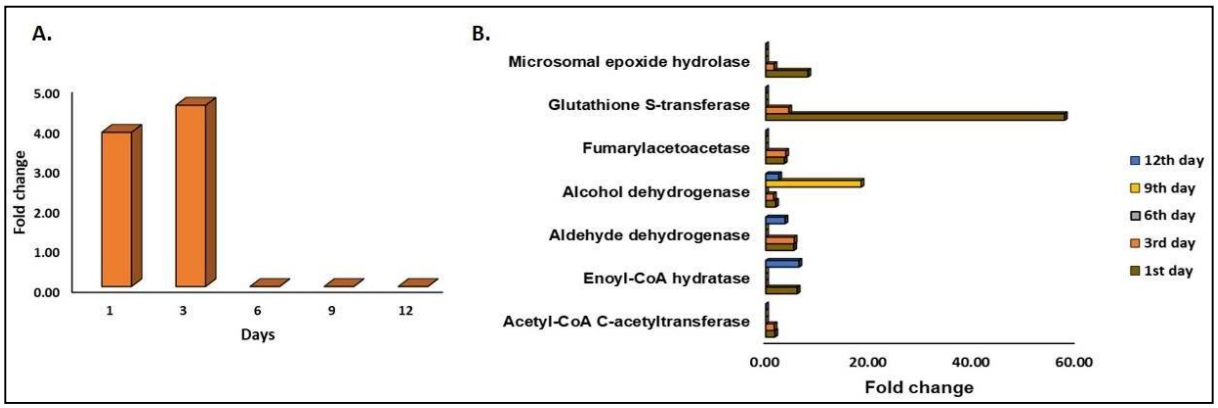


Fig.6.6. Proteins involved in xenobiotics and degradation

6.3.2.2.4. Effect of metabolism of transporters in response to HMF

Among the expressed transporters on HMF induction, putative MFS transporter (AC: A0A2P2HMK9) showed 21.84 fold up-regulation rather than other transporters (Fig. 6.7.A). This major facilitator superfamily (MFS) is from superfamily of secondary active transporters which transport different types of substrates. In a similar work, HMF and other intermediate's transport have been occurred though MFS transporters in *C. basilensis* HMF14 (Wierckx et al., 2015, Pham et al., 2020). Normally this transport is carried out either down substrate's concentration gradient or uphill by electrochemical gradients with the expense of energy (David et al., 2021).

6.3.2.3. Effect of oxidoreductases in response to HMF

Oxidoreductases are the class of enzymes normally convert toxic HMF into FDCA in microorganisms. Upon UniProt and KEGG analysis some of the major proteins under oxidoreductases were selected which may convert HMF into FDCA. For the analysis, heat map (Fig. 6.7.B) was generated for all the oxidoreductases with the help of Genesis software. Among them, aldehyde/histidinol dehydrogenase (AC: A0A5N6HBF9) was expressed greater than 3 fold change continuously for 1 to 12 days. According to the expression fold change of oxidoreductase family (Fig. 6.7.C), aldehyde dehydrogenase (aldh12) (AC: A0A2P2H760), putative aldehyde dehydrogenase (AC: A0A2P2HIK8), alcohol dehydrogenase 1 (AC: A0A364LPV9) and NAD binding Rossman fold oxidoreductase (AC: A0A364LU02) showed highest expressions like 28.66, 88.29, 18.46, 64.49 times for 1^{st}, 1^{st}, 9^{th} and 12^{th} days respectively. In a similar study of biotransformation of HMF into FDCA, expression of oxidoreductases like aryl alcohol oxidases and dehydrogenases have increased in *P. ostreatus* (Feldman et al., 2015).

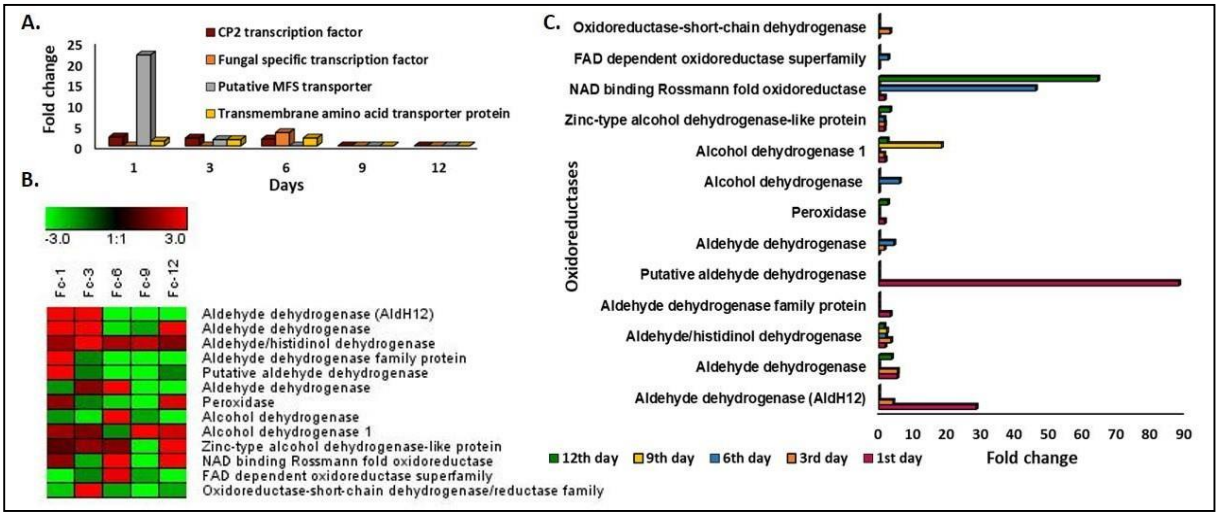


Fig.6.7.A. Relative expression of transporters, B. Heat map of oxidoreductases, C. Expression of oxidoreductases

6.3.3. Gene expression analysis in *A. flavus* APLS-1 for FDCA production

6.3.3.1. Analysis of differentially expressed genes

After the transcriptomic analysis, obtained sequences were compared with the annotated whole genome of *A. flavus* NRRL3357 as a reference genome and its gene ids (gene names) were identified. The gene names were converted by UniProt and its annotated proteins were predicted. Gene expression from HMF treated (test) and un-treated (control) samples were compared for different time intervals. According to the data, a total of 12,293 genes were differentially expressed in this organism. Among them, 3925 genes were up-regulated ($\log_2$ Fc $\geq$1) along with 4385 down-regulated ($\log_2$ Fc $\leq$ -1) after 24 h of HMF treatment in APLS-1 (Fig.6.8). However, this pattern of gene numbers changed after 144 h, in which 3166 genes were up-regulated and 1446 genes down-regulated. Also, the number of down-regulated genes ($\log_2$ Fc $\leq$ -1) were reduced drastically from 72 to 144 h. This means other genes were expressed in very minimal amount, which could not include even in the range of $\log_2$ Fc $\leq$ -1 down-regulated genes. This might be due to the absence of HMF and presence of HMF intermediates. Because, HMF is a toxic aldehyde and other acid intermediates are less toxic to the microbial

cells. Hence, even HMF is toxic it will be utilized as a better carbon source rather than its intermediates. Also, its crucial biotransformation aid the energy production of APLS-1. Hence, the organism bio-transform HMF for the survival and energy generation.

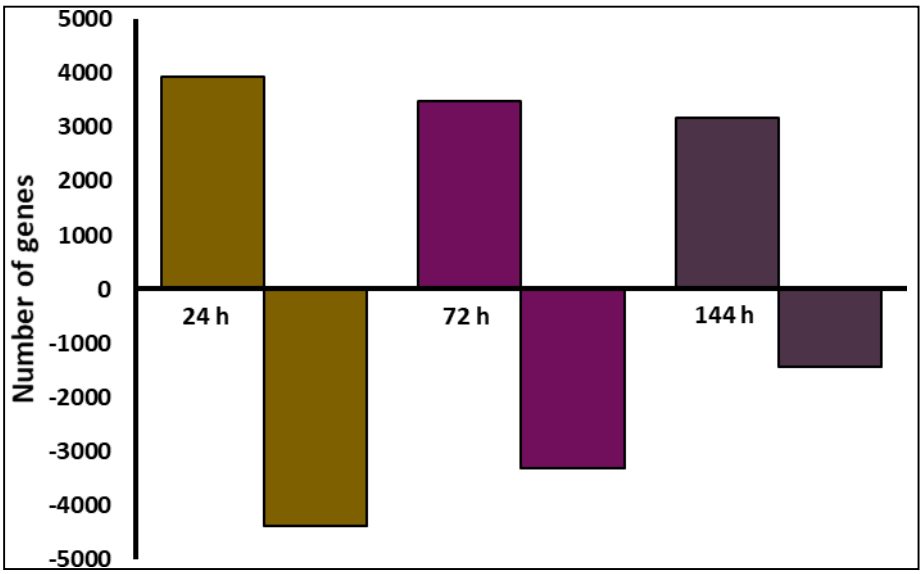


Fig.6.8. Up-regulated and down-regulated expressed genes in APLS-1

6.3.3.2. KEGG pathway analysis of expressed genes

KEGG pathway analysis of differentially expressed genes (>Log_2Fc) were carried out from HMF treated and un-treated APLS-1 for different time intervals (24-144 h). The percentage distribution of the obtained metabolisms is represented (Fig. 6.9). Almost 50% of the total up-regulated genes are distributed under protein families: genetic information processing, genetic and carbohydrate metabolisms after every time intervals. Also, less than 5% percentage of total genes are only distributed for glycan biosynthesis and metabolism, metabolism of terpenoids and polyketides, metabolism of other amino acids, xenobiotics and biodegradation, and biosynthesis of secondary metabolites.

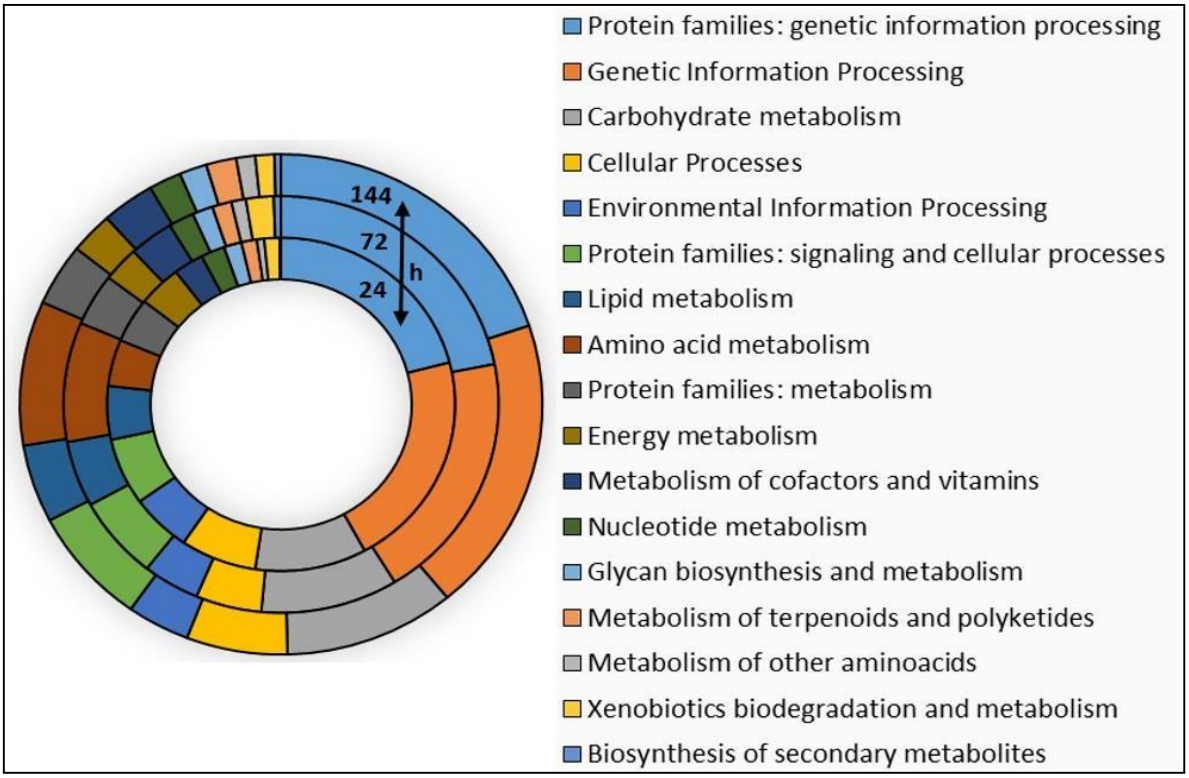


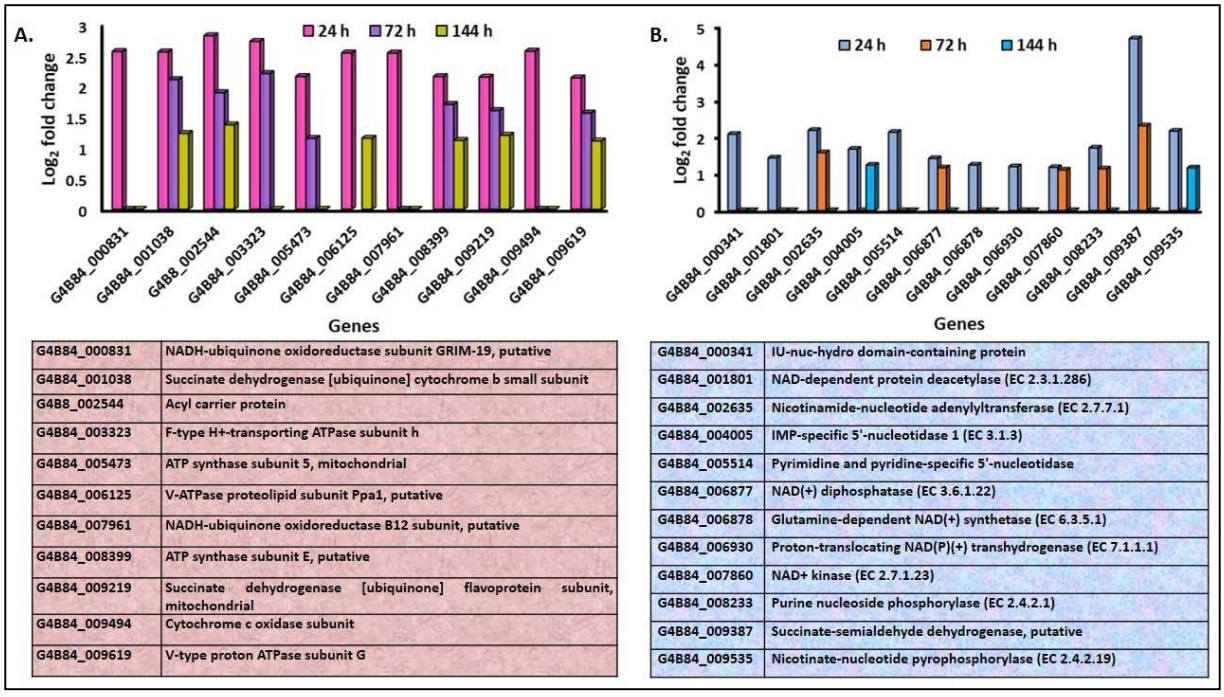
Fig.6.9. KEGG pathway analysis of up-regulated genes from HMF treated APLS-1 for different time intervals (percentage distribution)

6.3.3.2.1. Effect of energy metabolism in response to HMF

After HMF treatment, total 56 genes were up-regulated in the energy metabolism pathway for different time intervals. This genetic classification involved genes of energy synthesis and its hydrolysis. Among them, some of the genes were expressed (Fig.6.10.A) for every time intervals and these genes G4B84_001038, G4B84_002544, G4B84_006125, G4B84_008399, G4B84_009219 and G4B84_009619 annotated as Succinate dehydrogenase [ubiquinone] cytochome b small subunit, Acyl carrier protein, V-ATPase proteolipid subunit Ppa1 (EC 3.6.3.14), ATP synthase subunit E putative, Succinate dehydrogenase [ubiquinone] flavoprotein subunit mitochondrial (EC 1.3.5.1) and V-type proton ATPase subunit G respectively. Also, genes such as G4B84_001038, G4B84_008399 and G4B84_009219 take part in the energy synthesis (Sun et al., 2005), and G4B84_006125, G4B84_009619 that break

down ATP and pumps protons to intracellular compartments and acidify them (Toei et al., 2010).

According to the KEGG analysis, energy metabolism in APLS-1 was carried out by the action of nicotinate and nicotinamide metabolism (Fig.6.10.B). As the gene expression analysis, G4B84_009387 (Succinate-semialdehyde dehydrogenase, putative) was expressed in higher amount for different time intervals as compared to the other genes. All these genes are either participated in the metabolism of synthesis of energy precursors or synthesis of associated proteins related to energy.



G4B84_000831	NADH-ubiquinone oxidoreductase subunit GRIM-19, putative
G4B84_001038	Succinate dehydrogenase [ubiquinone] cytochrome b small subunit
G4B8_002544	Acyl carrier protein
G4B84_003323	F-type H+-transporting ATPase subunit h
G4B84_005473	ATP synthase subunit 5, mitochondrial
G4B84_006125	V-ATPase proteolipid subunit Ppa1, putative
G4B84_007961	NADH-ubiquinone oxidoreductase B12 subunit, putative
G4B84_008399	ATP synthase subunit E, putative
G4B84_009219	Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial
G4B84_009494	Cytochrome c oxidase subunit
G4B84_009619	V-type proton ATPase subunit G

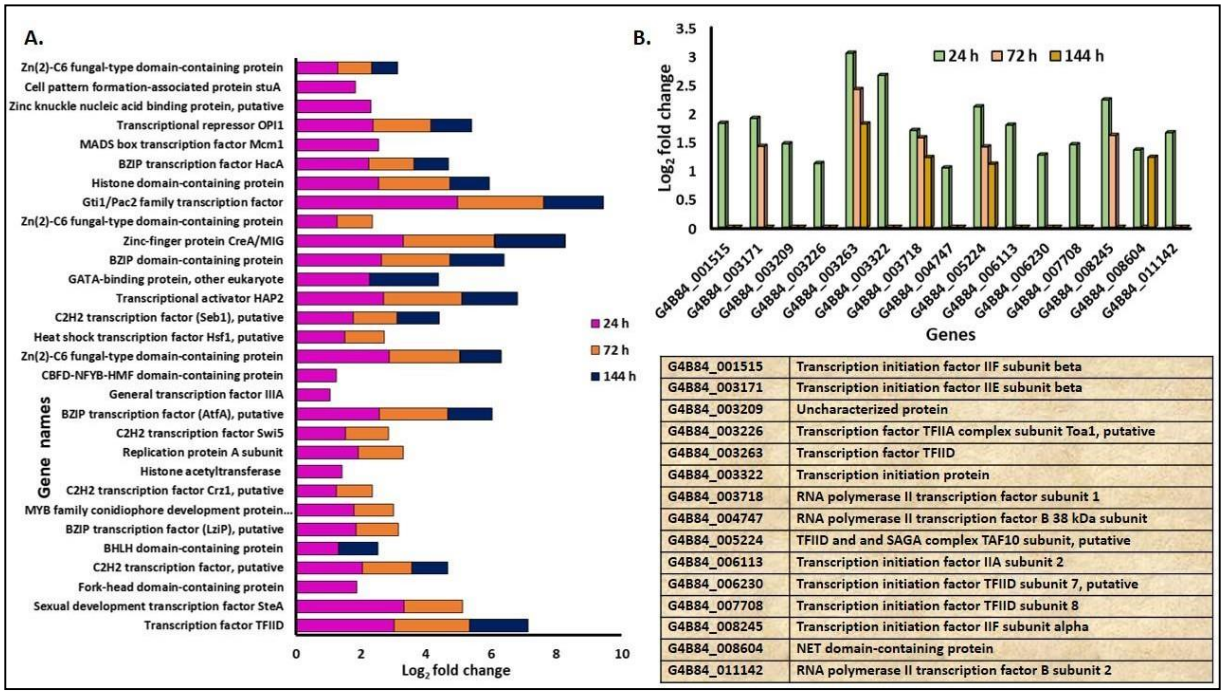
G4B84_000341	IU-nuc-hydro domain-containing protein
G4B84_001801	NAD-dependent protein deacetylase (EC 2.3.1.286)
G4B84_002635	Nicotinamide-nucleotide adenylyltransferase (EC 2.7.7.1)
G4B84_004005	IMP-specific 5'-nucleotidase 1 (EC 3.1.3)
G4B84_005514	Pyrimidine and pyridine-specific 5'-nucleotidase
G4B84_006877	NAD(+) diphosphatase (EC 3.6.1.22)
G4B84_006878	Glutamine-dependent NAD(+) synthetase (EC 6.3.5.1)
G4B84_006930	Proton-translocating NAD(P)(+) transhydrogenase (EC 7.1.1.1)
G4B84_007860	NAD+ kinase (EC 2.7.1.23)
G4B84_008233	Purine nucleoside phosphorylase (EC 2.4.2.1)
G4B84_009387	Succinate-semialdehyde dehydrogenase, putative
G4B84_009535	Nicotinate-nucleotide pyrophosphorylase (EC 2.4.2.19)

Fig.6.10.A. Up-regulated genes in the energy metabolism, B. nicotinate and nicotinamide metabolism

6.3.3.2.2. Effect of HMF treated genes on genetic information processing

Total 30 genes were actively expressed due to the action of HMF in APLS-1 for different time intervals (Fig.6.11.A). As the time increases the number of the genes for the transcription factors and their expression were reduced. Among them, Gti1/Pac2 family transcription factor

(G4B84_008139), Sexual development transcription factor SteA (G4B84_000535), Zinc-finger protein CreA/MIG (G4B84_005397), Transcription factor TFIID (G4B84_003263), Transcriptional activator HAP2 (G4B84_005117), BZIP domain-containing protein (G4B84_005297) were major transcription factors which showed higher log_2 expression fold after 24 h as 4.95, 3.33, 3.31, 3.03, 2.69 and 2.64 respectively. Almost 15 genes were involved as the basal transcription factors in the genetic information processing of RNA polymerase (Fig.6.11.B). Upon expression analysis, genes G4B84_003718 (RNA polymerase II transcription factor subunit 1) and G4B84_008604 (NET domain-containing protein) were showed significant up-regulation throughout the analysis.



