Bacterial Isolation and Bioconversion of Organic Materials to Industrial Platform Chemicals

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ABSTRACT

Careful selection/isolation of the suitable microbial consortium for enzymatic saccharification of organic matters is a critical step in biofuels production. We isolated strains EF2, OW1-1 and HK2 from intestine of *Eisenia fetida*, municipal organic waste and forest soil respectively. The strains EF2, OW1-1 and HK2 have higher potential to produce various extracellular enzymes including cellulase and xylose/glucose isomerase (GI). The qualitative screening of strains using plate assay techniques was performed in standard agar plates to obtaine a zone of clearance. The 16S rRNA gene sequences of strains EF2 and OW1-1were identified as gram (+ve) *Bacillus* sp. whereas HK2 was a gram (-ve) *Serratia marcescens*. The Carboxymethyl cellulase (CMCase) activities of EF2 and OW1-1 were 35.307 ± 0.08 IU/ml and 29.92 ± 0.01 IU/ml, respectively, when 2.5% (w/v) of lactose was used as a carbon source at their respective optimal pH and temperature. The co-culture of *Bacillus* sp. strains EF2 and OW1-1 in contrast to their monoculture, showed 15% and 35.71% increased in CMCase activity respectively. Similarly, the strain *S. marcescens* HK2 preferred the temperature of 35 to 40 °C and pH of 8 to 9 for efficient GI production. The GI activity was high when 1.5% xylose and 1:3 ratio of peptone and yeast extract were used in the culture medium. The SDS-PAGE and zymogram revealed that the molecular weight of CMCase and GI were 60 and 63 kDa in *Bacillus* sp. (both EF2 and OW1-1) and *S. marcescens* HK2 respectively. This study discovered a novel finding that the strain *S. marcescens* HK2 can utilize low cost agricultural residue for production of GI and improved activity was observed in whole cell immobilization which can further minimize the cost of down streaming processing. Thus, all three bacterial strains could be a promising candidate for biofuels industries.

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CHAPTER I

Bacterial Isolation and Synergistic Effect of Enzymes in Bioconversion of Organic Materials to Industrial Platform Chemicals: An Overview

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Abstract

Increasing energy demands are not only exploiting the fossil resources but, also depleting natural environment. Biofuels from lignocellulosic biomass is a renewable, ecofriendly, sustainable and could be a promising alternative to fossil fuels. However, pretreatment is an essential step to disarray the layers of lignocellulose prior to enzymatic hydrolysis. Among various pretreatments of lignocellulose, the biological pretreatment using microorganisms such as bacteria and fungi are gaining popularity due to its financial and environmental benefits. Careful selection of the suitable microbial consortium for efficient pretreatment of biomass is a critical step. The co-culture of bacteria and/or fungi in consolidated bioprocessing (CBP) is highly beneficial in the breakdown of complex biopolymers due to their high enzyme activity. The bacteria are appropriate for isolation and laboratory culture, an important step towards enzymatic bioconversion. It is very crucial to select efficient bacterial strain suitable to produce enzymes of our interest. Our selection of highly promising bacterial and/or fungal consortium can produce various extracellular enzymes including cellulase, hemicellulase, and xylose/glucose isomerase (GI). These strains can be used in CBP and the added advantage of co-culture and immobilization can help in biological pretreatment of lignocellulosic biomass following production of biofuels and bioproducts.

1. General Introduction

The biological process is performed by certain microorganisms and or enzymes for bioconversion of organic materials usually the plants or animal waste to produce energy or other value-added products. There is an undeniable fact that human reliance on fuels to quench the thirst of liquid energy (oil, biofuels, and other liquid fuels) is increasing progressively, resulted in resource depletion and environmental pollution. British Petroleum Global (2016) has estimated that increasing human population and rising prosperity associated with emerging economies accounted for 97% of the increase in global oil consumption. The rate of oil consumption grew by 1.9 million barrels per day (b/d) that is nearly double (1.9%) than the average of 1% seen in 2014. China accounted for the largest increment in demand of 6.3% (i.e. 770,000 b/d) in oil consumption (https://www.bp.com/). This increase in global fuel demand accompanied by depletion of fossil fuels over the years and various disadvantages attached to its use has lead in search for an innovative alternative energy from renewable source like lignocellulosic biomass (Hamelinck et al. 2005). The plant polysaccharides from lignocellulosic derivatives have been used as a potential cheap carbon feedstock for important enzymes (such as cellulase, hemicellulase and xylose isomerase) production from microorganism and for saccharification followed by microbial fermentation to produce biofuels such as bioethanol, biodiesel and other bioproducts including various chemicals, biofibers, biopulps, enzymes, etc. (Millati et al. 2011).