G4B84_001515	Transcription initiation factor IIF subunit beta
G4B84_003171	Transcription initiation factor IIE subunit beta
G4B84_003209	Uncharacterized protein
G4B84_003226	Transcription factor TFIIA complex subunit Toa1, putative
G4B84_003263	Transcription factor TFIID
G4B84_003322	Transcription initiation protein
G4B84_003718	RNA polymerase II transcription factor subunit 1
G4B84_004747	RNA polymerase II transcription factor B 38 kDa subunit
G4B84_005224	TFIID and and SAGA complex TAF10 subunit, putative
G4B84_006113	Transcription initiation factor IIA subunit 2
G4B84_006230	Transcription initiation factor TFIID subunit 7, putative
G4B84_007708	Transcription initiation factor TFIID subunit 8
G4B84_008245	Transcription initiation factor IIF subunit alpha
G4B84_008604	NET domain-containing protein
G4B84_011142	RNA polymerase II transcription factor B subunit 2

Fig.6.11.A. HMF treated genes on genetic information processing of RNA polymerase, B. genes of transcription factors on genetic information processing

6.3.3.2.3. Effect of HMF treated genes on transporters of signaling and cellular responses

Total 79 genes were expressed in the metabolism of signaling and cellular responses. Among them, major membrane transporter families were ABC transporters and MFS transporters. The genes from the family of ABC transporters (Fig.6.12.A) were G4B84_000547 (ABC multidrug

transporter Mdr1), G4B84_000672 (ATP-binding cassette subfamily G member 2), G4B84_001193 (ATP-binding cassette subfamily C (CFTR/MRP), member 1), G4B84_003554 (Peroxisomal ABC transporter (PXA1) putative), G4B84_003868 (ABC multidrug transporter Mdr2). Among them, gene G4B84_003354 showed highest expression as $\log_2$ Fc 2.48, 1.89 and 1.35 for 24, 72 and 144 h respectively. Its protein (Peroxisomal ABC transporter), is seen in the outer membrane of peroxisomes where detoxification of alcohol and other toxic substances take place with the help of oxidative enzymes. Another gene G4B84_003868 also showed good expression as $\log_2$ Fc 2.43, 2.2 and 1.9 for 24, 72 and 144 h respectively. Its protein is an ABC multidrug transporter Mdr2 which actively transport drugs or toxins out of the cells.

The genes from the family of MFS transporters (Fig.6.12.B) were G4B84_006140 (UMF1; MFS transporter UMF1 family), G4B84_004117 (MFS multidrug transporter, putative), G4B84_010386 (MFS transporter, SH family, lactate transporter), and G4B84_005887 (MFS transporter, SP family, general alpha glucoside:H+ symporter). Among them, G4B84_004117 showed highest expression as $\log_2$ Fc 2.91 after 24 h and involved in multidrug transport. These genes have a crucial role in HMF and other intermediates transport (Donoso et al., 2021). MFS multidrug transporters are actually antiporters, which bind to structurally unrelated drugs or toxins which have either charged or neutral or hydrophobic or aromatic moiety and extrude them from the cells using the energy released from the downhill transport of protons and associated electrochemical gradient. All other MFS transporters might be actively participated in sugar transport system for energy synthesis.

It has been reported that major facilitator superfamily transporters msf1 (GU556182 and GU556183) observed in the pathway of HMF/furfural metabolic pathway of *P. putida* ALS1267. (Crigler et al., 2020). These major facilitator superfamily transporters have showed 58% similarity to the HMF transporters, HmfT1 and HmfT2 in *C. basilensis* HMF 14, which

have been genetically expressed in *P. putida* S12-B38. This engineered strain is able to uptake higher HMF acid and FFCA and lead into increased FDCA production. (Wierckx et al., 2016). MFS multi drug resistant (mdr) transporters and ABC transporters are the two large families which actively participate in the drug or toxin resistance in fungi like *Aspergillus* sps (Drew et al., 2021). Hence, HMF and other intermediates might be transported into and out of the cells though ABC transporters and MFS multidrug transporters.

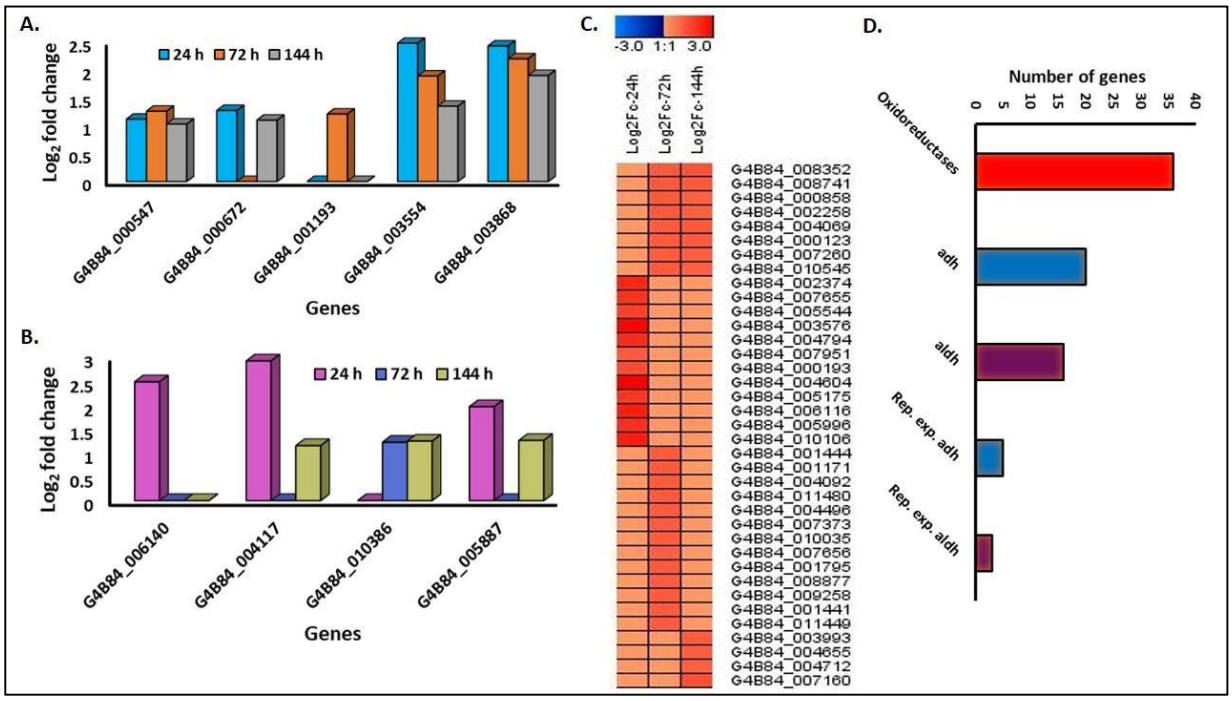


Fig.6.12. HMF treated genes of A. ABC superfamily transporters, B. MFS superfamily on signaling and cellular processes, C. Expressed oxidoreductase genes, D. Number of oxidoreductase genes

6.3.3.3. Effect of oxidoreductase in response to HMF

Oxidoreductases are the major class of enzymes participated in the conversion of HMF to FDCA. Almost 36 oxidoreductase genes were expressed when APLS-1 was treated with HMF (Fig.6.12.C). The genes under the classification of oxidoreductases obtained during biotransformation of HMF into FDCA were, alcohol dehydrogenase (*adh*) and aldehyde

dehydrogenase (*aldh*). There were 20 *adh* and 16 *aldh* genes expressed during this bioconversion (Fig.6.12.D). Among them, five *adh* and thee *aldh* genes were repeatedly expressed for different time intervals (Tab.6.1). Presence of alcohol dehydrogenases and oxidases were reported during conversion of HMF into FDCA in *P.ostreatus* (Feldman et al., 2015).

Sl. No.	Gene_id	Protein name	Log_2Fc-24 h	Log_2Fc-72 h	Log_2Fc-144 h
1	G4B84_008352	Alcohol dehydrogenase, putative	0	1.28	1.36
2	G4B84_008741	Alcohol dehydrogenase, putative	0	1.25	1.25
3	G4B84_000858	Alcohol dehydrogenase, putative	0	1.22	1.12
4	G4B84_002258	Alcohol dehydrogenase, putative	0	1.22	1.10
5	G4B84_004069	Alcohol dehydrogenase, putative	0	1.2	1.26
6	G4B84_000123	Aldedh domain-containing protein	0	1.22	1.22
7	G4B84_007260	Aldedh domain-containing protein	0	1.2	1.12
8	G4B84_010545	Aldehyde dehydrogenase family protein, putative	0	1.22	1.10

Tab.6.1. Repeatedly expressed alcohol and aldehyde dehydrogenase genes

6.3.3.4. Identification of presumptive decarboxylase in response to HMF

The genes responsible for the conversion of HMF into FDCA followed by FDCA to TCA cycle pathway is associated with the HMF gene clusters, *hmfABCDE* (Gen bank AC: GU556182) and *hmfFGH'H* (Gen bank AC: GU556183) in *C. basilensis* HMF 14. Also, in *C. basilensis* HMF 14, the conversion of FDCA into 2-furoic acid is taken place by the enzyme 2,5-furan-dicarboxylic acid decarboxylase with genes *hmfF* (2,5-furan-dicarboxylic acid decarboxylase

1) and *hmfG* (2,5-furan-dicarboxylic acid decarboxylase 2) (Koopman et al., 2010). In our study, genes responsible for 2,5-fdca decarboxylase was not expressed upon transcriptomic analysis of HMF treated *A. flavus* APLS-1. Even though, upon NCBI-BLAST analysis, *hmfF* from *C. basilensis* showed 26.97 and 28.28% similarity to the genes G4B84_003159 and G4B84_010172 in APLS-1. Both genes were identified as ferulic acid decarboxylase 1 (FDC1) (EC: 4.1.1.102) by UniProt analysis. Also, upon NCBI conserved domain search, both these genes were related to the UbiD family with decarboxylase activity on aromatic carboxylic acids. Hence, conversion of aromatic FDCA to 2-furoic acid might be carried out by the genes, G4B84_003159 and G4B84_010172 and its expression is shown in the table (Tab.6.2). The genes G4B84_003159 and G4B84_010172 were also showed 36 and 57.57% similarity to the gene AN7164 from *Aspergillus nidulans* FGSC A4. The AN7164 gene codes for an enzyme 3-octaprenyl-4-hydroxybenzoate carboxy-lyase which is also an UbiD family enzyme showed FDCA decarboxylase activity (Martins et al., 2020).

Sl.No.	Gene_id	Protein name	Log_2Fc-24 h	Log_2Fc-72 h	Log_2Fc-144 h
1	G4B84_003159	Ferulic acid decarboxylase 1 (FDC1)	0	1.09	1.134
2	G4B84_010172	Ferulic acid decarboxylase 1 (FDC1)	0	1.04	1.42

Tab.6.2. Expression of ferulic acid decarboxylase 1 on different time intervals

6.4. Summary

Enzymatic, and transcriptomic analysis of HMF treated APLS-1 were analyzed and desired proteins and respective genes identified. Also, possible pathway for the biotransformation of HMF into FDCA was elucidated (Fig.6.13). HMF and its associated intermediates might be transported though MFS and ABC drug transporters, by proton flux and stored ATP. Since HMF is toxic to APLS-1, when HMF is entered into cell cytoplasm, it may be converted into other non-toxic intermediates with the help of *adh* (alcohol dehydrogenase) and *aldh* (aldehyde dehydrogenase) genes with NADH production. This NADH will be utilized for ATP synthesis and might be utilized for HMF and its intermediate's transport and cell metabolic activities. This process will be continued until less stored energy and nutrient depletion. When there are not enough nutrients, the cells will start to uptake FDCA and it will be converted into 2-furoic acid by *FDC1* (ferulic acid decarboxylase 1) and enter into TCA cycle for energy synthesis. Eventually, after all FDCA is up taken by the cells, microorganism will go to its death phase.

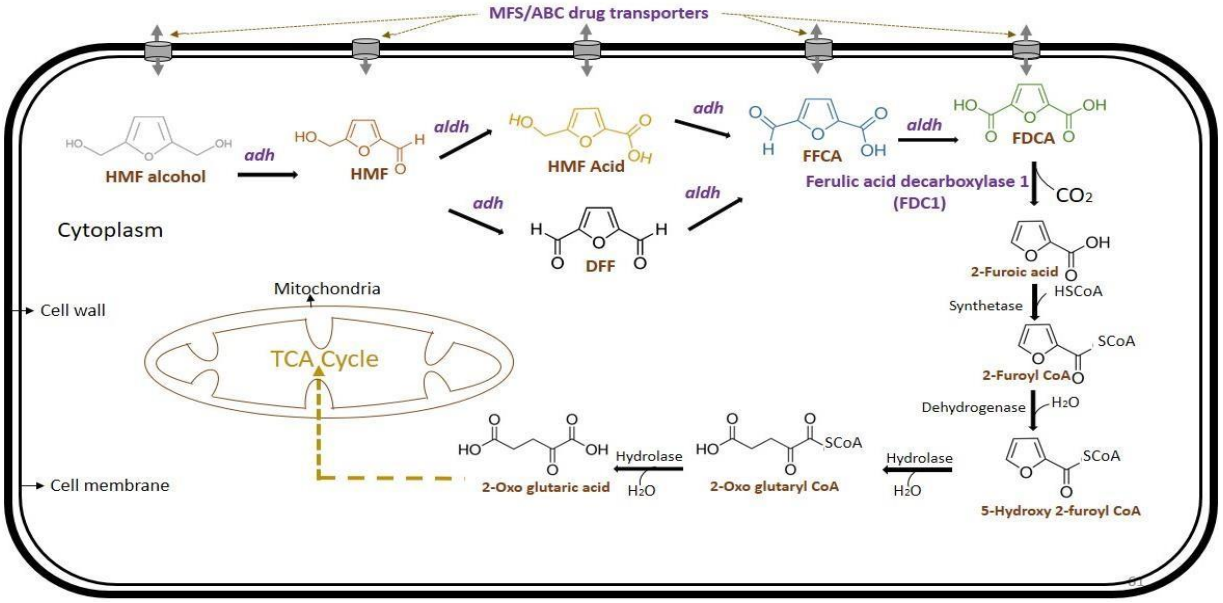


Fig.6.13. Proposed pathway for the biotransformation of HMF into FDCA in APLS-1

Chapter 7

Cell-free approach for FDCA production

7.1. Introduction

Biological production is an alternative to chemo-catalytic production of FDCA. The biological production of FDCA mainly involves two types, microbial and enzyme assisted. Among them, enzymatic reactions are fast as compared to the microbial cell assisted FDCA production. Even though, microbes catalyze the conversion of HMF into FDCA, other reaction media products or products secreted by cells may hinder the activity and makes the reaction slow and complex. Also, this makes the purification of FDCA as a hectic process. In order to avoid these, specific enzymes can be used for the conversion of HMF into FDCA. Enzymatic production of FDCA from HMF is not possible with single enzymes, as the aromatic intermediates have either aldehyde or acid or alcohol moiety (Fig.7.1). Hence, instead of single enzyme, multiple enzymes have to be used. This multiple enzymes are added either in tandem or one pot reactions. In our current study, we evaluated four different commercial enzymes like, two aldehyde dehydrogenase (ALDH), alcohol oxidase (AO) and galactose oxidase (GAO) and activity against different substrates were performed. Finally, FDCA production using multiple enzymes have been demonstrated.

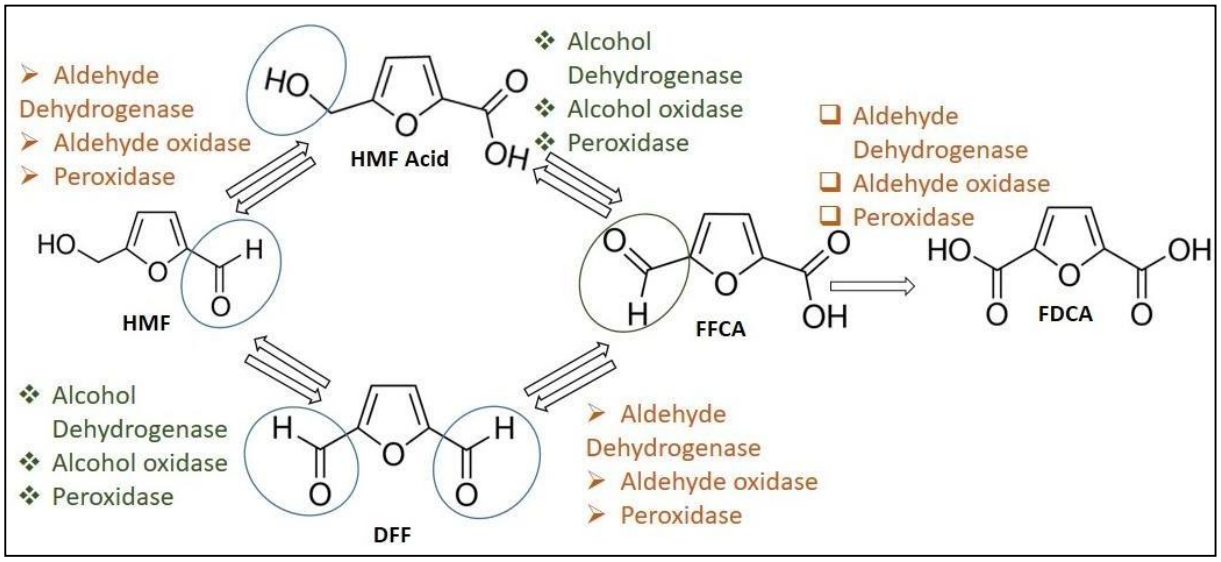


Fig.7.1. Action of multiple enzymes on FDCA production

7.2. Materials and methods

7.2.1. Enzymes

Commercially available enzymes ALDH from yeast (20 U/mg), ADH from yeast (300 U/mg), AO solution from *Pichia pastoris* (23 U/mg), GO from *Dactylium dendroides* (500 U/mg) were purchased from Sigma Aldrich India.

7.2.2. Oxidation of HMF, DFF and FFCA with ALDH

Oxidation activity (ALDH) was carried out using different amount of enzyme concentration (5- 15 U) on different substrates like HMF (50 mM), DFF (10 mM) and FFCA (10 mM) for the products HMF acid, FFCA and FDCA respectively. The experiments were conducted in 200 mM potassium phosphate buffer (KH_2PO_4) (pH 7) at 30 °C. For the reactions with HMF, 10 mM NAD^+ was used as a cofactor and 2.5 mM NAD^+ used for the substrates DFF and FFCA. The reactions without enzymes were kept as controls. Total reaction volume was 300 µl and reactions were conducted in five ml screw cap vials which were incubated in shaking incubator at 30 °C for six days at 200 rpm. The samples were taken for every 24 h intervals and the activity was stopped with 0.1 M Hydro chloric acid (HCl). The samples were centrifuged at 10,000 rpm and the collected supernatant were analyzed by HPLC.

Sl.No.	Reaction mixtures	C	T1	T2	T3	T4	T5
1	ALDH (U)	0	5	7.5	10	12.5	15
2	NAD^+ (mM)	10	10	10	10	10	10
3	HMF (mM)	50	50	50	50	50	50
4	DFF/FFCA (mM)	10	10	10	10	10	10
5	KH_2PO_4 (mM)	200	200	200	200	200	200

Tab.7.1. Enzyme (ALDH) reaction mixture

7.2.3. Oxidation of HMF and HMF acid with AO

Different amount of enzyme concentration (6-30 U) was used for checking the oxidation activity against substrates like HMF (50 mM) and HMF acid (10 mM) for the products DFF and FFCA respectively. Reactions were carried out using O_2 (atm. air) as cofactor in 200 mM KH_2PO_4 buffer (pH 7) at 30 °C. The reactions without enzymes were kept as controls. Tubes were incubated for five days. The reaction volume, sample processing and analysis were same as described above for all experiments unless otherwise stated.

Sl.No.	Reaction mixtures	C	T1	T2	T3	T4	T5
1	AO (U)	0	6	12	18	24	30
2	HMF (mM)	50	50	50	50	50	50
3	HMF acid (mM)	10	10	10	10	10	10
4	KH_2PO_4 (mM)	200	200	200	200	200	200

Tab.7.2. Enzyme (AO) reaction mixture

7.2.4. Oxidation of HMF and HMF acid with GAO

Different amount of GAO (4.5-22.5 U) was used for checking its oxidative action against substrates HMF (50 mM) and HMF acid (10 mM) for the products DFF and FFCA respectively with O_2 (atm. air) as cofactor in 200 mM KH_2PO_4 buffer (pH 7) at 30 °C. The reactions without enzymes were kept as controls. All the reactions were incubated for four days.

Sl.No.	Reaction mixtures	C	T1	T2	T3	T4	T5
1	GAO (U)	0	4.5	9	13.5	18	22.5
2	HMF (mM)	50	50	50	50	50	50
3	HMF acid (mM)	10	10	10	10	10	10
4	KH_2PO_4 (mM)	200	200	200	200	200	200

Tab.7.3. Enzyme (GAO) reaction mixture

7.2.5. Development of an enzyme cascade system for the production of FDCA

For the efficient oxidation of HMF to FDCA, better enzyme cascade system was needed to develop. So multiple commercial enzymes were used in a single pot reaction for the conversion of substrate into desired product (Tab.7.4). For efficient production of FDCA, three enzymes were selected based on their performance on HMF and its derivatives. Substrate, HMF was selected as 50 mM for the oxidation by enzymes. The enzymes like GAO, AO and ALDH were added in different concentration in each tubes, with 50 mM NAD^+ and O_2 (atm. air) as cofactor. All the reactions were performed in 200 mM KH_2PO_4 buffer (pH 7) at 30 °C and incubated in shaker for six days at 200 rpm. Total reaction volume was 300 µl and the reaction mixture without enzyme kept as control.

Sl.No.	Reaction mixtures	C	T1	T2	T3	T4
1	GAO (U)	0	22.5	22.5	22.5	22.5
2	AO (U)	0	0	30	0	30
3	ALDH (U)	0	0	0	10	10
4	HMF (mM)	50	50	50	50	50
5	NAD^+ (mM)	10	10	10	10	10
6	KH_2PO_4 (mM)	200	200	200	200	200

Tab.7.4. Enzyme cascade system reaction mixture

7.3. Results and discussion

7.3.1. Oxidation activity of ALDH on substrates HMF, DFF and FFCA

Different enzyme concentrations (5-15 U) were used to know its oxidation activity on substrates like HMF, DFF and FFCA for the products HMF acid, FFCA and FDCA respectively. After six days of incubation third test (T3) of experiment showed 1.82±0.05 mg/ml (12.97±0.38 mM) of HMF acid from 6.3 mg/ml (50 mM) of HMF with 10 U of enzyme (Fig.7.2.A). Even though, ALDH was acted efficiently on all the test experiments for the conversion of 1.24 mg/ml (10 mM) DFF, first set of experiment showed a production of 1.17±0.06 mg/ml (9.45±0.47 mM) FFCA after second day itself with 5 U of enzyme (Fig.7.2.B). For the conversion of 1.4 mg/ml(10 mM) FFCA to FDCA, fourth set (T4) of experiment showed best production of 1.06±0.05 mg/ml(6.8±0.34 mM) FDCA after six days of incubation with 12.5 U of enzyme (Fig.7.2.C). In the experiments, where desired products were formed in less concentration or declined was due tothe formation of other HMF intermediates by the multiple substrate oxidative action of ALDH.

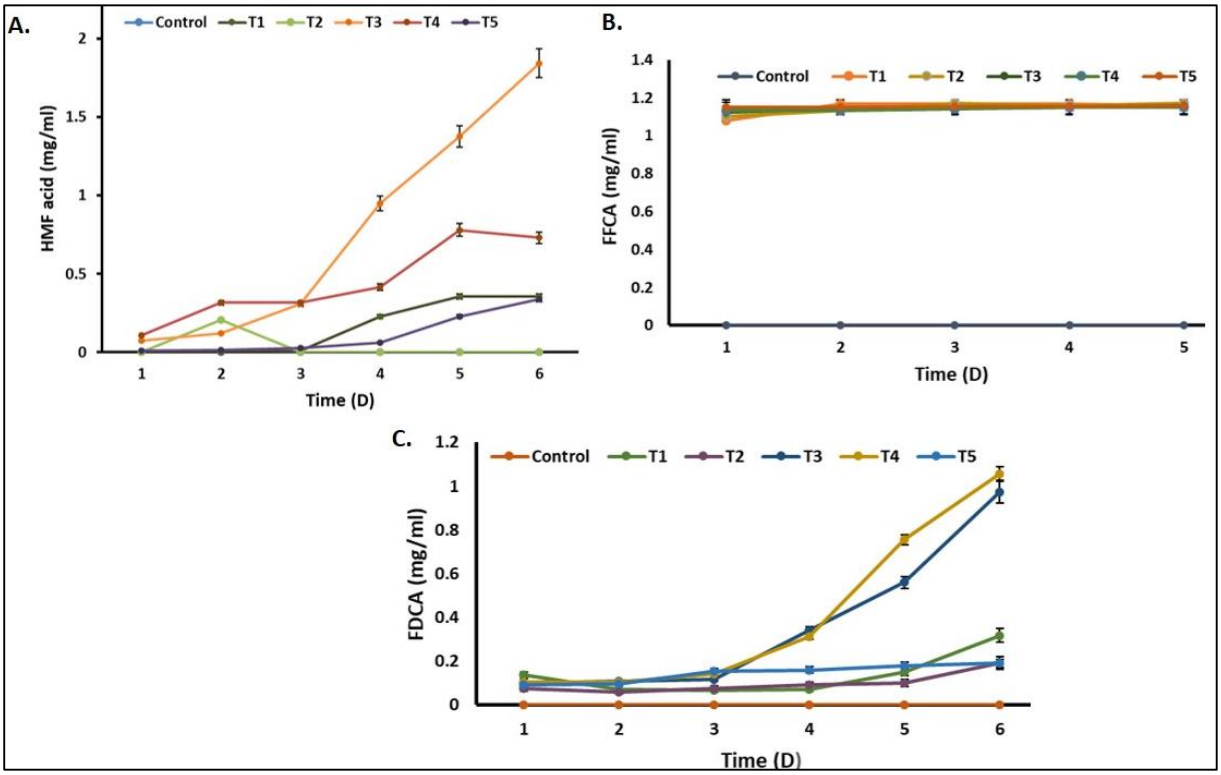


Fig.7.2. Oxidation on different substrates, A. HMF, B. DFF and C. FFCA with ALDH

7.3.2. Oxidation activity of AO on substrates HMF and HMF acid

Oxidation activity of AO (6-30 U) was performed against substrates, HMF and HMF acid for the products DFF and FFCA respectively. When the oxidation activity was checked against 6.3 mg/ml (50 mM) HMF, fourth test (T4) showed 1.52±0.08 mg/ml (12.25±0.61 mM) of DFF production after first day of incubation with 24 U of enzyme and it declined afterwards (Fig.7.3.A). Upon oxidation on 1.42 mg/ml (10 mM) HMF acid with 24 U of enzyme, produced 1±0.05 mg/ml (7.18±0.36 mM) of FFCA after five days of incubation as fourth set (T4) of experiment (Fig.7.3.B).Product declining on certain experiments were due to inter conversion of intermediates by themultiple substrate specificity of AO. It has been reported that oxidation on 20 mM HMF with a commercial AO has produced 19.5% DFF with a *Km* of 14 (Cajnko et al., 2020).

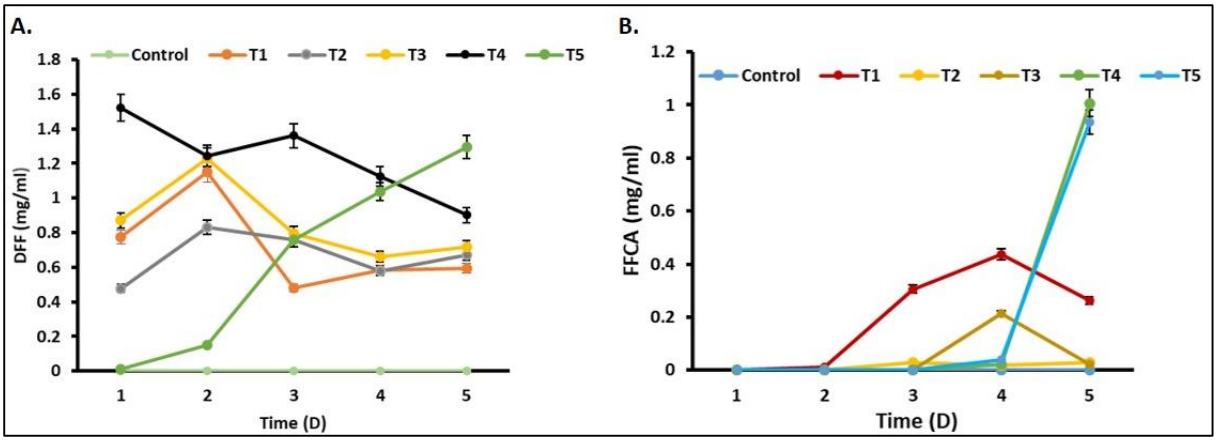


Fig.7.3. Oxidation on different substrates, A. HMF, B. HMF acid with AO

7.3.3. Oxidation activity of GAO on substrates HMF and HMF acid

Oxidation activity of GAO (4.5-22.5 U) was tested against substrates HMF and HMF acid (10 mM) for the products DFF and FFCA respectively. When the enzyme was tested against 6.3 mg (50 mM) of HMF, the fifth test (T5) showed best production after first day itself (Fig.7.4.A). In this, 22.5 U enzyme showed a production of 3.8±0.19 mg/ml (30.64±1.53 mM) DFF from 6.3 mg/ml (50 mM) HMF immediately and declined its amount afterwards due to other intermediates formation. Also, for the conversion of (10 mM) HMF acid, fourth test (T4) with 18 U of enzymeproduced 1.12±0.06 mg/ml (8.02±0.4 mM) FFCA after third days of incubation followed by a decline (Fig.7.4.B). Activity of a commercial GAO has been reported that converted 10 mM HMF andHMF acid into 5.1% DFF and 2.7% FFCA respectively after three days of incubation (Cajnkoet al., 2020)..

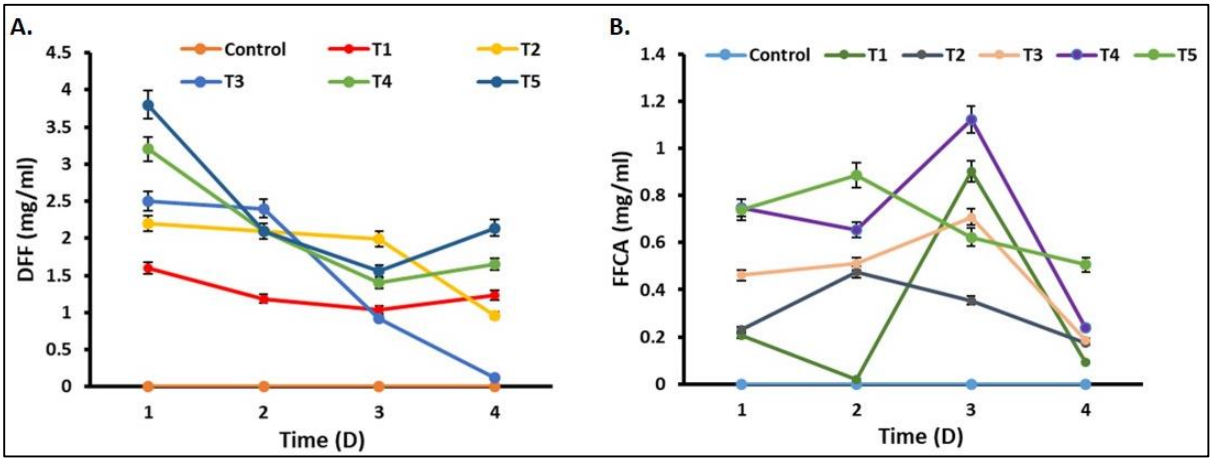


Fig.7.4. Oxidation on different substrates, A. HMF, B. HMF acid with GAO

7.3.4. One pot conversion of HMF into FDCA using enzyme cascade system

For increasing the FDCA production from HMF three enzymes ALDH, AO and GAO were selected based on their activity. Four tests were prepared using three enzymes and incubated for five days. Among them second (T2) test showed best FDCA production followed by first test (T1) (Fig.7.5.A).

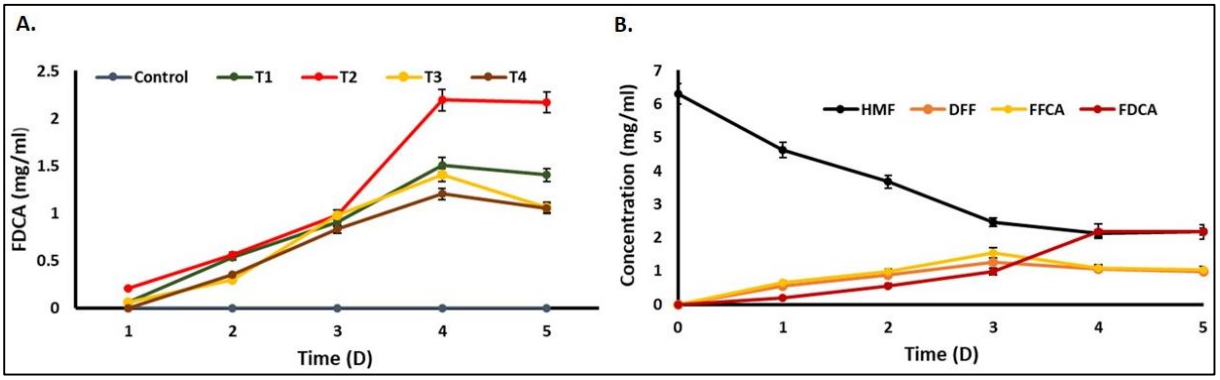


Fig.7.5. A. Production of FDCA by different enzyme cascade systems, B. Concentration of intermediates during conversion of HMF into FDCA in third test

Second test showed FDCA production of 2.19±0.11 mg/ml (14.03±0.70 mM) from 6.3 mg (50 mM) HMF after four days with 22.5 U of GAO and 30 U of AO after four days with a yield of 25.4±1.27%. In the intermediate analysis HMF was utilized for the reaction until four days and it attained a stationary phase in the fifth day. FDCA production was also stopped in fourth day

and reached to stationary level after that. An enzyme cascade system with multiple enzymes have been published in which wild-type enzyme like aryl alcohol oxidase (AAO) from *P .eryngii*, peroxygenase from *Agrocybe aegerita* (*Aae*UPO) and recombinant GAO have converted 10 mM HMF into 7.9 mM FDCA with 80% yield (Karich et al., 2018). Also, in a previous study, one pot study of GAO M_{3-5} and aldehyde oxidase PaoABC, Horse Radish peroxidase (HRP) and catalase converted 100 mM HMF into 100% FDCA after 6 h (Mckenna et al., 2013). Sameauthors have converted 50 mM HMF into FDCA in 74% yield with GAO M_{3-5} and aldehyde oxidase PaoABC (Mckenna et al., 2015). Also, in our work second set showed 1.5±0.08 mg/ml (9.86±0.5mM) FDCA with 22.5 U of GAO only (single enzyme) after four days. It means that GAO can oxidize all the HMF intermediates in the pathway and eventually end up in FDCA production. In a previous study HMF oxidase gene from *Methylovorus sp*. MP688 has expressed in *E. coli* and this HMF oxidase (HMFO) converted 2 mM HMF into 92% FFCA and 8% FDCA after 5 h reaction (Dijkman et al., 2014).