The lignocellulosic biofuel is renewable, cost efficient, ecofriendly and thus creating a global priority. However, the main hurdles in utilizing lignocellulosic materials lie in the crystalline nature of cellulose sheathed by hemicellulose, degree of polymerization, biomass particle size and recalcitrance of their bonding due to protective covering of lignin which allow very less surface

area for enzymatic hydrolysis (Horn et al. 2012; Zhang et al. 2007; Zavrel et al. 2009). Thus, to increases the digestibility of cellulose and hemicellulose, the removal or efficient breakdown of lignin from lignocellulosic biomass is usually a targeted step in pretreatment. The physical pretreatment such as milling, grinding, chipping, ultrasonic, etc. and chemical pretreatment with acids, alkali or oxidative delignification can efficiently breakdown the recalcitrant bonding in a short time thus are being extensively used in several industries. However, it requires high energy and operational cost along with chances of high risk of chemical hazards on environment. The biological pretreatment on the other hand has its very wide application and gaining its popularity because it requires low energy, has no chemicals, less pollution and cost effective. The naturally occurring bacteria and fungi secret different cellulolytic enzymatic complexes including endoglucanase, exoglucanases and β-glucosidases which act synergistically to disarray the recalcitrant bonding of lignocellulose and release monomeric sugar molecules (figure 1). The glucose molecules so formed after hydrolysis can be further utilized for production of biofuels after alcoholic fermentation (Zhou and Ingram 2000).

On the other hand, glucose can be easily converted into fructose by enzymatic isomerization using xylose/glucose isomerase (GI). Further, the fructose can be utilize to produce other various platform chemicals such as glycerol, levulinic acid, xylitol, sorbitol etc. by biocatalytic conversion which in turn converted into a fine chemical, polymers and fuels (Jäger and Büchs 2012). Thus, many efforts have been made on catalytic conversion of glucose to fructose using different organic solvents and metal chlorides by altering their chemical composition and other physiochemical parameters. However, relatively a new approach of utilizing fructose as a reactive chemical feed stock has been practicing in several smaller and larger scale facilities for the production of an

industrially important platform chemical, 5-hydrolymethyle furfural (Yong et al. 2008; Liu et al. 2012; Melo et al. 2014; Thombal and Jadhav 2014).

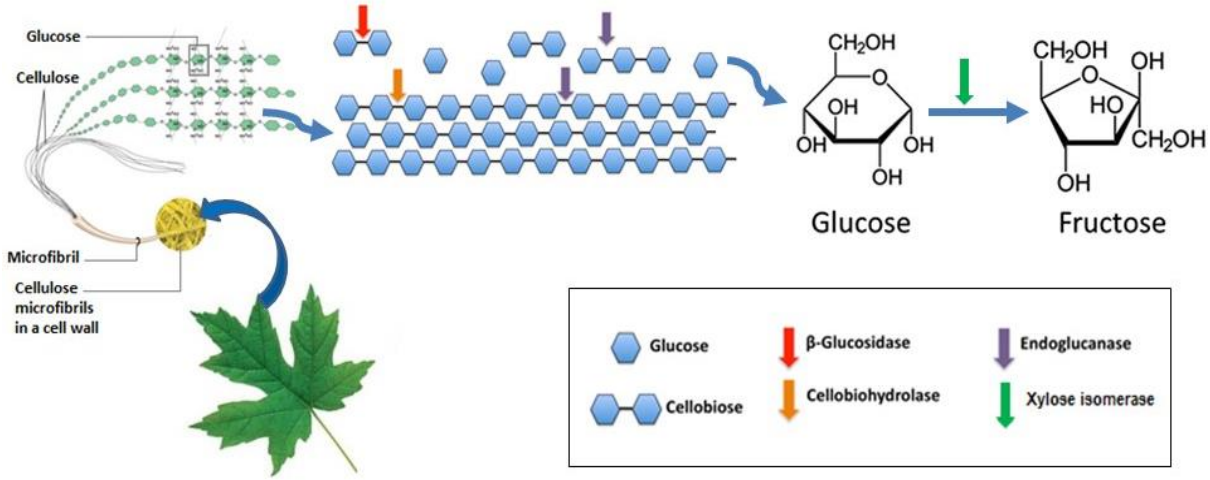

Fig 1. Disruption of lignocellulosic biomass and synergistic catalysis of different enzymes in hydrolysis of polysaccharide.

Since, the enzymatic conversion using efficient microbes is more suitable, environment friendly and cost-effective approach, the study mainly focused on to explore biological pretreatment methods and isolation of efficient bacterial strains which could give higher yield of cellulase and xylose/glucose isomerase (GI) enzymes. These enzymes play a vital role in bioconversion process which have greater application in biofuels industries. The study also explored the optimum conditions for co-culture and enzyme production. The attempts have been made on whole cell immobilization of efficient strain in calcium alginate beads to improve the enzyme activity which could also economize the downstream production. The study not only provides the baseline data on cellulase and GI enzymes activity assays but also provide future recommendations which might be useful to biofuel industries and help to mitigate the fuel crisis.