7.4. Summary

Oxidation of HMF (6.3 mg/ml), DFF (1.24 mg/ml), and FFCA (6.3 mg/ml) was conducted with ALDH and produced HMF acid (1.8±0.05 mg/ml), FFCA (1.17±0.06 mg/ml), and FDCA (1.05±0.05 mg/ml) respectively. Also, AO converted HMF (6.3 mg/ml) and HMF acid (1.42 mg/ml) into DFF (1.52±0.08 mg/ml), and FFCA (1±0.05 mg/ml) respectively. Also, activity of GAO was tested which converted HMF (6.3 mg/ml) and HMF acid (1.42 mg/ml) into DFF (3.8±0.19 mg/ml), and FFCA (1.12±0.06 mg/ml) respectively. After enzymatic optimization, 2.19±0.11 mg/ml FDCA was produced from 6.3 mg/ml HMF with the mixture of enzymes GAO and AO with cofactors NAD^+ and O_2 (atm. air) at pH 7 after 4 days. The overall yield of one pot synthesis with multiple enzymes was 25.4±1.27%. Also, Single enzyme GAO produced 1.5±0.08 mg/ml of FDCA with O_2 (atm. air) as cofactor.

Chapter 8

Summary and conclusion

Depletion of resources of petroleum based products and its associated greenhouse gas emission are the major concerns for the whole world today. Also, fluctuating price of fossil products for energy, fuel and chemicals are increasing day by day which hinder the growth and economy of developing and undeveloped countries. Apart from the conventional non-renewable sources, renewable source like biomass can be used as an alternative feed stock for the development of energy, fossil and other valuable chemical products. Also, burning of lignocellulosic biomass makes increase in the atmospheric CO_2 which affect whole flora and fauna due to the greenhouse gas emission and global warming. To avoid these problems, generation of value added products from lignocellulosic biomass is very essential. This will lead the world to a sustainable circular economy with net zero carbon emission.

One of the value added chemical from biomass is FDCA which is a monomer of PEF can be used for plastic applications. This FDCA is synthesized from HMF which is derived from fructose or glucose or lignocellulosic biomass. Chemical processes for the production of FDCA from HMF normally require carcinogenic and expensive chemicals, and organic solvents which conduct in high pressure and temperature. To avoid the high expenditure and environmental hazard of chemical processes, biological processes of FDCA is crucial in the recent scenario. Hence, in our work HMF was synthesized by chemo-catalytic routes from pineapple waste and the biological production of FDCA carried out with microbial (whole cell) and industrial enzymes (cell free). The major findings from the work are summarized below.

8.1. Significant findings from the study

HMF (0.29±0.02 g/g) was synthesized from pineapple peel using $CrCl_3$ in 1 h at 100 °C. After isolation and screening, *A. flavus* APLS-1 was selected for further FDCA production studies. Using APLS-1 (whole cell) optimization of media conditions for the synthesis of FDCA were

carried out. After statistical optimization of most effective parameters, yield of FDCA was 64.5% in MSM. Upon validation, production of (0.8 g/g) FDCA (58 h, 6.66 gL^{-1} CDW, pH 6.5) was increased up to 67% yield. FDCA production in 2 L fermentor resulted 0.5 g/L FDCA from 1 g/L HMF after 12 days with a yield of 40%. In Fed batch approach, in a strategy of initial 1 g/L HMF followed by 0.25 g/L HMF in every 12 h up to 48 h yielded 1.17±0.09 g/L FDCA after 14 days. The yield of FDCA for this approach was (0.59±0.05) 51%. Also, pineapple derived HMF was utilized for the production of FDCA using APLS-1 and 0.16±0.01 g/L FDCA was obtained after 14 days.

For the future improved bioprocesses, identification of pathway for the conversion of HMF into FDCA in *A. flavus* APLS-1 would be very much crucial. Hence, proteomic and transcriptomic analysis of HMF treated APLS-1 were analyzed and desired proteins and respective genes identified. The up-regulated group of genes include oxidoreductases, energy synthesis, membrane transporters, stress proteins, redox and cofactors, transcription and DNA repair proteins. HMF may be transported through MFS/ABC transporters and oxidized to FDCA with the help of proteins from the genes *adh* and *aldh* for the energy production. The FDCA may be decarboxylated by *fdc1* to form 2-furoic acid and enter into TCA cycle. Also, synthesis of FDCA was carried out through enzymatic processes. To develop an enzyme (cell free) process, commercial enzymes like ALDH, AO and GAO were purchased and their activity checked on HMF to FDCA production. After enzymatic optimizations 2.19±0.11 mg/ml FDCA was produced from 6.3 mg/ml HMF with the mixture of enzymes GAO and AO in onepot with cofactor O_2 (atm. air) at pH 7 after 4 days. The overall yield of FDCA from one pot synthesis was (0.34±0.02 g/g) 25.4±1.27%.

8.2. Conclusions

Sustainable bioprocess of industrially valued FDCA from a biomass is essential today. Our study is a first report of a bioprocess of FDCA using *A. flavus* APLS-1 from HMF and pineapple waste. Also, enzymes and genes responsible for the biotransformation of HMF into FDCA are known for the first time in APLS-1, which will benefit for the future improved FDCA production. After enzymatic optimization, FDCA was produced from HMF with the mixture of enzymes GAO and AO with cofactor O_2 (atm. air), which is a first report of enzymatic synthesis of FDCA using these industrial enzymes. Over all the present study demonstrates that toxic and expensive chemical processes can be eliminated by biological process for the production of FDCA using *A. flavus* APLS-1 and industrial enzymes. However improved bioprocesses may be developed in future to increase the yield of FDCA.

8.3. Future perspectives

For improving our bioprocess, the genes in the pathway of HMF to FDCA can be metabolically engineered in *A. flavus* APLS-1. Also, the genes, *adh* and *aldh* can be expressed in suitable host and the purified enzymes used as the enzyme cascade system for the conversion of HMF into FDCA. Reports suggest that acid pretreated liquor (APL) contains HMF which inhibits ethanol production of *Saccharomyces cerevisiae*. Therefore, necessary genes can be incorporated into *Saccharomyces cerevisiae* which will uptake the HMF and convert into FDCA along with ethanol. Hence, our study will pave the way for future improved bioprocesses for FDCA production.

References

Alamillo R, Crisci AJ, Gallo JMR, Scott SL, Dumesic JA. A tailored microenvironment for catalytic biomass conversion in inorganic–organic nanoreactors. Angew. Chem. Int. Ed 2013; 52:10349-51.

Allen SA, Clark W, McCaffery JM, Cai Z, Lanctot A, Slininger PJ, et al. Furfural induces reactive oxygen species accumulation and cellular damage in *Saccharomyces cerevisiae.* Biotechnol. Biofuels. 2010; 3:2.

Almeida JR, Bertilsson M, Gorwa-Grauslund MF, Gorsich S, Lidén G. Metabolic effects of furaldehydes and impacts on biotechnological processes. Appl. Microbiol. Biotechnol. 2009; 82:625.

Amzel LM, Bianchet MA, Leyva JA. Understanding ATP synthesis:structure and mechanism of the F1-ATPase. Mol. Membr. Biol. 2003; 20 (1):27–33

Andrews, Simon. FastQC:a quality control tool for high throughput sequence data. 2010.

Arnaud MB, Cerqueira GC, Inglis DO, Skrzypek MS, Binkley J, Chibucos MC, Crabtree J, Howarth C, Orvis J, Shah P, Wymore F, Binkley G, Miyasato SR, Simison M, Sherlock G, Wortman JR. The Aspergillus Genome Database (AspGD):recent developments in comprehensive multispecies curation, comparative genomics and community resources. Nucleic Acids Res. 2012; 40(D1):D653-9. doi:10.1093/nar/gkr875

Becker GMaHG. 2, 5-furan dicarboxylate derivatives, and use thereof as plasticizers. WO pat, 2011:1-54.

Boisen A, Chistensen TB, Fu W, Gorbanev YY, Hansen TS, Jensen JS, et al. Process integration for the conversion of glucose to 2,5-furandicarboxylic acid. Chem. Eng. Res. Des 2009; 87:1318-27.

Brodin M, Vallejos M, Opedal MT, Area MC, Chinga-Carrasco G. Lignocellulosics as sustainable resources for production of bioplastics–A review. J. Clean. Prod. 2017; 162:646-64.

Cajnko, M.M., Novak, U., Grilc, M. and Likozar, B. Enzymatic conversion reactions of 5-hydroxymethylfurfural (HMF) to bio-based 2, 5-diformylfuran (DFF) and 2, 5-furandicarboxylic acid (FDCA) with air:mechanisms, pathways and synthesis selectivity. Biotechnol. Biofuels. 2020; 13(1):1-11.

Campos, D.A., Ribeiro, T.B., Teixeira, J.A., Pastrana, L., Pintado, M.M. Integral Valorization of Pineapple (Ananas comosus L.) By-Products though a Green Chemistry Approach towards Added Value Ingredients.Foods. 2020; 9:60.

Carro J, Fernandez-Fueyo E, Fernandez-Alonso C, Canada J, Ullrich R, Hofrichter M, et al. Self-sustained enzymatic cascade for the production of 2,5-furandicarboxylic acid from 5-methoxymethylfurfural. Biotechnol. Biofuels. 2018; 11:86.

Carro J, Ferreira P, Rodriguez L, Prieto A, Serrano A, Balcells B, et al. 5-hydroxymethylfurfural conversion by fungal aryl-alcohol oxidase and unspecific peroxygenase. FEBS J. 2015; 282:3218-29.

Chen S, Zhou Y, Chen Y, Gu J. fastp:an ultra-fast all-in-one FASTQ preprocessor. J. Bioinform. 2018; 34(17):1884-90.

Cleveland TE, Yu J, Fedorova N, Bhatnagar D, Payne GA, Nierman WC, Bennett JW. Potential of Aspergillus flavus genomics for applications in biotechnology. Trends Biotechnol. 2009; 27:151-7.DOI:10.1016/j.tibtech.2008.11.008

Collias DI, Harris AM, Nagpal V, Cottrell IW, Schultheis MW. Biobased terephthalic acid technologies:a literature review. Ind. Biotechnol. 2014; 10:91-105.

Crigler, J., Eiteman, M.A. and Altman, E. Characterization of the furfural and 5-hydroxymethylfurfural (HMF) metabolic pathway in the novel isolate Pseudomonas putida ALS1267. Appl. Biochem. Biotechnol. 2020; 190(3):918-30.

Dai, H., Huang, Y., Zhang, Y., Zhang, H., Huang, H. Green and facile fabrication of pineapple peel cellulose/magnetic diatomite hydrogels in ionic liquid for methylene blue adsorption. Cellulose. 2019; 26:3825–44.

Dalal S, Kapoor M, Gupta MN. Preparation and characterization of combi-CLEAs catalyzing multiple non-cascade reactions. J. Mol. Catal. B Enzym. 2007; 44:128-32.

Datsenko KA, Wanner BL. One-step inactivation of chomosomal genes in *Escherichia coli* K-12 using PCR products. Proc. Natl. Acad. Sci. 2000; 97:6640-5.

David Drew, Rachel A. North, Kumar Nagarathinam, and Mikio Tanabe. Structures and General Transport Mechanisms by the Major Facilitator Superfamily (MFS). Chem. Rev. 2021; 121 (9), 5289-35

De Bont Johannes Adrianus Maria WHJR, Doorn ; Jan Werij , Gorssel. Fungal Production of FDCA. In:Application USP, ed., 2018:1-179.

Del Poeta M, Schell WA, Dykstra CC, Jones S, Tidwell RR, Czarny A, et al. Structure-in vitro activity relationships of pentamidine analogues and dication-substituted bis-benzimidazoles as new antifungal agents. Antimicrob Agents Chemother. 1998; 42:2495-502.

Dhepe PL, Sahu R. A solid-acid-based process for the conversion of hemicellulose. Green Chem. 2010; 12:2153-6.

Dijkman WP, de Gonzalo G, Mattevi A, Fraaije MW. Flavoprotein oxidases:classification and applications. Appl. Microbiol. Biotechnol. 2013; 97:5177-88.

Dijkman WP, Groothuis DE, Fraaije MW. Enzyme-catalyzed oxidation of 5-hydroxymethylfurfural to furan-2, 5-dicarboxylic acid. Angew. Chem. Int. Ed Engl. 2014; 53:6515-8.

Dijkman, W.P. and Fraaije, M.W. Discovery and characterization of a 5-hydroxymethylfurfural oxidase from Methylovorus sp. strain MP688. Appl. Environ. Microbiol. 2014; 80(3):1082-90.

Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, Batut P, Chaisson M, Gingeras TR. STAR:ultrafast universal RNA-seq aligner. J. Bioinform. 2013:29(1):15-21.

Donoso, R.A., González-Toro, F. and Pérez-Pantoja, D. Widespread distribution of hmf genes in Proteobacteria reveals key enzymes for 5-hydroxymethylfurfural conversion. Comput. Struct. Biotechnol. J. 2021; 19:2160-9.

Drew, D., North, R.A., Nagarathinam, K. and Tanabe, M. Structures and general transport mechanisms by the major facilitator superfamily (MFS). Chem. Rev. 2021; 121(9):5289-35.

Eerhart A, Faaij A, Patel MK. Replacing fossil based PET with biobased PEF; process analysis, energy and GHG balance. Energ. Env. Sci. 2012; 5:6407-22.

Ewels P, Magnusson M, Lundin S, Käller M. MultiQC:summarize analysis results for multiple tools and samples in a single report. J. Bioinform. 2016:32(19):3047-8.

Fang Z, Smith RL, Qi X. Production of platform chemicals from sustainable resources. Springer, 2017.

Feldman D, Kowbel DJ, Glass NL, Yarden O, Hadar Y. Detoxification of 5-hydroxymethylfurfural by the *Pleurotus ostreatus* lignolytic enzymes aryl alcohol oxidase and dehydrogenase. Biotechnol. Biofuels 2015; 8:63.

Fittig R, Heinzelmann. Announcements from the Chemical Institute of the University of Strasbourg. Rep Ger Chem Soc. 1876; 9:1189-99.

Gadda G. Hydride transfer made easy in the reaction of alcohol oxidation catalyzed by flavin-dependent oxidases. Biochem. J. 2008; 47:13745-53.

Gilkey MJ, Balakumar R, Vlachos DG, Xu B. Adipic acid production catalyzed by a combination of a solid acid and an iodide salt from biomass-derived tetrahydrofuran-2, 5-dicarboxylic acid. Catal. Sci. Technol. 2018; 8:2661-71.

Godan TK, Rajesh R, Loreni PC, Rai AK, Sahoo D, Pandey A, et al. Biotransformation of 5-hydroxymethylfurfural by *Acinetobacter oleivorans* S27 for the synthesis of furan derivatives. Bioresour. Technol. 2019; 282:88-93.

Gomes M, Gandini A, Silvestre AJ, Reis B. Synthesis and characterization of poly (2, 5-furan dicarboxylate) s based on a variety of diols. J Polym. Sci. Part A:Polym. Chem. 2011; 49:3759-68.

Han, X., Li, C., Liu, X., Xia, Q., Wang, Y. Selective oxidation of 5-hydroxymethylfurfural to 2, 5 furandicarboxylic acid over MnO x–CeO 2 composite catalysts. Green. Chem. 2017; 19, 996-1004.

Hatmaker EA, Zhou X, Mead ME, Moon H, Yu JH, Rokas A. Revised transcriptome-based gene annotation for Aspergillus flavus Strain NRRL 3357. Microbiol. Resour. Announc. 2020; 9(49):e01155-20.

Hossain Gazi Sakir, Haibo Yuan, Jianghua Li, Hyun-dong Shin, Miao Wang, Guocheng Du, Jian Chen, Long Liu Metabolic engineering of Raoultella ornithinolytica BF60 for the production of 2, 5 furandicarboxylic acid from 5-hydroxymethylfurfural Appl. Environ. Microbiol. 2016; 1-42 doi:10.1128/AEM.02312-16

Hossain GS, Yuan H, Li J, Shin H-d, Wang M, Du G, et al. Metabolic engineering of *Raoultella ornithinolytica* BF60 for production of 2, 5-furandicarboxylic acid from 5-hydroxymethylfurfural. Appl. Environ. Microbiol. 2017; 83:e02312-16.

Huang R, Qi W, Su R, He Z. Integrating enzymatic and acid catalysis to convert glucose into 5-hydroxymethylfurfural. Chem. Commun. 2010; 46:1115-7.

Jang Y-S, Lee JY, Lee J, Park JH, Im JA, Eom M-H, et al. Enhanced butanol production obtained by reinforcing the direct butanol-forming route in *Clostridium acetobutylicum*. mBio. 2012; 3:e00314-12.

Jia Z, Wang J, Sun L, Zhu J, Liu X. Fully bio-based polyesters derived from 2, 5-furandicarboxylic acid (2, 5-FDCA) and dodecanedioic acid (DDCA):From semicrystalline thermoplastic to amorphous elastomer. J. Appl. Spectrosc. 2018; 135:46076.

Johnson RG, Beers MF, Scarpa A. H+ ATPase of chromaffin granules. Kinetics, regulation, and stoichiometry. J. Biol. Chem. 1982; 257 (18):10701–7

Jung, D., Körner, P. and Kruse, A. Kinetic study on the impact of acidity and acid concentration on the formation of 5-hydroxymethylfurfural (HMF), humins, and levulinic acid in the hydrothermal conversion of fructose. Biomass Convers. Biorefin. 2021; 11(4):1155-70.

Karberg J, M, Lambowitz AM. Targeted and random bacterial gene disruption using a group II intron (targetron) vector containing a retrotransposition-activated selectable marker. Nucleic Acids Res. 2003; 31:1656-64.

Karich A, Kleeberg SB, Ullrich R, Hofrichter M. Enzymatic Preparation of 2,5-Furandicarboxylic Acid (FDCA)-A Substitute of Terephthalic Acid-By the Joined Action of Thee Fungal Enzymes. Microorganisms. 2018; 6(1), 5.

Kenney WC. The reaction of N-ethylmaleimide at the active site of succinate dehydrogenase. J. Biol. Chem. 1975; 250 (8):3089–94

Kobayashi H, Fukuoka A. Synthesis and utilisation of sugar compounds derived from lignocellulosic biomass. Green Chem. 2013; 15:1740-63.

Koopman F, Wierckx N, de Winde JH, Ruijssenaars HJ. Efficient whole-cell biotransformation of 5-hydroxymethylfurfural into FDCA, 2,5-furandicarboxylic acid. Bioresour. Technol. 2010; 101:6291-6.

Koopman F, Nick Wierckx, Johannes H. de Winde, and Harald J. Ruijssenaars. Identification and characterization of the furfural and 5-(hydroxymethyl)furfural degradation pathways of Cupriavidus basilensis HMF14. Proc. Natl. Acad. Sci. U.S.A. 2010; 107 (11):4919-24

Kroh LW. Caramelisation in food and beverages. Food Chem. 1994; 51:373-9.

Larsen DB, Sønderbæk-Jørgensen R, Duus JØ, Daugaard AE. Investigation of curing rates of bio-based thiol-ene films from diallyl 2, 5-furandicaboxylate. Eur. Polym. J. 2018; 102:1-8.

Leimkühler S, Iobbi-Nivol C. Molybdenum enzymes, their maturation and molybdenum cofactor biosyntehsis in Escherichia coli. Biochim. Biophys. Acta. 2013; 1827:1086-101.

Lewkowski J. Synthesis, chemistry and applications of 5-hydroxymethyl-furfural and its derivatives. Arch. Org. Chem. 2001; 1:17-54.

Li W, Zhang T, Xin H, Su M, Ma L, Jameel H, et al. p-Hydroxybenzenesulfonic acid–formaldehyde solid acid resin for the conversion of fructose and glucose to 5-hydroxymethylfurfural. RSC Adv. 2017; 7:27682-8.

Marques S, Moreno AD, Ballesteros M, Gírio F. Starch Biomass for Biofuels, Biomaterials, and Chemicals. Biomass and Green Chemistry:Springer, 2018:69-94.