2. Lignocellulose degrading enzymes

2.1. Cellulolytic enzymes

Cellulase consists of endoglucanase, exoglucanase or cellobiohydrolase (CBH), and β-glucosidase, all these hydrolytic enzymes belong to glycosyl hydrolase (GH) family (Henrissat and Davies 1997). There are 128 GH families consisting of different cellulase enzymes and the synergistic actions of these hydrolytic enzymes catalyze the cellulose into monomeric sugar units. The endo- and exo-glucanases hydrolyze the glycosidic bonds from chain ends of cellulose to release cellobiose and some glucose. The β-glucosidases finally cleave cellobiose to glucose (Himmel et al. 1996). Various bacteria and fungi are known to secrete endo or exo-acting cellulases that act on cellulose, resulting in release of glucose and cellobiose. So far, cellodextrin and cellobiose have their inhibitory activities during cellulose hydrolysis, the β-glucosidase is essential to break the final glycosidic bonds of cellobiose so as to produce sufficient glucose molecules (Maki et al. 2009; Dashtban et al. 2010).

2.2. Hemicellulolytic enzymes

Hemicellulases can be categories into glycoside hydrolase (GH) groups found in about 29 GH families and carbohydrate esterase (CE) groups found in about 9 CE families (Sweeney and Xu 2012). The GH groups hydrolyze the glycosidic bonds whereas the CE hydrolyze the ester bonds of acetate or ferulic acid groups. There are wide array of interdependent hemicellulases involve synergistically during hydrolysis of hemicellulose to form several monomeric sugars and also liberate cellulase (Pérez et al. 2002; Sweeney and Xu 2012). The enzymes like endo- and exo-xylanases hydrolyze the cross-linked of hemicelluloses that cleave the xylen to generate oligosaccharides (Pérez et al. 2002). The other enzymes like β-xylosidases, α-arabinofuranosidase,

and esterases hydrolyze xylooligosaccharides to xylose; arabinose into furanose and pyranose forms; acetyl group into arbinose and ferulic acids respectively (Zhang et al. 2012).

2.3. Ligninolytic enzymes

The ligninolytic enzymes are a group of enzymes that degrade highly complex and recalcitrant lignin. Most of the White rot fungi possess enzymatic system to degrade the lignin (Plácido and Capareda 2015). They produce laccase and various peroxidases such as manganese peroxidase (MnP), lignin peroxidase (LiP) and versatile perioxidase (Singh nee' Nigam et al. 2009; Niladevi 2009). The white rot fungi are well-known producer of ligninolytic enzymes, followed by brown rot and soft rot fungi (Niladevi 2009). Unlike fungi, the bacteria are considered as low potential for lignin degradation. However, the three groups of bacteria namely, actinomycetes, α-proteobacteria and γ-proteobacteria are known to have ligninolytic system (Bugg et al. 2011). The bacterial lingninolytic enzymes such as laccase, lignin peroxidase (LiP), dye-decolorizing peroxidases (DyP), β-etherases, superoxide dismutases, etc. has already been discovered in different bacteria (De Gonzalo et al. 2016). Among these above enzymes some of the most significant ligninolytic enzymes are laccase and peroxidases. Laccase is a multicopper oxidases having four copper molecules and act as oxidizing agent and cofactor. Similarly, various peroxidases have their potential to degrade different aromatic structure by involved in redox reaction (Plácido and Capareda 2015).

2.4. Lytic polysaccharide monooxygenases (LPMO)

Lytic polysaccharide monooxygenase (LPMO) was initially discovered for its activity on chitin degradation (Horn et al. 2012; Vaaje-Kolstad et al. 2010) however recently it has been known to

disrupt the glycoside bonds in cellulose (Horn et al. 2012). LPMO is copper-dependent monooxygenases (Hemsworth et al. 2013; Aachmann et al. 2012), belongs to the auxiliary activities (AA) enzyme classes. The carbohydrate-active enzyme of LPMO is classified into four AAs families AA9, AA10, AA11 and AA13 (Villares et al. 2017). AA9 is found exclusively in fungi (*Arthrobotrys oligospora*, *Aspergillus nidulans, Coprinopsis cinerea***,** etc), AA10 is predominantly found in bacteria (*Bacillus cellulosilyticus, Streptomyces halstedii*, *Serratia marcescens*, etc.) whereas AA11 and AA13 LPMOs are found in wider groups of fungi and some bacteria (www.cazy.org) (Levasseur et al. 2013). LPMO carry out oxidative disintegration of recalcitrant polysaccharide chains in their crystalline regions so as to release oxidized oligosaccharides (Vaaje-Kolstad et al. 2010; Eibinger et al. 2014). LPMO works synergistically with hydrolytic enzymes, boost up the hydrolytic activity and increases the sugar production from lignocellulosic biomass (Patel et al. 2016).

3. Xylose/glucose isomerase (GI) enzyme

The enzyme belongs to isomerase family commonly called as fructose isomerase, xylose isomerase and glucose isomerase. Its systematic name is d-xylose ketol-isomerase EC 5.3.1.5 which can catalyze the interconversion of glucose and fructose (Khalilpour and Roostaazad 2008). Several bacteria such as *E. coli, Aerobacter* sp., *Pseudomonas* sp., *Sarcina* sp., *Arthrobacter* sp., *Streptomyces murinus* etc. are known to secret GI enzyme (Suekane and Iizuka 1981). It is a tetramer having four subunits and two substrate binding sites. The histidine (His 53) of enzyme catalyze the ring opening step to form an open chain conformation of sugar molecule which is followed by hydride shift isomerization between C2 and C1 to form the isomers (Blow et al. 1992; Asbóth and Náray-Szabó 2000). The enzyme is used to produce pentose and hexose sugars

including fructose, xylulose, etc. Conversion of glucose to fructose for the production of high fructose corn syrup (HFCS) is industrially established process where efficient GI from microbes play a vital role in isomerization.

4. Lignocellulosic biomass

Lignocellulose is the plant biomass composed of carbohydrate polymers: cellulose (40-60%), hemicellulose (20-40%), and an aromatic polymer: lignin (10-24%) as main composition of plants cell walls (Putro et al. 2016). The composition of lignocellulosic biomass varies from one plant species to another and their sources such as hardwoods, softwoods, and grasses (Table 1). Moreover, the composition within a single plant also differs with age, stage of growth, and conditions under which plant grows (Jeffries 1994; Chen 2014). The sources of lignocellulosic biomass not only include crop and forest residues, but also found in municipal solid waste, animal manures, papermill sludge, bioenergy crops and forest products. It has been estimated that about 10 – 50 billion ton of lignocellulosic biomass is produced annually worldwide (Sánchez and Cardona 2008). It can be farmed for energy purposes thereby enabling higher production per unit land area and thus increasing land-use efficiency (Larson 2008). It is an abundantly available renewable resource on the Earth that reduces reliance on fossil fuels by production of biofuels which is carbon neutral, alternative to petroleum and can mitigate the greenhouse gas emission. Thus, the lignocellulosic biomass has promising future and well chosen as predictable, feasible and sustainable resource for biofuels and other value added products (Saritha et al. 2012).

4.1. Cellulose

Cellulose is the structural material in cell wall and composed of D-glucose subunits linked by β-1, 4 glycosidic bonds (Pérez et al. 2002). The long polysaccharide chains are unbranched and arranged parallelly to form cellulose microfibrils. These cellulose molecules are the most abundant natural biopolymers found in earth. The cellulose microfibrils are tightly bound each other by inter- and intra-molecular hydrogen bonds which allow a rigid crystalline or amorphous structure. The CP-MAS study reveal the crystalline structure of cellulose has two form called Iα and Iβ (Atalla and VanderHart 1984; VanderHart and Atalla 1984; O'Sullivan 1997).

4.2. Hemicellulose

Hemicellulose is a complex carbohydrate, branched polymer consists of heterogeneous mixture of pentoses (xylose, arbinose), hexoses (mannose, glucose, galactose) and sugar acids (4-O-methyl-glucuronic, galacturonic and glucuronic acids). These sugars are linked together by β-1, 4-glycosidic and sometimes by β-1, 3-glycosidic bonds (Joy, J., Jose, C., Mathew, P. L., Thomas, S., Khalaf 2016). Its composition varies in hardwood which contain xylans and glucomannans; and softwood that contain glucomannans, xylans, arabinogalactans, xyloglucans and glucans (Saha 2003; Zhang et al. 2012). Hemicelluloses bind with cellulose microfibrils, lignin and pectin to form a cross-linked network of heterogeneous mixture of pentoses and hexoses in the cell walls (Zhang et al. 2012).