Martins SI, Van Boekel MA. Kinetics of the glucose/glycine Maillard reactionpathways:influences of pH and reactant initial concentrations. Food Chem. 2005; 92:437-48.

Martins, C., Hartmann, D.O., Varela, A., Coelho, J.A., Lamosa, P., Afonso, C.A. and Silva Pereira, C. Securing a furan-based biorefinery:disclosing the genetic basis of the degradation of hydroxymethylfurfural and its derivatives in the model fungus Aspergillus nidulans. Microb. Biotechnol, 2020; 13(6):1983-96.

Mascal M, Nikitin EB. Direct, High-yield conversion of cellulose into biofuel. Angew. Chem. Int. Ed 2008; 47:7924-6.

Matsumura S, Hlil AR, Lepiller C, Gaudet J, Guay D, Shi Z, et al. Ionomers for proton exchange membrane fuel cells with sulfonic acid groups on the end groups:novel branched poly (ether− ketone) s. Macromolecules. 2008; 41:281-4.

McKenna, S.M., Leimkühler, S., Herter, S., Turner, N.J. and Carnell, A.J. Enzyme cascade reactions:synthesis of furandicarboxylic acid (FDCA) and carboxylic acids using oxidases in tandem. Green Chem. 2015; 17(6):3271-75.

McKenna, S.M., Mines, P., Law, P., Kovacs-Schreiner, K., Birmingham, W.R., Turner, N.J., Leimkühler, S. and Carnell, A.J. The continuous oxidation of HMF to FDCA and the immobilisation and stabilisation of periplasmic aldehyde oxidase (PaoABC). Green Chem. 2017; 19(19):4660-65.

McNeff CV, Nowlan DT, McNeff LC, Yan B, Fedie RL. Continuous production of 5-hydroxymethylfurfural from simple and complex carbohydrates. Appl Catal A:General 2010; 384:65-9.

Morales-delaRosa S, Campos-Martin JM, Fierro JL. Optimization of the process of chemical hydrolysis of cellulose to glucose. Cellulose. 2014; 21:2397-407.

Moscoviz R, Trably E, Bernet N, Carrère H. The environmental biorefinery:state-of-the-art on the production of hydrogen and value-added biomolecules in mixed-culture fermentation. Green Chem. 2018; 20:3159-79.

Neumann M, Mittelstädt G, Iobbi-Nivol C, Saggu M, Lendzian F, Hildebrandt P, et al. A periplasmic aldehyde oxidoreductase represents the first molybdopterin cytosine dinucleotide cofactor containing molybdo-flavoenzyme from *Escherichia coli*. FEBS J. 2009; 276:2762-74.

Nierman WC, Yu J, Fedorova-Abrams ND, Losada L, Cleveland TE, Bhatnagar D, Bennett JW, Dean R, Payne GA. Genome sequence of Aspergillus flavus NRRL 3357, a strain that causes aflatoxin contamination of food and feed. Genome Announc. 2015; 3(2):e00168-15.

Ning C, Su E, Tian Y, Wei D. Combined cross-linked enzyme aggregates (combi-CLEAs) for efficient integration of a ketoreductase and a cofactor regeneration system. J. Biotechnol. 2014; 184:7-10.

Omari KW, Besaw JE, Kerton FM. Hydrolysis of chitosan to yield levulinic acid and 5-hydroxymethylfurfural in water under microwave irradiation. Green Chem. 2012; 14:1480-7.

Pacheco JJ, Davis ME. Synthesis of terephthalic acid via Diels-Alder reactions with ethylene and oxidized variants of 5-hydroxymethylfurfural. Proc. Natl. Acad. Sci. 2014; 111:8363-7.

Peng F, Ren JL, Xu F, Sun R-C. Chemicals from hemicelluloses:A review. ACS Publications, 2011:219-59.

Perlack Robert D., Lynn L. Wright, Anthony F. Turhollow, Graham RL. Biomass as feedstock for a bioenergy and bioproducts industry:the technical feasibility of a billion-ton annual supply. 2005:1-79.

Petkovic M, Seddon KR, Rebelo LPN, Pereira CS. Ionic liquids:a pathway to environmental acceptability. Chem. Soc. Rev. 2011; 40:1383-403.

Pham NN, Chen C-Y, Li H, Nguyen MTT, Nguyen PKP, Tsai S-L, et al. Engineering Stable Pseudomonas putida S12 by CRISPR for 2,5-Furandicarboxylic Acid (FDCA) Production. ACS Synth Biol. 2020; 9(5):1138–49

Qi X, Watanabe M, Aida TM, Smith Jr RL. Catalytical conversion of fructose and glucose into 5-hydroxymethylfurfural in hot compressed water by microwave heating. Catal. Commun. 2008; 9:2244-9.

Qin Y-Z, Li Y-M, Zong M-H, Wu H, Li N. Enzyme-catalyzed selective oxidation of 5-hydroxymethylfurfural (HMF) and separation of HMF and 2,5-diformylfuran using deep eutectic solvents. Green Chem. 2015; 17:3718-22.

Rajesh R, Godan TK, Pandey A, Binod P. Whole cell based biocatalytic production of 2, 5-furan dicarboxylic acid. Ind. J. Exp. Biol. 2018; 56:493-7.

Rajesh RO, Godan TK, Rai AK, Sahoo D, Pandey A, Binod P. Biosynthesis of 2, 5-furan dicarboxylic acid by *Aspergillus flavus* APLS-1:Process optimization and intermediate product analysis. Bioresour. Technol. 2019; 284:155-60.

Richter DT, Lash TD. Oxidation with dilute aqueous ferric chloride solutions greatly improves yields in the '4+ 1'synthesis of sapphyrins. Tetrahedron Lett. 1999; 40:6735-8.

Rosatella AA, Simeonov SP, Frade RF, Afonso CA. 5-Hydroxymethylfurfural (HMF) as abuilding block platform:Biological properties, synthesis and synthetic applications. GreenChem. 2011; 13:754-93.

Sajid M, Zhao X, Liu D. Production of 2,5-furandicarboxylic acid (FDCA) from 5-hydroxymethylfurfural (HMF):recent progress focusing on the chemical-catalytic routes. Green Chem. 2018; 20:5427-53.

Seppey, M., Manni, M., Zdobnov, E.M. BUSCO:Assessing Genome Assembly and Annotation Completeness. In:Kollmar, M. (eds) Gene Prediction. Methods in Molecular Biology. 2019; 1962:227–45 https://doi.org/10.1007/978-1-4939-9173-0_14

Seri K-i, Inoue Y, Ishida H. Catalytic activity of lanthanide (III) ions for the dehydration of hexose to 5-hydroxymethyl-2-furaldehyde in water. BCSJ. 2001; 74:1145-50.

Skerker JM, Pianalto KM, Mondo SJ, Yang K, Arkin AP, Keller NP, Grigoriev IV, Louise Glass NL. Chromosome assembled and annotated genome sequence of Aspergillus flavus NRRL 3357. G3:Genes Genomes Genet. 2021; 11(8):jkab213. doi:10.1093/g3journal/jkab213.

Sousa AF, Vilela C, Fonseca AC, Matos M, Freire CS, Gruter G-JM, et al. Biobased polyesters and other polymers from 2, 5-furandicarboxylic acid:a tribute to furan excellency. Polym. Chem. 2015; 6:5961-83.

Sturn, A., Quackenbush, J. and Trajanoski, Z. Genesis:cluster analysis of microarray data. J. Bioinform. 2002; 18(1):207-8.

Sun F, Huo X, Zhai Y, Wang A, Xu J, Su D, Bartlam M, Rao Z. Crystal structure of mitochondrial respiratory membrane protein complex II. Cell J. 2005; 121(7):1043-57

Sznida, E. The EU's Path Toward Sustainable Development Goals–Responsible Consumption and Production. 2018. Available SSRN 3292067.

Tang X, Zuo M, Li Z, Liu H, Xiong C, Zeng X, et al. Green processing of lignocellulosic biomass and its derivatives in deep eutectic solvents. Chem. Sus. Chem. 2017; 10:2696-706.

The UniProt Consortium, UniProt:the universal protein knowledgebase in 2021, Nucleic Acids Res. 2021; 49 (D1):D480–D489

Toei M, Saum R, Forgac M. Regulation and isoform function of the V-ATPases. Biochem. 2010; 49(23):4715-23

Tomaszewska J, Bieliński D, Binczarski M, Berlowska J, Dziugan P, Piotrowski J, et al. Products of sugar beet processing as raw materials for chemicals and biodegradable polymers. RSC Adv. 2018; 8:3161-77.

Tong X, Ma Y, Li Y. Biomass into chemicals:conversion of sugars to furan derivatives by catalytic processes. Appl. Catal. A:General. 2010; 385:1-13.

Van de Vyver S, Geboers J, Jacobs PA, Sels BF. Recent advances in the catalytic conversion of cellulose. Chem. Cat. Chem. 2011; 3:82-94.

Vineetha, R.C., Hariharan, S., Jaleel, A., Chandran, M., Nair, R.H. L-ascorbic acid –tocopherol synergistically triggers apoptosis inducing antileukemic effects of arsenic trioxide via

oxidative stress in human acute promyelocytic leukemia cells. Front. Oncol. 2020; 21:10:65

Voet D, Voet JG, Pratt CW. Fundamentals of biochemistry:life at the molecular level (4th ed). Hoboken, NJ:Wiley. 2013; Chapter 18:581–620

Wang P, Yu H, Zhan S, Wang S. Catalytic hydrolysis of lignocellulosic biomass into 5-hydroxymethylfurfural in ionic liquid. Bioresour. Technol. 2011; 102:4179-83.

Weingarten R, Conner WC, Huber GW. Production of levulinic acid from cellulose by hydrothermal decomposition combined with aqueous phase dehydration with a solid acid catalyst. Ener. Env. Sci. 2012; 5:7559-74.

Werpy T, Petersen G. Top value added chemicals from biomass:volume I--results of screening for potential candidates from sugars and synthesis gas. National Renewable Energy Lab., Golden, CO (US), 2004.

Wierckx N, Elink Schuurman TD, Blank LM, Ruijssenaars HJ. Whole-Cell Biocatalytic Production of 2,5-Furandicarboxylic Acid. In:Kamm B. (eds) Microorganisms in Biorefineries. Microbiology Monographs, Springer, Berlin, Heidelberg. 2015; 6:207-23

Wierckx, N. J., Schuurman, T. D., Kuijper, S. M., & Ruijssenaars, H. J. Genetically modified cell and process for use of said cell. 2016; United States pat. 9,309,546.

Xu, S., Zhou, P., Zhang, Z., Yang, C., Zhang, B., Deng, K., Bottle, S., Zhu, H. Selective oxidation of 5-hydroxymethylfurfural to 2, 5-furandicarboxylic acid using O2 and a photocatalyst of Co-thioporphyrazine bonded to g-C3N4. J. Am. Chem. Soc. 2017; 139:14775-82.

Y, Tschaplinski TJ, Engle NL, Hamilton CY, Rodriguez M, Liao JC, et al. Combined inactivation of the *Clostridium cellulolyticum* lactate and malate dehydrogenase genes substantially increases ethanol yield from cellulose and switchgrass fermentations. Biotechnol. Biofuels. 2012; 5:2.

Yan D, Xin J, Zhao Q, Gao K, Lu X, Wang G, et al. Fe–Zr–O catalyzed base-free aerobic oxidation of 5-HMF to 2, 5-FDCA as a bio-based polyester monomer. Catal. Sci. Technol. 2018; 8:164-75.

Yang CF, Huang CR. Biotransformation of 5-hydroxy-methylfurfural into 2,5-furan-dicarboxylic acid by bacterial isolate using thermal acid algal hydrolysate. Bioresour. Technol. 2016; 214:311-8.

Yang CF, Huang CR. Isolation of 5-hydroxymethylfurfural biotransforming bacteria to produce 2,5-furan dicarboxylic acid in algal acid hydrolysate. J Biosci. Bioeng. 2018; 125:407-12.

Yang Y, Xiang X, Tong D, Hu C, Abu-Omar MM. One-pot synthesis of 5-hydroxymethylfurfural directly from starch over SO42-/ZrO2–Al2O3 solid catalyst Bioresour. Technol. 2012; 116:302-6.

Yuan H, Li J, Shin H-d, Du G, Chen J, Shi Z, et al. Improved production of 2, 5-furandicarboxylic acid by over expression of 5-hydroxymethylfurfural oxidase and 5-hydroxymethylfurfural/furfural oxidoreductase in *Raoultella ornithinolytica* BF60. Bioresour. Technol. 2018; 247:1184-8.

Yuan H, Liu Y, Li J, Shin Hd, Du G, Shi Z, et al. Combinatorial synthetic pathway fine-tuning and comparative transcriptomics for metabolic engineering of *Raoultella ornithinolytica* BF60 to efficiently synthesize 2, 5-furandicarboxylic acid. Biotechnol. Bioeng. 2018; 115:2148-55.

Yuan H, Liu Y, Lv X, Li J, Du G, Shi Z, et al. Enhanced 2,5-Furandicarboxylic Acid (FDCA) Production in *Raoultella ornithinolytica* BF60 by Manipulation of the Key Genes in FDCA Biosynthesis Pathway. J. Microbiol. Biotechnol. 2018; 28:1999-2008.

Zhang, Z., & Deng, K. Recent advances in the catalytic synthesis of 2, 5-furandicarboxylic acid and its derivatives. ACS. Catal. 2015; 5:6529-44.

Zhu S, Wang J, Fan W. Graphene-based catalysis for biomass conversion. J Adv. Catal. Sci. 2015; 5:3845-58.

Media Composition

1. Mineral Salt Medium (MSM) gL^{-1}

$MgSO_4$	0.2
$CaCl_2$	0.02
K_2HPO_4	0.5
KH_2PO_4	0.5
NH_4Cl	0.5
Trace element solution	10 ml/L

Adjust the pH to 7.0 by 1 N HCl/ NaOH, and sterilized by autoclaving

2. Potato Dextrose Broth (PDB) gL^{-1}

Potatoes, infusion from	200
Dextrose	20.0
Final pH (at 25°C)	5.1±0.2

Adjust the pH to 5.1±0.2 by 1 N HCl/ NaOH, and sterilized by autoclaving

3. Potato Dextrose Agar (PDA) gL^{-1}

Potatoes	4.0
Dextrose	20.0
Agar (for solid medium)	25.0

Final pH 5.6 ± 0.2 and sterilized by autoclaving

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	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

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	Biodegradable polymers	Completed
	Level 400		
1.	BIO-NIIST-4-0001	Project proposal	Completed
2.	BIO-NIIST-4-0002	Review article	Completed
	Level 800		
1.	BIO-NIIST-4-0003	Societal Project	Completed

ABSTRACT

Name of the Student: **Rajesh R.O.** Registration No. : **10BB16A39019**

Faculty of Study: **Biological Science** Year of Submission: **2022**

AcSIR academic centre/CSIR Lab: **CSIR-NIIST** Name of the Supervisor: **Dr. Binod P.**

Title of the thesis: **Whole cell and cell-free synthesis of 2,5-furandicarboxylic acid from pineapple waste**

Dependence of non-renewable resources for value added products lead the world into greenhouse gas emission followed by global warming. Hence, new value added products have been developed from renewable resources like lignocellulosic biomass. One of the platform chemical synthesized from biomass is FDCA which is a monomer used in plastic industries. Current chemical processes for the production of FDCA involve carcinogenic, toxic and expensive chemical catalysts in high pressure and temperature. This create huge environment hazard and severely affect flora and fauna. Our study was to develop a bioprocess for the synthesis of FDCA from pineapple waste using microorganism, *A. flavus* APLS-1 and commercial enzymes. HMF (0.29±0.02 g/g) was synthesized from pineapple peel using $CrCl_3$ at 100 °C. After optimizations, 0.8 g/g FDCA was produced by APLS-1 from HMF at inoculum age 58 h, inoculum size 6.66 gL^{-1} CDW and pH 6.5 with 67% yield. Also, pineapple derived HMF was converted into 0.16±0.01 gL^{-1} FDCA by APLS-1 after 14 days. One pot reaction using commercial enzymes GAO and AO produced 0.34±0.02 g/g FDCA with 25.4±1.27% yield with cofactor O_2 (atm. air) at pH 7 after 4 days. However, our bioprocesses may be improved to increase the yield of FDCA which will pave the way for renewable value added products in future.

List of publications emanated from thesis

R.O. Rajesh, Tharangaattumana Krishnan Godan, Amit Kumar Rai, Dinabandhu Sahoo, Ashok Pandey & Parameswaran Binod. Biosynthesis of 2,5-furandicarboxylic acid by Aspergillus flavus APLS-1:Process optimization and intermediate product analysis. Bioresource Technology. 2019; 284:155–160

R.O. Rajesh, Tharangattumana Krishnan Godan, Ashok Pandey, Parameswaran Binod. Whole cell based biocatalytic production of 2,5-furandicarboxylic acid. Indian Journal of Experimental biology. 2018; 56:493-497

Conference proceedings

R.O. Rajesh, Parameswaran Binod 'Bioprocess development for the production of 2,5-furandicarboxylic acid'. ETBWC-2017. Oct 8-10. CSIR-NEERI, Nagpur

R.O. Rajesh, Tharangattumana Krishnan Godan, Parameswaran Binod. 'Bioprocess development for the production of 2,5-furandicarboxylic acid using Aspergillus flavus'. BioSD – 2018. Nov 22 – 25. CSIIR – IICT, Hyderabad

R.O. Rajesh, Tharangattumana Krishnan Godan, Parameswaran Binod. 'Biocatalytic production of 2,5-furandicarboxylic acid by Aspergillus flavus APLS-1: fermentation modes, media optimization, kinetics and intermediate product analysis'. NHBT-2019. Nov 20-24. CSIIR – NIIST, Trivandrum (Received best poster award)

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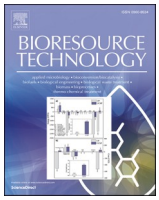

Corrigendum to "Biosynthesis of 2,5-furan dicarboxylic acid by Aspergillus flavus APLS-1: Process optimization and intermediate product analysis" [Bioresour. Technol. 284 (2019) 155–160]

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Biosynthesis of 2,5-furan dicarboxylic acid by *Aspergillus flavus* APLS-1: Process optimization and intermediate product analysis

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ABSTRACT

The aim of the present study was to develop an eco-friendly biological process for the production of 2,5-furan dicarboxylic acid (FDCA) from 5-hydroxy methylfurfuraldehyde (HMF) using microorganisms. Microorganisms were isolated from the soil samples and evaluated for its biotransformation efficiency. Among the isolates, *Aspergillus flavus* APLS-1 was found to be potent for efficient conversion of HMF to FDCA. The bioconversion parameters were optimized by Box-Behnken design. The optimization resulted in 67% conversion efficiency where 1 g/L HMF (8 mM) was transformed to 0.83 g/L (6.6 mM) FDCA in 14 days at pH6.5 with biomass size of 5.7 g/L and biomass age 60 h. This is the first report on *Aspergillus* sp., capable of detoxifying HMF and produces FDCA.