4.3. Lignin

Lignin is a complex, amorphous hetero-biopolymer, insoluble in water and consisting of phenylpropane units joined together by carbon-carbon and aryl-ether linkages. Lignin along with

cellulose is considered the most abundant biopolymer in nature (Pérez et al. 2002). It is formed by oxidative coupling of three monolignols namely: trans-p-coumaryl alcohol, trans-coniferyl alcohol and trans-sinapyl alcohol. These monomers when form polymer, the phenylpropane units are called p-hydroxyphenyl, guaiacyl and syringyl units (Lewis and Yamamoto 1990; Cesarino et al. 2012). This phenylpropanoid unit of lignin is the main bottleneck of breakdown of lignocellulosic biomass because it provides structural support, impermeability, and protection against microbial invasion (Mussatto 2016).

Table 1**.** Composition of lignocellulose on dry basis modified from (Kim and Day 2011; Sun and Cheng 2002)

Lignocellulosic materials	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Hardwoods stems	40–55	24–40	18–25
Softwood stems	45–50	25–35	25–35
Corn cobs	45	35	15
Wheat straw	30	50	15
Switchgrass	45	31.4	12
Sugarcane bagasse	42	25	20

4. Biomass derived biofuels and value-added chemicals

Biomass is an organic matter derived from living organisms. Biomass like wood, charcoal or dried animal waste has traditionally been used as unprocessed primary fuel whereas the processed biofuels have been increasingly used for transportation. The fuels derived from biological carbon fixation rather than geological process are called biofuels. The application of thermal, chemical, and/or biochemical conversion of biomass (mainly the plants or plants derived materials) can

results in production of biofuels such as bioethanol, biobutanol, biodiesel, etc. and some other value-added chemicals (Table 2). These are hydrocarbon fuels which can be used to produce energy in different mechanical setting. Wider range of microbial strains have been used in biofuels production however the *Saccharomyces cerevisiae*, a species of yeast is primarily used in industrial scale production of ethanol using starch and sugars as major feedstock (Bai et al. 2008; Balat and Balat 2009). The most common carbon feedstock in biofuels production used so far in the fermentation are agricultural products, mainly the corn in the United States, wheat in the European Union, and sugar cane in Brazil (Balat and Balat 2009).

Table 2. Biomass derived biofuels and value added platform chemicals (Jiang et al. 2016; Werpy et al. 2004; http://www.ieabioenergy.com/)

Carbon no.	Value added chemicals
C1	Methanol, formic acid, methane, syngas
C2	Ethylene, ethyl acetate, ethanol, glycolic acid, oxalic acid, glycine, acetic acid, acetaldehyde
C3	Lactic acid, acrylic acid, malonic acid, propylene, serine, glycerol, epichlorohydrin, 3-hydroxy propionic acid, ethyl lactate, 1,3-propanediol, isopropanol, 1,2-propanediol, acetone
C4	Butanol, 1,4-butanediol, iso-butene, succinic acid, malic acid, iso-butanol, methyl methacrylate, threonine, acetoin
C5	Furfural, itaconic acid, glutamic acid, levulinic acid, xylitol, arabinitol, isoprene
C6	Sorbitol, adipic acid, fructose, lysine, FDCA, isosorbide, glucaric acid, citric acid, ascorbic acid, aconitic acid
Cn	Polyhydroxy-alkanoates, para-xylene, dicarboxylic acids, fatty acid derivatives

Biofuels are non-fossil fuels, can be divided into primary and secondary biofuels. The primary (unprocessed) biofuels such as firewood, wood chips and pellets are directly combusted in their natural form mainly for heating, cooking or electricity production. The secondary (processed) biofuels such as charcoal, bioethanol, biodiesel and biogas are produced from biomass. Depending upon the sources of feedstock used and their technological innovation, the secondary biofuels are further divided into first, second and third generation biofuels (Singh Nigam and Singh 2011).

4.1. First generation biofuels

The first-generation biofuels are made from the food crops such as: sugarcane in Brazil, corn in the United State of America (USA) and beet or wheat in Europe and biodiesel made from plant oil such as: oilseed in France and Germany and from palm oil in Indonesia, Malaysia, Central America, Thailand, Africa and some other parts of the world. USA and Brazil together produced 85% (i.e. 21793 million gallons) of ethanol and rest of the world produced only 15% (i.e. 3783 million gallons). Of which USA alone produced 14700 million gallons (57%) and Brazil produced 7093 million gallons (28%) of ethanol (http://www.ethanolrfa.org/) (Renewable Fuels Association 2016). However, it has some conflicting issue because of its intrinsic parts in the food chain.

4.2. Second generation biofuels

The second-generation biofuels are manufactured from agriculture and forest residues and non-food crop feedstock including wood, organic waste, food waste and specific bioenergy crops. The study of U.S. Environmental Protection Agency (https://www.epa.gov/) showed, USA produced 2.18 million gallons of cellulosic ethanol in 2015. Similar, high potential of cellulosic ethanol can be noticed from Gao et al. (Gao et al. 2016) who estimated that 66% of agricultural residue and

34% of forest residue in China make a total of 12693 petajoule biomass available for energy production. However, several concerns including competition and impact on arable land uses remain unchanged.