1. Introduction

2,5-furan dicarboxylic acid (FDCA; $C_6H_4O_5$) is a building block chemical reported as one of the top 12 value added chemicals by the U.S. Department of energy (Werpy and Peterson, 2004; Bozell & Petersen, 2010). It is a monomer that is synthetically produced from 5-hydrooxymethylfurfuraldehyde (HMF) by subsequent oxidations through chemical processes. The substrate, HMF, is a natural product produced by the dehydration of fructose or glucose (Wang et al., 2015). FDCA can be used for the synthesis of polyethylene furanoate (PEF) which can be used as analogs of polyethylene terephthalate (PET) and polybutylene terephthalate (PBT). Use of PEF polymers can reduce the dependence of nonrenewable fossil fuel products by almost 45–55% in the future (Gandini et al., 2009). Also, FDCA can be used for the synthesis of poly(decylene-2,5-furanoate) (Tsanaktsis et al., 2015). Polymers of FDCA can be synthesized for polyesters, polyamides and polyurethanes which can be used for making bottles, containers, nylons, films, coating and resins. Even though large scale FDCA synthesis through chemical catalysts are reported (Zhang & Deng, 2015), these production methods involve metal salts and organic solvents using high temperature and high pressure which is non-eco-friendly and expensive. As an alternative to chemical processes (Davis et al., 2012; Han et al., 2017; Xu et al., 2017), greener and environmentally friendly approaches are more attractive and advised nowadays to solve the environmental and global warming issues. In this context, the biological conversion of HMF to FDCA through microbial routes is highly important and acceptable as this method solves most of the environmental related issues. But the biological conversion methods pose issues related to economic challenges which could be partially addressed by various optimization approaches.

The currently used industrial chemical processes for the production of FDCA could be replaced with the biological conversion methods by microorganisms or enzymes. Several microorganisms are reported to convert HMF to FDCA. Recently, bacterial production of FDCA from HMF have been reported by *Cupriavidus basilensis* HMF14, *Pseudomonas putida* S12 (Koopman et al., 2010a; Koopman et al., 2010b), *Burkholderia cepacia* H-2 (Yang & Huang, 2016) *Methylovorus* sp (Dijkman et al., 2014), *Raoultella ornithinolytica* BF60 (Hossain et al., 2017; Yuan et al., 2018a; Yuan et al., 2018b) and *Enterobacter* sp (Rajesh et al., 2018). Fungal FDCA production and HMF degrading associated enzymes have been reported in *Pleurotus ostreatus* PC9 (Feldman et al., 2015). HMF degradation and the enzymes involved in the HMF catabolism along with FDCA production have been reported recently in *Penicillium brasilianum* C1 and its transformed variants. Same authors

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have reported FDCA production in recombinant strains like *Saccharomyces cerevisiae* CEN.PK/$_P$TT2 and *Yarrowia lipolytica* Pol g also (De Bont et al., 2017). The HMF and furfuraldehyde degradation by microorganisms happen through 2-furoic acid and 5-Hydroxymethyl-2-furan carboxylic acid (HFCA) degradation routes (Kakinuma and Yamatodani, 1964; Trudgill, 1969). The HMF degradation pathway in *Cupravidus basilensis* HMF14 has been reported (Koopman et al., 2010b; Wierckx et al., 2010). In the case of *Saccharomyces cerevisiae*, HMF has directly inhibited alcohol dehydrogenase, pyruvate dehydrogenase and aldehyde dehydrogenase along with the yeast energy repairing mechanism by intracellular ATP and NADP(H) levels. This process is occurring by enzymatic inhibition or cofactor consumption or regeneration (Almeida et al., 2007). Reports show that HMF is getting converted into HMF alcohol, maleic anhydride (MA), 5-Hydroxymethyl-2-furan carboxylic acid (HFCA), 2,5-diformylfuran (DFF), 5-formyl furan carboxylic acid (FFCA), and FDCA. Among them, FDCA is industrially more relevant than HFCA and DFF.

In this study, a potent HMF degrading fungal strain *Aspergillus flavus* APLS-1 were enriched and isolated from drainage soil and the bioconversion of HMF to FDCA has been enhanced using single parameter as well as multi parameter optimization methods.

2. Materials and methods

2.1. Chemicals

5-Hydroxymethyl furfuraldehyde and 2,5-furan dicarboxylic acid were procured from Sigma Aldrich, India. 5-(Hydroxymethyl)furfuryl Alcohol (HMF alcohol), 5–Hydroxymethyl-2-furan carboxylic acid (HMF acid), Diformyl furan (DFF), Formyl furan carboxylic acid (FFCA) and 2-furoic acid were procured from Carbosynth Chemicals, UK.

2.2. Isolation of microorganisms by enrichment technique in the presence of HMF

Soil samples were collected from the acid pretreatment liquor disposal site of biofuel pilot plant of the CSIR-National Institute for Interdisciplinary Science and Technology, Kerala, India. Microorganisms were isolated based on the enrichment culture technique. One gram of soil was suspended in 99 ml of distilled water along with 4 g/L (32 mM) HMF in 250 ml conical flasks. It has been kept for three days in an incubator shaker at 200 rpm at 30 °C. After three days, one ml from this flask was transferred to 99 ml of distilled water and diluted serially. Single colonies were isolated in nutrient agar and potato dextrose agar plates supplemented with 3 g/L (24 mM) HMF and kept at 37 °C and 30 °C for 5 days in incubators for isolating bacterial and fungal colonies respectively. The pure cultures were kept at 4 °C and sub-cultured in frequent intervals.

2.3. Screening of microorganisms for FDCA production

Isolated microorganisms were pre-cultured before inoculating into appropriate media. Single colony of pure culture bacteria was inoculated into 50 ml nutrient broth and kept for 14 h at 37 °C at 200 rpm. From this one percentage inoculum was transferred into 100 ml nutrient broth in 250 ml conical flasks and kept for 24 h in an incubated shaker at 30 °C at 200 rpm. From this, 10 percentage inoculum was collected and centrifuged at 8000 rpm for 10 min. The obtained biomass pellet was collected and washed three times in phosphate buffer saline solution and transferred into 50 ml of Mineral Salt Media (MSM) (pH 7.2) containing 0.5 g/L HMF in 250 ml conical flasks and kept for 14 days in an incubator shaker at 200 rpm at 37 °C (Yang & Huang, 2016). The samples were withdrawn in every 24hrs, filtered and HMF and FDCA concentrations were analysed using HPLC.

In the case of non-sporulating fungus, 3 mm potato dextrose agar disc containing pre-grown fungus was inoculated into 100 ml potato dextrose broth in 250 ml conical flasks and kept in an incubator shaker at 200 rpm at 30 °C for 72 h (Rajesh et al., 2018). For sporulating fungus, 1×10^6 spores were inoculated into 100 ml potato dextrose broth in 250 ml conical flasks and kept in an incubator shaker at 200 rpm at 30 °C for 72 h. In both cases after 72 h, 20 ml of the sample was collected, filtered using Whatman filter paper. Collected biomass was transferred into 50 ml of Mineral Salt Media (MSM) ($_P$H 7.2) containing 0.5 g/L HMF in 250 ml conical flasks and kept for 14 days in an incubator shaker at 200 rpm at 30 °C. The samples were withdrawn in every 24 h, filtered and HMF and FDCA concentrations were analysed using HPLC.

2.4. Selection of FDCA production media for the positive isolates

The fungus APLS-1 which showed highest FDCA production was selected and checked for better production media. Since it is a sporulating fungus, 1×10^6 spores of APLS-1 were inoculated into 100 ml potato dextrose broth in 250 ml conical flasks and kept in an incubator shaker at 200 rpm at 30 °C for 72 h. After the specified time of incubation, 20 ml of the sample was collected and centrifuged. This biomass pellet was transferred into 50 ml of Mineral Salt Media (MSM) (pH 7.2) containing 0.5 g/L HMF in 250 ml conical flasks and kept for 14 days in an incubator shaker at 200 rpm at 30 °C. The production media used in the screening procedure was kept as control.

In first set of experiment, $1x10^6$ spores were inoculated into 100 ml of potato dextrose broth supplemented with 0.5 g/L HMF. In second set of experiment $1x10^6$ spores were inoculated in 100 ml mineral salt media with one percentage of glucose (carbon source) supplemented with 0.5 g/L HMF. In third set of experiment $1x10^6$ spores were inoculated in 100 ml mineral salt media with one percentage of peptone (nitrogen source) supplemented with 0.5 g/L HMF. In fourth set of experiment $1x10^6$ spores were inoculated in 100 ml mineral salt media with one percentage of glucose (carbon source) and one percentage of peptone (nitrogen source) supplemented with 0.5 g/L HMF. These experiments were done in 250 ml conical flasks and kept for 14 days in an incubator shaker at 200 rpm at 30 °C. In the fifth set of experiment, 1×10^6 spores of APLS-1 were inoculated into 100 ml potato dextrose broth in 250 ml conical flasks and kept in an incubator shaker at 200 rpm at 30 °C for 72 h. After 72 h, 20 ml of the sample was collected and centrifuged to separate the biomass. This biomass pellet was transferred into 50 ml of potassium phosphate buffer (50 mM) (pH 7.0) containing 0.5 g/L HMF in 250 ml conical flasks and kept for 14 days for FDCA analysis in a shaker at 200 rpm at 30 °C.

2.5. Growth analysis of APLS-1 in different HMF concentration

In order to check the tolerance of APLS-1 towards HMF, disc (3 mm) was cut from pre-grown culture and placed in the center of petri dish in such a way that top position of the cut disk to downward direction. First experimental media contained mineral salt media with2% agar for solidification supplemented with different concentration of HMF (1 g/L to 3 g/L). The second experimental media contained distilled water and 2% agar for solidification supplemented with different concentration of HMF (1 g/L to 3 g/L). Both set were kept for seven days for observing the changes in the growth morphology.

2.6. Identification of the fungus APLS-1

Fungal spores (1×10^6 spores) were inoculated in 100 ml potato dextrose broth and kept for 48 h in an incubator shaker at 200 rpm at 30 °C. The biomass was filtered using Whatman filter paper and fungal DNA was isolated according to the universal phenol-chloroform protocol. The Internal Transcribed Spacer region was amplified in a thermal cycler (Biorad) using universal primers ITS 1F: 5′ CTTGGTCA TTTAGAGGAAGTAA 3′ and ITS 4R: 5′ CAGACTTGTATATGGTCCAG 3′. Conditions of the PCR cycle were initial denaturation at 95 °C for 5 min

followed by 30 cycles of 95 °C for 1 min 15 s, 65 °C for 1 min and 72 °C for 2 min. and the final step extension at 72 °C for 10 min. PCR products were purified using a QIA quick gel extraction kit (Qiagen) and kept at −20 °C. Sequencing was done on an ABI model 3130 automatic DNA sequencer using a Big Dye terminator cycle sequencing kit (Applied Biosystems).

2.7. Optimization of pH, inoculum size, inoculum age and substrate concentration for FDCA production

To optimize the pH, the reaction was carried in 50 ml mineral salt media inoculated with 72 h grown APLS-1 biomass of 1.75 g/L at 30 °C. The ranges of pH used were from 6.0 to 8.0. For the optimization of biomass size, the reaction was done in 50 ml mineral salt media with 72 h grown biomass with an optimized pH of 6.5 at 30 °C. The range of inoculums size (biomass size) selected was 4 g/L to 8.7 g/L cell dry weight (CDW). To optimize inoculum age (biomass age), the reaction was performed in 50 ml mineral salt media inoculated with optimized 6 g/L biomass and pH 6.5 at 30 °C. The range of inoculum age used was from 48 h to 72 h. For the optimization of substrate concentration, the reaction was done in 50 ml mineral salt media inoculated with optimized 6 g/L biomass at an age 60 h and pH 6.5 at 30 °C. Substrate (HMF) concentration used was ranging from 0.25 g/L to 2 g/L. All the experiments were done in duplicates in 250 ml flasks kept in an incubator shaker at 200 rpm for 14 days.

2.8. Optimization of FDCA production using Box-Behnken design

A Box-Behnken design was used to evaluate the influence of three major parameters like pH, biomass size and biomass age on FDCA production (Table.1). The pH used were 6, 7 and 8. The biomass size and biomass age were 3.9 ± 0.06 g/L, 5.7 ± 0.1 g/L and 8.9 ± 0.51 g/L and 48 h, 60 h and 72 h respectively. Total of 15 experiments were conducted. Linear regression and analysis of variance were conducted to know the significant effect of variables on the response ($p < 0.05$). Regression relation between all the process variables and the response variable were plotted. All these statistical analysis were done in Minitab 17 software (Ver. 15).

2.9. Validation of FDCA production

The optimum conditions obtained from the Box–Behnken design was validated based on predicted and experimental values (Table 2).

Table 1
Box-Behnken experimental design and FDCA production by *A. flavus* APLS-1 after 14 days of incubation.

Sl.No	pH	Inoculum Size (CDW-g/L) ± SD	Inoculum age (h)	FDCA production (14th Day)
1	6	3.9 ± 0.06	60	0.74
2	6	5.7 ± 0.09	48	0.53
3	8	3.9 ± 0.06	60	0.54
4	6	5.7 ± 0.09	72	0.08
5	7	3.9 ± 0.06	72	0.05
6	7	5.7 ± 0.09	60	0.80
7	7	8.9 ± 0.51	48	0.40
8	7	5.7 ± 0.09	60	0.79
9	8	5.7 ± 0.09	48	0.13
10	8	3.9 ± 0.06	60	0.51
11	8	5.7 ± 0.09	72	0.05
12	7	3.9 ± 0.06	60	0.79
13	7	5.7 ± 0.09	72	0.69
14	6	3.9 ± 0.06	60	0.79
15	7	5.7 ± 0.09	48	0.58

Table 2
Validation of Box-Behnken experimental design and FDCA production by *A. flavus* APLS-1after 14 days of incubation.

Sl.No	pH	Biomass size (CDW-g/L) ± SD	Biomass age (h)	FDCA (g/L)
1	6	6.6 ± 0.1	58	0.783
2	6	6.6 ± 0.1	60	0.792
3	6	8.9 ± 0.5	62	0.69
4	6.5	6.6 ± 0.1	58	0.83

Table 3
Selection of production media based on intermediates in the conversion of HMF to FDCA by *A. flavus* APLS-1after 14 days of incubation.

Sl.No	Media name	HMF alcohol (g/L)	HMF (g/L)	HFCA (g/L)	DFF (g/L)	FFCA (g/L)	FDCA (g/L)	2-Furoic acid (g/L)
1	First set	0.07	0	0.42	0	0	0	0
2	Second set	0.13	0.31	0	0	0	0	0
3	Third set	0.07	0.07	0.35	0	0	0	0
4	Fourth set	0.39	0	0.2	0	0	0	0
5	Fifth set	0.15	0	0.06	0	0	0	0
6	Control	0	0	0.3	0	0.05	0.13	0

2.10. Analysis of HMF alcohol, HMF, HFCA, DFF, FFCA, FDCA and 2-furoic acid

The amount of HMF alcohol, HMF, HFCA, DFF, FFCA, FDCA and 2-furoic acid in the reaction mixture was determined by using gradient RP-HPLC (Shimadzu, Japan). The column used for the analysis of these compounds was C–18 (Phenomenex) with pore size 5 µm and size 150 × 4.6 mm kept at 40 °C using a column oven. Mobile phases used were 0.06N H_2SO_4 (mobile phase A) and Acetonitrile (mobile phase B). Acetonitrile was run at 6–2% for 1–2 min and 2–6% next 2 to 15 min at a flow rate of 1 ml/min for 20 min.

3. Results and discussion

3.1. Isolation and screening of microorganisms for FDCA production

Total of seven microorganisms were isolated from the acid pretreatment liquor drainage site of the pilot plant. Based on the morphological differences, microorganisms comprise bacteria (4 Nos.) and fungus (3 Nos.). Pure colonies of bacterial and fungal cultures were stored in a refrigerator at 4 °C. These strains were selected for the screening of FDCA production. Among them, a fungus APLS-1 showed better FDCA production as compared to other isolates and it was selected for further studies.

3.2. Selection of FDCA production media for the selected fungus APLS-1

The fungus APLS-1 with highest FDCA production was selected and checked for better production media (Table 3). In the first set of experiment where spores were inoculated into 100 ml of potato dextrose broth supplemented with 0.5 g/L HMF showed no FDCA production. Instead of FDCA production APLS-1 transformed HMF into HMF alcohol and HMF acid. Since the potato dextrose broth media had enough nutrient components for its metabolic activities, organism did not utilize HMF for its metabolic activities and it just detoxified HMF and expelled out with less toxic substances HMF alcohol and HFCA after 14 days of incubation. In the second set of experiment where spores were inoculated into mineral salt media with one percentage of glucose along with 0.5 g/L HMF, HMF was not completely utilized by the organism. The utilized HMF was converted into HMF alcohol. So instead of the forward reaction it was going to reductase mechanism. In the third set

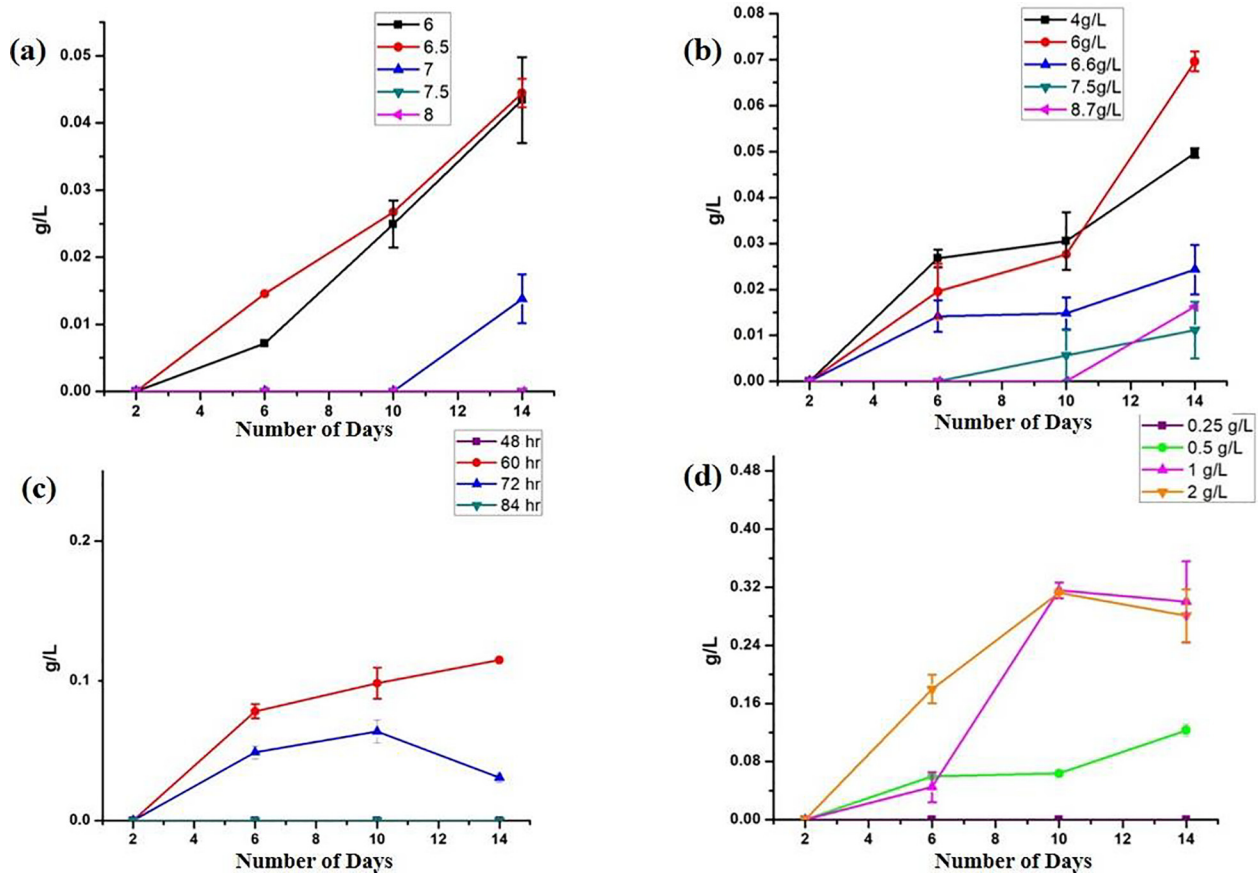

Fig. 1. Optimization of FDCA production by using single parameters optimization (a) pH; (b)inoculum size; (c), inoculum age; (d) substrate concentration. Experiments were done in duplicates and error bars are plotted based on the standard deviation of the mean.

of experiment where spores were inoculated into mineral salt media with one percentage of yeast extract supplemented with 0.5 g/L HMF, the utilization of HMF was faster as compared to second set as there was no other carbon source present in it. Also, the reaction went into forward direction where less amount of HMF and HMF alcohol was noticed and more HFCA was present. In the fourth set of experiment where spores were inoculated in mineral salt media with one percentage of glucose and yeast extract supplemented with 0.5 g/L HMF, HMF was completely used by the organism and transformed into more HMF alcohol and less HFCA. In this condition, the organism followed reductase mechanism rather than oxidation as in the case of the second set of experiments. In the fifth set of experiment, already grown biomass was transferred into 50 ml potassium phosphate buffer (50 mM) (pH 7.0) containing 0.5 g/L HMF, the HMF utilization was less and less amount of HFCA and HMF alcohol was observed. Already grown biomass was transferred into 50 ml of Mineral Salt Media (pH 7.2) containing 0.5 g/L HMF was used as the control and only this control set showed the complete detoxification and oxidation reaction of HMF into HFCA, FFCA and FDCA. This was the best production media selected for further studies.

3.3. HMF tolerance of APLS-1

The HMF intolerance of the organism on a solid medium was observed in which the organism was growing well and reached its reproductive stage in mineral salt media with a low concentration of HMF (1 g/L). When the agar medium was increased with high concentration of HMF like 3 g/L HMF organism's reproductive stage was minimized. In the second set of experiment where only distilled water was used, the organism maintained its primary growth rather than going to its reproductive stage and spore formation. So it concludes that HMF and some minerals are also needed for the organism for its primary metabolic activities, growth and reproduction rather than HMF alone. It is similar to the study of the growth of *P. ostreatus* PC9 on a solid glucose-peptone (GP) medium supplemented with different concentrations of HMF. The IC-50 of HMF to *P.ostreatus* has been shown as 12.5 mM (1.57 g/L), where the growth is declined with increased concentration of HMF up to 40 mM (Feldman et al., 2015).

3.4. Identification of the fungus APLS-1

Internal Transcribed Spacer region of APLS-1 was amplified and sequenced. Obtained sequence was compared with NCBI – BLAST and it showed 100% similarity to *Aspergillus flavus*. The sequence was submitted in GenBank under the Accession number MK434161.

3.5. Optimization of pH, biomass size, biomass age and substrate concentration for FDCA production

Reaction conditions like pH, biomass size, biomass age and substrate concentration were optimized for the FDCA production. Results revealed that the optimum pH of the reaction was found to be 6.5 in which 0.5 g/l of HMF was converted into 0.040 ± 0.002 g/L (Fig. 1a). At pH 8 a drastic declining in the FDCA production was noticed. Optimum biomass size was found as 6 g/L which converted 0.5 g/L HMF into 0.06 ± 0.002 g/L of FDCA (Fig. 1b). As the biomass size increased the production rate was reduced. It might be because of fast metabolic reaction in which the reaction continued by using the formed FDCA into its metabolic pathway. It has been reported that higher cell concentrations cannot transform HMF into FDCA in *R. ornithinolytica* (Hossain et al., 2016). The FDCA production was less when the organism was in 48 h and 84 h of age. The maximum activity of biomass

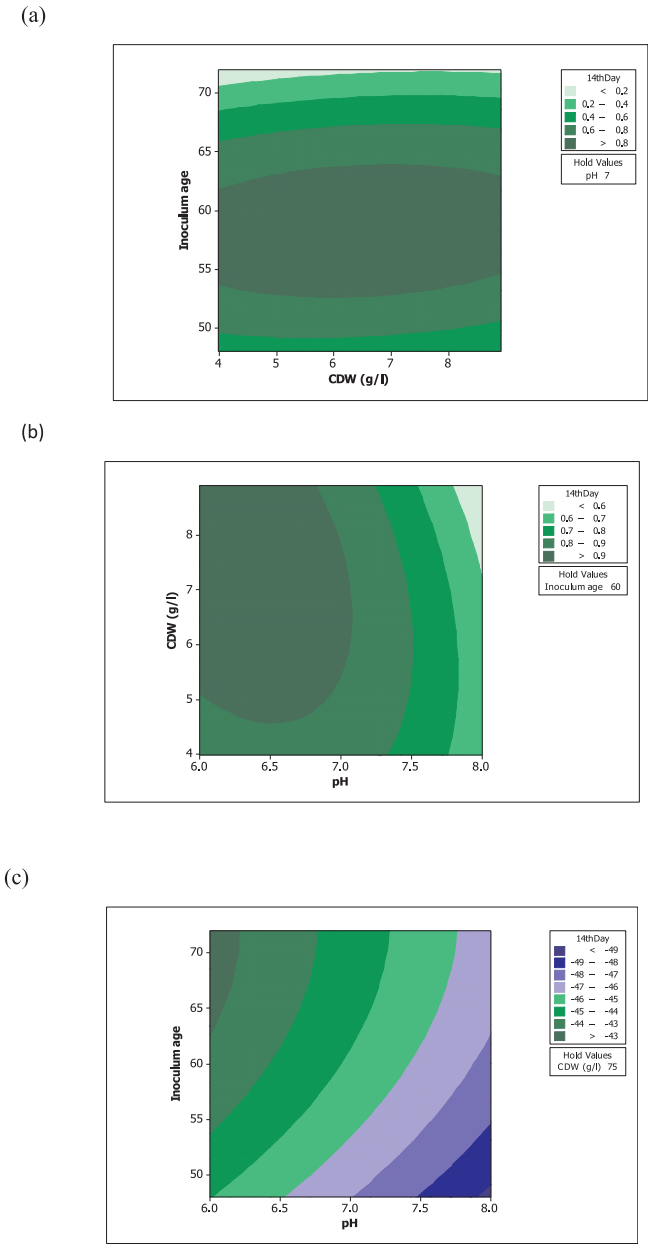

Fig. 2. Contour plot of showing the interaction of (a) inoculum size and inoculum age; (b), pH and inoculums size; (c) pH and inoculum age.

age was 60 h which gave 0.12 ± 0 g/L of FDCA from 0.5 g/L HMF (Fig. 1c). This was the late exponential phase of the organism. Harvesting the cells at late exponential phase has been shown better FDCA production in *R. ornithinolytica* (Hossain et al., 2016). The HMF concentrations ranging from 0.25 g/L to 2 g/L were evaluated for the biotransformation and maximum production of FDCA was found as 0.32 ± 0.06 g/L in the medium supplemented with 1 g/L HMF after 14 days of incubation (Fig. 1d).

3.6. Optimization of FDCA production using Box-Behnken design

Three major parameters like pH, inoculum size (in Cell Dry Weight) and inoculum age were selected to know the multi-parameter influence on HMF biotransformation and FDCA production. According to the Box-Behnken design, 15 set of experiments were done in which 6th, 8th, 12th, and 14thset showed better FDCA production from 1 g/L HMF (kept same for all experiments) after 14 days (Table 1). Contour plot

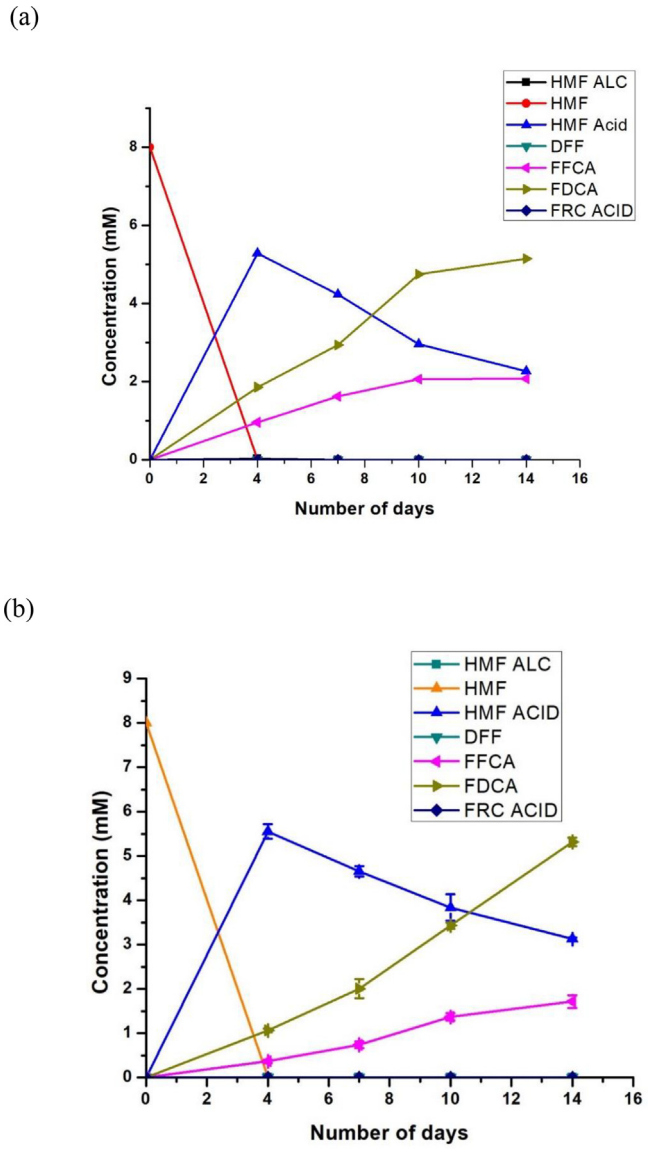

Fig. 3. Intermediates in the bioconversion of HMF to FDCA by *A. flavus* APLS-1 (a) 6th run in Box–Behnken design; (b) 4th run in the validation study. Experiments were done in duplicates and error bars are plotted based on the standard deviation of the mean.

was drawn for the FDCA production after 14 days in terms of inoculum size and inoculum age (Fig. 2a), pH and inoculum size (Fig. 2b), pH and inoculum age (Fig. 2c). Results revealed that biomass size and biomass age and pH influenced highly in the production of FDCA. Among them, 6th experimental run showed 0.8 g/L (6.4 mM) FDCA from 1 g/L HMF (8 mM) after 14 days (Fig. 3a) where the conditions were pH 7, biomass size 5.7 g/L and biomass age 60 h. According to theoretical conversion 1 g/L HMF (8 mM) should give 1.24 g/L (8 mM) FDCA and the present study resulted in conversion efficiency of approximately 67%. The recent report suggests that higher cell concentrations might make oxygen as a limiting factor by generating reducing power. This reducing power may be oxidized again to sustain the flux and do not enhance the FDCA production (Hossain et al., 2016).

3.7. Validation of FDCA production

According to the validation design results (Table 3) the 4th set of experiments with pH 6.5, biomass size 6.6 ± 0.1 g/L and biomass age 60 h showed FDCA production of 0.83 g/L (6.6 mM) from 1 g/L (8 mM)

HMF with a yield of 67% after 14 days (Fig. 3b). So during extracellular sampling analysis, no DFF peaks were observed in the HPLC profile after biotransformation by *A. flavus* APLS-1. The intermediate compound analysis showed that there occurs rapid conversion of HMF to HFCA and further conversion of HFCA to FFCA was delayed. It might be due to the absence of channel proteins or by the limitation of oxygen uptake for the sequential oxidation.

4. Conclusions

The present study describes the potential of *A. flavus* APLS-1 for the bioconversion of HMF to FDCA. After the optimization of bioconversion parameters, the organism could able to produce 0.83 g/L FDCA from 1 g/L HMF. The HMF concentration higher than 1 g/l was found to be toxic to the organism and higher cell concentration making the oxygen limitation and reducing the yield of FDCA. Since this is a first report on the production of FDCA by *A. flavus,* further studies on understanding the mechanism and the enzymes involved in the pathway of HMF biotransformation are planned as future activities.

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References

Almeida, J.R., Modig, T., Petersson, A., Hähn Hägerdal, B., Lidén, G., Gorwa-Grauslund, M.F., 2007. Increased tolerance and conversion of inhibitors in lignocellulosic hydrolysates by *Saccharomyces cerevisiae*. J. Chem. Technol. Biotechnol. 82, 340–349.

Bozell, J.J., Petersen, G.R., 2010. Technology development for the production of biobased products from biorefinery carbohydrates—the US Department of Energy's "Top 10" revisited. Green. Chem. 12, 539–554.

Davis, S.E., Zope, B.N., Davis, R.J., 2012. On the mechanism of selective oxidation of 5-hydroxymethylfurfural to 2, 5-furandicarboxylic acid over supported Pt and Au catalysts. Green. chem. 14, 143–147.

De Bont, J.A.M., Ruijssenaars, H.J., Jan, W., 2017. Fungal production of FDCA. Patent. WO 2017/050815 A1.

Dijkman, W.P., Groothuis, D.E., Fraaije, M.W., 2014. Enzyme-catalyzed oxidation of 5-hydroxymethylfurfural to furan-2,5-dicarboxylic acid. Angew. Chem. Int. Ed. Engl. 53, 6515–6518.

Feldman, D., Kowbel, D.J., Glass, N.L., Yarden, O., Hadar, Y., 2015. Detoxification of 5-hydroxymethylfurfural by the *Pleurotus ostreatus* lignolytic enzymes aryl alcohol oxidase and dehydrogenase. Biotechnol. Biofuels. 8, 63.

Gandini, A., Silvestre, A.J.D., Neto, C.P., Sousa, A.F., Gomes, M., 2009. The furan counterpart of poly(ethylene terephthalate): An alternative material based on renewable resources. J. Polym. Sci. Pol. Chem. 47, 295–298.

Han, X., Li, C., Liu, X., Xia, Q., Wang, Y., 2017. Selective oxidation of 5-hydroxymethylfurfural to 2, 5-furandicarboxylic acid over MnO x–CeO 2 composite catalysts. Green. Chem. 19, 996–1004.

Hossain, G.S., Yuan, H., Li, J., Shin, H.D., Wang, M., Du, G., Chen, J., Liu, L., 2017. Metabolic Engineering of *Raoultella ornithinolytica* BF60 for Production of 2,5-Furandicarboxylic Acid from 5-Hydroxymethylfurfural. Appl. Environ. Microbiol. 83, 1–15.

Kakinuma, A., Yamatodani, S., 1964. L-Glutamic acid formation from 2-furoic acid by soil bacteria. Nature 201, 420.

Koopman, F., Wierckx, N., de Winde, J.H., Ruijssenaars, H.J., 2010a. Efficient whole-cell biotransformation of 5-(hydroxymethyl)furfural into FDCA, 2,5-furandicarboxylic acid. Bioresource. Technol. 101, 6291–6296.

Koopman, F., Wierckx, N., de Winde, J.H., Ruijssenaars, H.J., 2010b. Identification and characterization of the furfural and 5-(hydroxymethyl) furfural degradation pathways of *Cupriavidus basilensis* HMF14. P. Natl. Acad. Sci. 1–6.

Rajesh, R., Godan, T.K., Pandey, A., Binod, P., 2018. Whole cell based biocatalytic production of 2, 5-furan dicarboxylic acid. Indian. J. Exp. Biol. 56, 493–497.

Trudgill, P., 1969. The metabolism of 2-furoic acid by *Pseudomonas* F2. Biochem. J. 113, 577–587.

Tsanaktsis, V., Bikiaris, D.N., Guigo, N., Exarhopoulos, S., Papageorgiou, D.G., Sbirrazzuoli, N., Papageorgiou, G.Z., 2015. Synthesis, properties and thermal behavior of poly (decylene-2, 5-furanoate): a biobased polyester from 2, 5-furan dicarboxylic acid. RSC Adv. 5, 74592–74604.

Wang, S., Zhang, Z., Liu, B., 2015. Catalytic conversion of fructose and 5-hydroxymethylfurfural into 2,5-furandicarboxylic acid over a recyclable Fe3O4–CoOx magnetite nanocatalyst. ACS. Sustain. Chem. Eng. 3, 406–412.

Werpy, T., Peterson, G., 2004. Top value added chemicals from biomass. NREL report FDCA, 1, 26-28.

Wierckx, N., Koopman, F., Bandounas, L., De Winde, J.H., Ruijssenaars, H.J., 2010. Isolation and characterization of *Cupriavidus basilensis* HMF14 for biological removal of inhibitors from lignocellulosic hydrolysate. Microb. Biotechnol. 3, 336–343.

Xu, S., Zhou, P., Zhang, Z., Yang, C., Zhang, B., Deng, K., Bottle, S., Zhu, H., 2017. Selective oxidation of 5-hydroxymethylfurfural to 2, 5-furandicarboxylic acid using O2 and a photocatalyst of Co-thioporphyrazine bonded to g-C3N4. J. Am. Chem. Soc. 139, 14775–14782.

Yang, C.F., Huang, C.R., 2016. Biotransformation of 5-hydroxy-methylfurfural into 2,5-furan-dicarboxylic acid by bacterial isolate using thermal acid algal hydrolysate. Bioresour. Technol. 214, 311–318.

Yuan, H., Li, J., Shin, H.D., Du, G., Chen, J., Shi, Z., Liu, L., 2018a. Improved production of 2,5-furandicarboxylic acid by overexpression of 5-hydroxymethylfurfural oxidase and 5-hydroxymethylfurfural/furfural oxidoreductase in *Raoultella ornithinolytica* BF60. Bioresour. Technol. 247, 1184–1188.

Yuan, H., Liu, Y., Li, J., Shin, H.D., Du, G., Shi, Z., Chen, J., Liu, L., 2018b. Combinatorial synthetic pathway fine-tuning and comparative transcriptomics for metabolic engineering of *Raoultella ornithinolytica* BF60 to efficiently synthesize 2,5-furandicarboxylic acid. Biotechnol. Bioeng. 115, 2148–2155.

Zhang, Z., Deng, K., 2015. Recent advances in the catalytic synthesis of 2, 5-furandicarboxylic acid and its derivatives. ACS. Catal. 5, 6529–6544.

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Whole cell based biocatalytic production of 2,5-furan dicarboxylic acid

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Polyethylene furanoate (PEF), made of 2,5-furan dicarboxylic acid (FDCA), has immense application value and can play a role in reducing the dependence of non-renewable energy sources by replacing the petroleum based products such as polyethylene terephthalate (PET) and polybutylene terephthalate (PBT). The conventional chemical process for production of FDCA via oxidation of 5-hydroxymethyl furfuraldehyde (HMF) requires high temperature, high pressure, metal salts, organic solvents and toxic chemicals. Further, purification of FDCA makes the processes expensive as well as generates considerable pollutants and eco-friendly. The alternative bio-based approach, microbial conversion of substrates such as HMF, fructose and renewable biomass to FDCA sounds promising. In the present study, several soil isolates were evaluated for production of FDCA and one isolate, *Enterobacter* sp., showed potential for conversion of 5-(hydroxymethyl)furfural (HMF) to FDCA with an yield of 0.07g/L FDCA from 0.5g/L HMF in mineral salt media at 14 days of incubation. Changing the production media with 0.25% glucose and 0.25% glycerol showed an inhibition in the FDCA production by 7- and 2-folds, respectively.