4.3. Third generation biofuels

The third-generation biofuels are bioethanol and biodiesel manufactured from algae and sea weeds. It is of low-cost, possess high-energy, and completely renewable sources of energy. The algae-based biofuels and bioproducts have immense potentiality to replace fossil fuel and thus have promising future because of production of sustainable green energy. It has been estimated that the most efficient microalgae grown in optimized photobioreactors can produce 19000 to 57000 liters of algal oil per acre per year (Demirbas 2010). It can grow in areas unsuitable for first and second generation crops using sewage, wastewater, and saltwater which would minimize impacts/competition on water and arable land uses. However, it has high operational cost and thus required intensive research on its further technological innovation and efficient utilization.

5. Pretreatment of lignocellulosic biomass

There are various pretreatment methods like physical, chemical, biological, and/or their combination. The purpose of any pretreatment method is to disintegrate the cellulose, hemicellulose and lignin so that the polymers are converted into smaller fragments (Figure 2) readily accessible for enzymatic hydrolysis and other bio-refinery process to produce greater yield of various platform chemicals and value-added products (Figure 3).

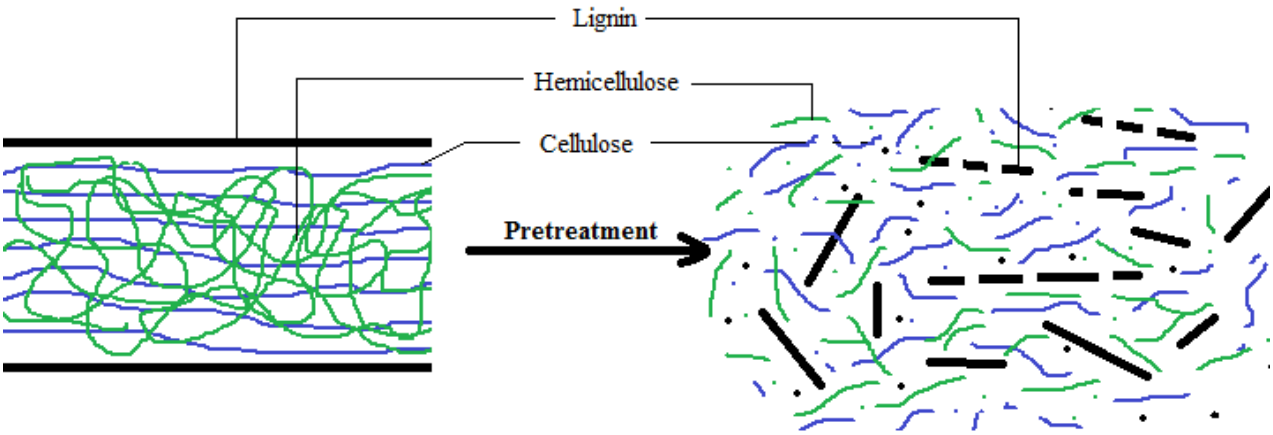

Fig 2. Lignocellulosic biomass subjected to pretreatment

However, each pretreatment method has its own advantages and disadvantages (Table 3). The physical methods (such as chipping, grinding or milling) are for mechanical breakdown of biomass that reduces the particle size and increase exposed surface area for further hydrolysis. But, it required high energy and is not cost efficient. Similarly, the application of chemicals like acids, alkalis, ozone, or peroxide in pretreatment is faster but may produce toxic substances and involves extra financial circumstances for chemicals recovery to sustain the system. Nevertheless, a combined mechanical and chemical method like steam explosion, and hot water treatments have reported a relatively cost-effective technique (Mosier et al. 2005). The biological pretreatment on the other hand is comparatively slower process and cannot easily control but in some circumstances where time is not always a major concern, it is cost effective technique, requires low energy input, no chemicals and ecofriendly (Wan and Li 2012; Shi et al. 2011). However, the biological method has been less investigated due to low industrial significance and limited technological progress.