Keywords: Biopolymer, FDCA, Green chemicals, 5-(hydroxymethyl)furfural (HMF)

The hydroxymethyl furan derivative, 2,5-furandicarboxylic acid (FDCA), is reported to be one of the most valuable top 12 'green chemicals' of future by the US Department of Energy[1]. This valuable building block monomer is combined with monoethylene glycol and the polymer obtained through the esterification process is known as polyethylene furanoate (PEF). Polyethylene furanoate polymer can be used as analogs of petroleum based products, such as polyethylene terephthalate (PET) and polybutylene terephthalate (PBT). The FDCA based PEF can reduce the dependence of non-renewable energy sources and can reduce the PET and PBT use by 45-55%[2]. FDCA monomers can be used for synthesis of polyesters, polyamides and polyurethanes. The polymers made of FDCA are valuable for the synthesis of bottles, containers, nylons and films. It is used as an ingredient of fire foams, plastics and resin coatings also. FDCA market size is expected to an estimated production of 9 billion lb/yr, and it may yield a revenue of USD 498.20 million by 2020 with a CAGR of 36.70%[3].

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Currently, industries following chemical routes for production of FDCA via oxidation of 5-hydroxy-methyl furfuraldehyde (HMF)[4]. The chemical production process requires high temperature, high pressure, metal salts, organic solvents and toxic chemicals. Also, purification of FDCA after the chemical reactions makes the processes expensive and it generate lots of chemical pollutants which are not eco-friendly. Hence, industries are looking for an alternative environmental friendly method for FDCA production. A bio-based microbial conversion of substrates, such as HMF, fructose and renewable biomass to FDCA seems to be a promising approach.

HMF is a type of toxic furan aldehyde which inhibits primary metabolic activities of micro-organisms and eventually leading to its death. These aldehydes form reactive oxygen species which damage proteins, nucleic acids, and other cell organelles[5]. Some microorganisms have the property to convert toxic HMF into other furan intermediate chemicals such as HMF alcohol, maleic anhydride (MA), 5-hydroxymethyl-2-furancarboxylic acid (HFCA or HMF acid), 2,5-diformylfuran (DFF), 5-formyl furoic

acid (FFA) or 2,5-furandicarboxylicacid (FDCA) which are less toxic to them and tends to survive in the medium. The HMF biotransformation or degradation pathway was completely reported by Kakinuma and Yamatodani[6]; Trudgill[7]. First biological production of this chemical using a microorganism was reported by Wierckx *et al*.[8] using *Cupriavidus basilensis* HMF14 which had a furfural/ HMF oxidoreductase. Engineered *Pseudomonas putida* expressing this furfural/HMF oxidoreductase was reported by Koopman *et al*.[9]

In the present work, a bacterial strain was isolated from a HMF containing acid pretreated liquor waste soil from the premises of bioethanol pilot plant of CSIR-National Institute for Interdisciplinary Science and Technology, Thiruvananthapuram, India. In the primary screening, this strain showed the ability to convert HMF to FDCA. This organism may contain the oxidative mechanisms for conversion of HMF and its intermediate products and survive well in the HMF medium, and finally converts them into FDCA. The growth and bioconversion was also checked in other media with different carbon sources.

Materials and Methods

Isolation of microorganisms by enrichment culture technique

Soil samples were collected from the acid pretreated liquor drainage site (CSIR-National Institute Interdisciplinary Science and Technology, Thiruvananthapuram, India). For Enrichment culture technique, 5 g of soil sample was suspended in 95 mL of mineral salt media of pH 7.2 (MSM) containing 5-hydroxy methyl furfuraldehyde (HMF) ranging from 0.25-3g/L in 250 mL Erlenmeyer flasks and kept in incubated shaker at 200 rpm for 72 h at 30°C. One mL of the above suspension culture was diluted in 99 mL sterile distilled water and a series of dilutions (10^{-2}-10^{-10}) were performed. Single colonies of different morphologies were isolated on 5-HMF agar plates (Nutrient and Potato dextrose agar) having HMF concentrations 0.25-3g/L[10]. The plates were incubated for 2-5 days at 30 and 37ºC for fungal and bacterial growth, respectively. Isolated single colonies were stored in refrigerator at 4ºC.

Preparation of the biomass for the screening of FDCA production

Single pure culture colony of the above microorganisms were inoculated into 50 mL Nutrient Broth (for bacteria) and 50 mL Potato Dextrose Broth [For fungus (3mm disc)] in conical flasks and agitated at 200 rpm in incubated shaker at 30 and 37ºC for fungus and bacteria, respectively. After 48 h of incubation, the pellet of 20% bacterial broth was harvested by centrifugation at 7500 rpm for 10 min. The fungal cultures were incubated for 72 h and the pellet 20% of fungal broth was harvested by centrifugation at 8000 rpm for 10 min. The obtained pellet was washed three times in 50 mM phosphate buffer and used for the screening of FDCA production.

Whole-cell biocatalytic activity of microorganisms for FDCA production

Erlenmeyer flasks (150 mL) were used for the bioconversion of HMF to FDCA. The reaction mixture (50 mL) contained 50 mL mineral salt media (MSM) with 0.5 g/L HMF and pellet from the respective microorganisms. This reaction mixture incubated in a shaker at 200 rpm at 30°C for 20 days. The reaction was stopped by centrifugation at 7500 rpm for 10 min, and the supernatant was recovered and analyzed by HPLC.

Analysis of HMF utilization and FDCA production by microorganisms

The amount of HMF and FDCA in the reaction mixture was determined by HPLC (Shimadzu, Japan) on phenomenex C-18 column (250 mm × 4.6 mm × 5 µm) at 30°C, 0.06N H_2SO_4 (60%) and methanol (40%) as mobile phase. The flow rate was 0.6 mL/min and standards were detected with a Diode Array Detector at a wave length of 280 nm for HMF and 263 nm for FDCA. The HPLC retention time values of the standards of HMF and FDCA were 3.8 and 5.2 min, respectively.

Study of Intolerance of positive isolate in HMF and other carbon sources

Pure single colony of the positive isolate was inoculated in MSM containing 0.5 g/L HMF and growth was observed for five days. Experiments were done in presence of 0.25% glucose and 0.50% glucose.

Biocatalytic activity of positive isolate for FDCA production in presence of different carbon sources

The carbon sources used in this study was 0.25% glucose and 0.25% glycerol apart from 0.5g/L HMF in 50 mL MSM. Production media contained MSM with 0.5 g/L HMF and the microbial pellet was inoculated. This reaction mixture was incubated in a shaker at 200 rpm at 30°C for 20 days. Quantification of FDCA was done by HPLC method as mentioned earlier.

Identification of microorganisms

Bacterial DNA was isolated according to the manufactures protocol (Gene Elute™ Bacterial Genomic DNA Kit, Sigma). The 16S rRNA gene was amplified in a Biorad Mycycler thermal cycler using universal primers 27F: 5'-AGAGTTTGATCMTGGC TCAG-3' and 1492R: 5'-TACGGYTACCTTGTTAC GACTT-3'. Conditions of the PCR cycle was initial denaturation at 94°C for 2 min, denaturation at 94°C for 1 min, annealing at 54°C for 1 min, and extension at 72°C for 2 min. PCR products were purified using a QIA quick gel extraction kit (Qiagen) followed by sequencing on an ABI model 3130 automatic DNA sequencer using a Big Dye terminator cycle sequencing kit (Applied Biosystems).

Chemicals

HMF and FDCA standards of analytical grade were purchased from Sigma-Aldrich, India.

Results

Isolation of microorganisms

Based on the morphological differences, total six microorganisms were isolated which comprises of bacteria (4) and fungus (2). Pure colonies were stored in refrigerator at 4ºC. These microorganisms were able to grow in 0.25g/L of HMF (in MSM) agar plates.

Screening of microorganisms for FDCA production

Among six strains four bacterial strains were showed positive for HMF utilization and FDCA production. But fungi were not much effective as compared to bacteria. Among the six isolates, the strain AS-3 (which was later identified as *Enterobacter* sp.) was selected, based on FDCA production, for further studies.

Identification of microorganisms

The culture AS-3 was identified by sequencing 16S rRNA. Sequence similarity search was done in NCBI-BLAST and the organism showed 99% similarity to *Enterobacter* sp.

Analysis of HMF utilization and FDCA production by microorganisms

The HPLC retention time values of the standards of HMF and FDCA in 0.06N H_2SO_4 (60%) and methanol (40%) mobile phase were 3.8 and 5.2 min, respectively (Fig 1A, B and C). The reaction mixture inoculated with *Enterobacter* sp. (AS-3) showed the utilization of HMF (peak reduction 3.8 min) and production of FDCA (peak at 5.2 min) (Fig 1D).

Study of Intolerance of *Enterobacter* sp. in HMF and other carbon sources

When the pure culture of *Enterobacter* sp. was inoculated in the mineral salt media containing 0.5 g/L

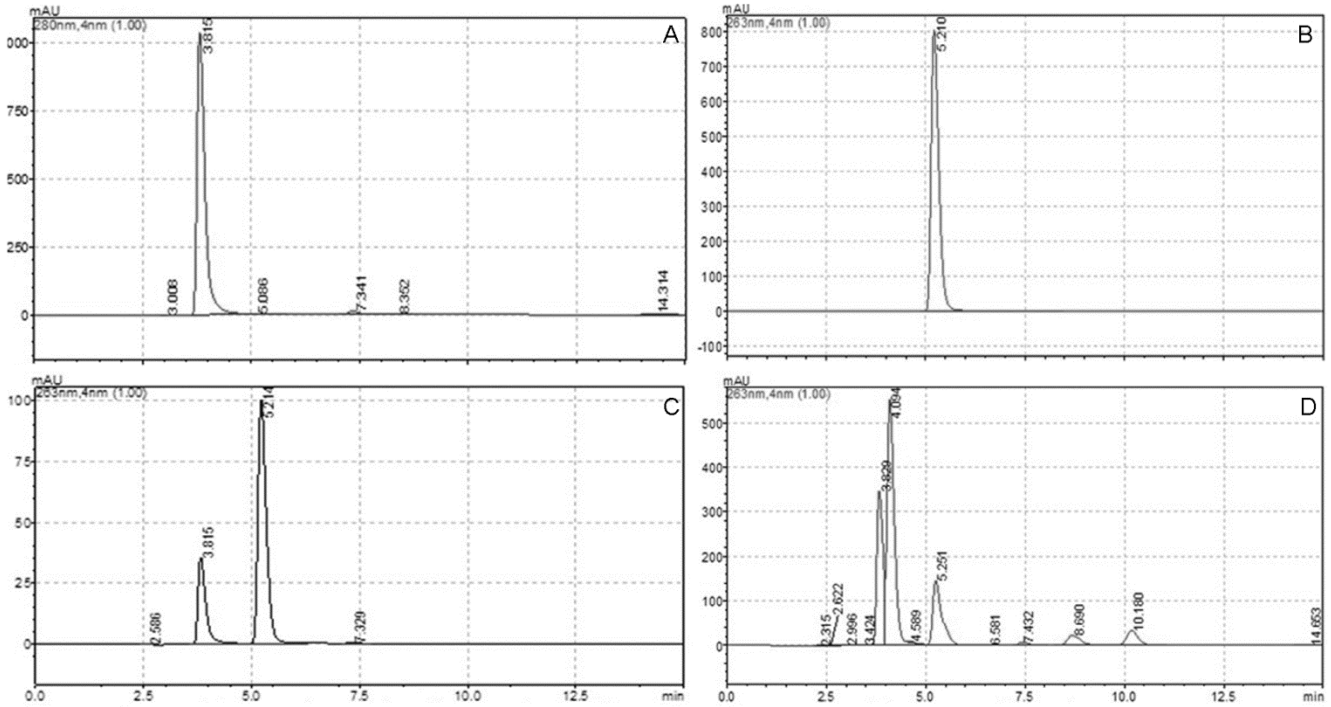

Fig. 1—HPLC analysis of 5-(hydroxymethyl)furfural (HMF) and 2,5-furan dicarboxylic acid (FDCA) in same mobile phase [0.06N H_2SO_4 (60%) and methanol (40%)]. (A) HMF standard with peak at 3.8 mt; (B) FDCA standard with peak at 5.2 mt; (C) HMF (3.8 mt) and FDCA (5.2 mt) standard in same mobile phase; and (D) Biotransformation of HMF and production of FDCA by *Enterobacter* sp.

HMF, the organism showed positive growth until 48 h, and then the growth declined (Fig. 2). When same media (MSM and 0.5g/L HMF) was added with 0.25% glucose and 0.50% glucose, turbidity was higher as compared to the media with HMF alone. There was a steep decrease in the turbidity and growth in 0.50% glucose media after 48 h. From 24-120 h turbidity was almost same for 0.25% glucose media.

Biocatalytic activity of *Enterobacter* sp. for FDCA production in presence of HMF and other carbon sources

Among the six positive isolates, *Enterobacter* sp. showed better production of FDCA in the MSM containing 0.5 g/L HMF. Maximum FDCA production (0.07 g/L) was observed after 14 days of incubation, without any additional carbon sources (Fig. 3). When carbon sources like 0.25% glucose and 0.25% glycerol was added to the media, the FDCA production rate decreased. It seems that this carbon sources supported growth of microorganism rather than the production of FDCA. The FDCA production was noted only after 14 days of incubation and further increase in incubation time does not resulted the increase in production.

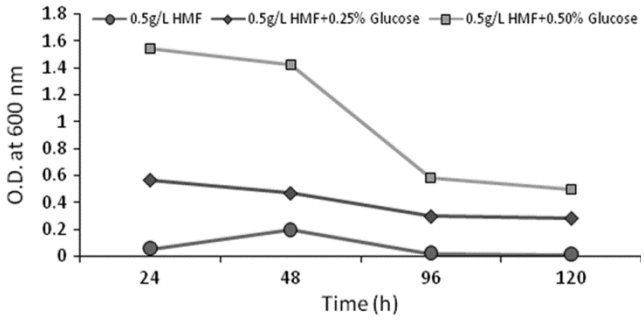

Fig. 2 — Intolerance of *Enterobacter* sp. to 5-(hydroxymethyl)furfural (0.5g/L)

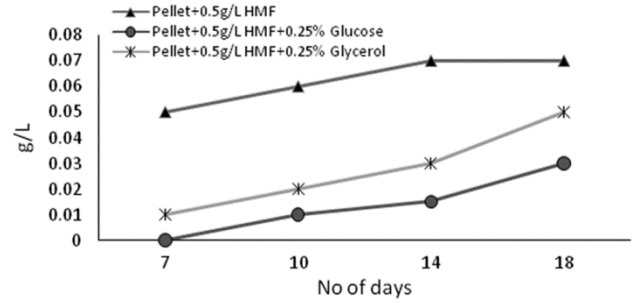

Fig. 3 — FDCA production by *Enterobacter* sp. in different carbon sources

Discussion

The six isolated microorganisms had the capability to grow in 0.25g/L HMF (in MSM) agar plates. It means that all these organisms have the capability to grow in the media containing HMF as the sole carbon source, but it does not mean that all these microorganisms have FDCA production capability. In one of the earlier reports, the authors isolated six strains from the campus soil of National Yunlin University of Science and Technology, Taiwan by enriched mixed culture isolation technique. After screening, five strains grown in the media containing HMF as the carbon source[10]. Among the six isolated strains, *Enterobacter* sp. showed positive for the bioconversion of HMF to FDCA. It showed 0.07g/L FDCA production from 0.5 g/L HMF. The organism showed other intermediate products like hydroxy- methyl furfuroic acid, 2, 5-diformyl furan and formyl furan carboxylic acid apart from FDCA. There are reports on the production of similar intermediate products by a genetically engineered strain of *Raoultella ornithinolytica* BF60[11]. *Enterobacter* sp. showed highest growth in 0.50% glucose as carbon source instead of 0.25% glucose and HMF alone in the media. Since it is a biocatalytic reaction using whole cells, the highest growth does not mean that it would show a higher amount of FDCA production. Earlier studies have shown that *Burkholderia cepacia* H-2 transform 2.0 g/L HMF to 1.28 g/L FDCA[10]. Hossain *et al.*[12] reported 51.0% (mol/mol) conversion ratio for 100 mM HMF to FDCA at 45 g/L using whole cell biocatalyst at 30°C. They used 50 mM phosphate buffer [pH 8.0] as a production media[12]. From the present study, it is concluded that the isolated strain of *Enterobacter* sp. could be used for the biological production of FDCA from HMF.

To summarize, our initial studies have demonstrated that *Enterobacter* sp. is a potential microorganism which can convert HMF to FDCA. It is necessary to go for further optimization for maximizing the bioconversion rate. Even though biological production of FDCA is still in laboratory scale there is much scope for research in this area for its commercialization.

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References

1 Bozell Joseph J & Gene R Petersen, Technology Development for the Production of Bio based Products from Biorefinery Carbohydrates—the US Department of Energy's 'Top 10' Revisited. *Green Chem*, 12 (2010) 539.

2 Gandini Alessandro, Armando JD Silvestre, Carlos PascoalNeto, Andreia F Sousa & Moonica Gomes, The Furan Counterpart of Poly(ethylene Terephthalate): An Alternative Material Based on Renewable Resources. *J Polymer Sci Part A: Polymer Chem*, 47 (2009) 295.

3 Grand view Research Inc, Furandicarboxylic Acid (FDCA) market potential analysis and segment forecasts to 2020, (Report ID: 978-1-68038-043-9), 2015, 80.

4 Wang Shuguo, Zhang Zehui& Liu Bing, Catalytic conversion of fructose and 5hydroxymethylfurfural into 2,5-furandicarboxylic acid over a recyclable Fe3O4-CoOx magnetite nanocatalyst. *ACS Sustain Chem Eng*, 3 (2015) 406.

5 Zaldivar J, A Martinez & LO Ingram, Effect of Selected Aldehydes on the Growth and Fermentation of Ethanologenic *Escherichia Coli*. *Biotechnol Bioeng*, 65 (1999) 24.

6 KakinumaA&Yamatodani S, L-Glutamic acid formation from 2-furoic acid by soil bacteria. *Nature*, 201 (1964) 420.

7 Trudgill PW, The metabolism of 2-furoic acid by pseudomanas F2.*Biochem J*, 113 (1969) 577.

8 Wierckx N, Koopman F, Ruijssenaars HJ & de Winde JH, Microbial degradation of furanic compounds: biochemistry, genetics, and impact. *ApplMicrobiolBiotechnol*, 92 (2011) 1095.

9 Koopman F, Wierckx N, Ruijssenaars HJ & de Winde JH, Efficient Whole-Cell Biotransformation of 5-(hydroxyl-methyl)furfural into FDCA, 2,5-Furandicarboxylic Acid. *Bioresour Technol*, 101 (2010) 6291.

10 Yang, Chu Fang &CiRuei Huang, Biotransformation of 5-Hydroxy-Methylfurfural into 2,5Furan-Dicarboxylic Acid by Bacterial Isolate Using Thermal Acid Algal Hydrolysate, *Bioresour Technology*, 214 (2016) 311.

11 Yuan H, Li J, Shin HD, Du G, Chen J, Shi Z & Liu L, Improved production of 2,5furandicarboxylic acid by over expression of 5-hydroxymethylfurfural oxidase and 5-hydroxy-methylfurfural/furfural oxidoreductase in *Raoultella ornithinolytica* BF60. *Bioresour Technol*, 247 (2017) 1184.

12 Hossain GaziSakir, Haibo Yuan, Jianghua Li, Hyun-dong Shin, Miao Wang, Guocheng Du, Jian Chen & Long Liu, Metabolic engineering of *Raoultella ornithinolytica* BF60 for the production of 2,5-furandicarboxylic acid from 5-hydroxymethylfurfural. *Appl Environ Microbiol*, 1 (2016) e02312.