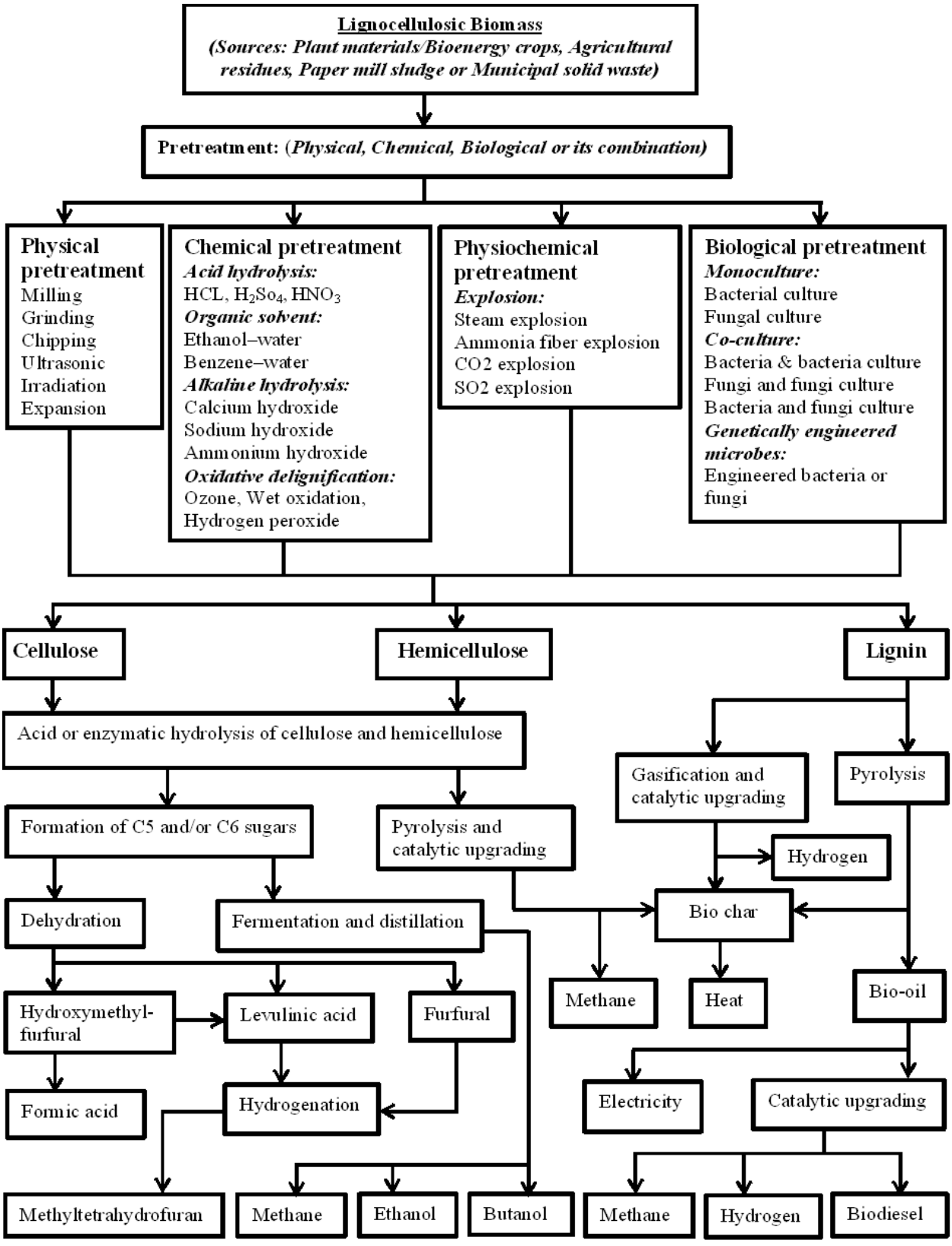

Fig 3. Pretreatment of biomass to value-added end products.

Table 3. Comparison of pretreatment methods (Harmsen et al. 2010; Taherzadeh and Karimi 2008; Conde-Mejía et al. 2012; Maurya et al. 2015; Singh et al. 2014; Kim et al. 2014; Bensah and Mensah 2013).

Pretreatment	Advantages	Disadvantages
Physical pretreatment		
Milling, grinding, chipping, ultrasonic pretreatment, irradiation	Useful to get desired particle size by increasing the surface area. No chemical required. Effective in reducing cellulose crystallinity, help enzymatic hydrolysis.	High operating costs. High chances of equipment depreciation. Not suitable for lignin removal. High energy requirement
Chemical pretreatment		
Liquid hot water	No catalyst and chemical involved. Reduction of feedstock size by disrupting the lignocellulosic components, mainly the hemicellulose. Hydrates the cellulose and make it more accessible to hydrolytic enzymes. It also removes part of lignin and have high xylose recovery	High water and energy demand. Multi-stage pretreatment at low temperature and long residence time is required to recover hemicellulose and its valuable sugars.
Acid hydrolysis: HCL, H_2So_4, HNO_3	A powerful agent for removal of hemicelluloses and lignin. Concentration of acids has its significant role in pretreatment. Dilute acids are more favored in pretreatment that affectively remove hemicellulose, maximize sugars yield and can alter the lignin structure, while strong acids can hydrolyze cellulose. Some acids such as H_2SO_4 and HCl are cheap.	Acids are corrosive, and it is crucial to recycle in order to lower cost. The formation of degradation products such as furfural, 5-hydroxymethylfufural, levulinic acids and formic acid formed from cellulose and hemicellulose together with organic acids from lignin degradation act as inhibitors, that affect the subsequent stages of enzymatic hydrolysis and fermentation. Requires high temperature and specific reaction vessels which is costly.

Organic solvent: Methanol, ethanol, ethylene glycol, acetone, oxalic acid, salicylic acid, acetylsalicylic acid	Help in removal of lignin and hemicellulose, improve retention and enzymatic digestibility of the cellulose.	High cost of solvent and catalyst. Greater chances of environmental impact. Some are inflammable and causes fire and explosion.
Alkaline hydrolysis: Calcium hydroxide Sodium hydroxide ammonium hydroxide	Important in removal of lignin from the biomass and exposed the polysaccharides, sometime also breaks the crystalline cellulose. Increase surface area and makes the hydrolysis faster.	High operational cost, formation of inhibitors. Generally, not suitable for woody biomass. It requires chemicals and generally has harsh conditions
Oxidative delignification: Ozone, wet oxidation, hydrogen peroxide peracetic acid	Removes hemicellulose and lignin from biomass. Improve retention and enzymatic digestibility of the cellulose. Very low formation of enzyme-inhibiting compounds.	High operational cost. Acids formed in the process act as inhibitor in fermentation. Parts of hemicellulose are lost.
Physiochemical pretreatment		
Explosion: Steam explosion, ammonia fiber explosion, CO_2 explosion, SO_2 explosion	Low chemicals and energy consumption. Hemicellulose and lignin disruption. Acids help to improve hydrolysis. Increases the assessable surface area and enzymatic digestibility of the cellulose. Suitable for industrial application	Degradation products may inhibit further processes. Need high pressure. Low yield but high energy consumption. Chances of chemical hazard.
Biological pretreatment		
Bacteria Fungi	Environment friendly, low energy requirement, cost effective, sustainable, no chemical required. Useful in hydrolysis of cellulose, hemicellulose and lignin.	Slow process, partial hydrolysis of hemicellulose. Chances of health hazard

6. Biological pretreatment

The naturally found wide taxonomic array of microorganisms are used in biological pretreatment. They alter or degrade lignocellulose extracellularly by secreting hydrolytic enzyme (such as hydrolases); and ligninolytic enzyme, which depolymerizes lignin (Pérez et al. 2002). Due to this the cell wall structure open up and allowing the subsequent hydrolysis of biopolymers. In biological pretreatment, the cellulose and hemicellulose are usually hydrolyzed into monomeric sugars using cellulolytic and hemicellulolytic microorganisms. The simultaneous degradation of lignocellulosic biomass followed by fermentation process are initiated at the same time which result in formation of biofuels such as ethanol, hydrogen, methane, furfural, etc. and bioproducts such as several enzymes, lactate, acetate, organic acids, etc. (Reguera et al. 2015; Zhao et al. 2011; Faik 2013). Some bacteria (such as *Clostridium sp., Cellulomonas sp., Bacillus sp., Thermomonospora sp., Streptomyces sp. etc.*) and several fungi (such as *P. chrysosporium*, *Trichoderma reesei, T. viride, Aspergillus niger etc.*) are known to hydrolyze the natural biopolymers.

5.1. Bacterial pretreatment

There are many bacteria producing various biomass degrading enzymes used in biological pretreatment. The selection of the most efficient bacterial strains in pretreatment of lignocellulosic biomass followed by enzymatic hydrolysis and fermentation are the crucial steps during biofuel production. Unlike lignin, the cellulose and hemicellulose are comparatively easier to degrade. The cellulolytic bacteria for example *Cellulomonas fimi* and *Thermomonospora fusca* have been extensively studied for cellulase production. Similarly, cellulolytic bacteria, *Paenibacillus campinasensis* can survive in harsh conditions and has good potential for the pretreatment of

lignocellulosic biomass (Maki et al. 2009). There are at least 30 predominant rumen cellulolytic bacterial species (for example *F. succinogenes, R. flavefaciens,* and *R. albus,* etc.) which have a specific mechanism of adhesion to cellulose and its hydrolysis (Miron et al. 2001). Although there are many cellulolytic anaerobic bacteria such as *Clostridium thermocellum* and *Bacteroides cellulosolvens* that produce high cellulase activity, they do not secret enough enzymatic concentration (Duff and Murray 1996). However, anaerobic bacteria like *Zymomonas mobilis* is a notable cellulolytic candidate and can be used in fermentation of sucrose, glucose and fructose to give high yield of ethanol (Dien et al. 2003). The Gram-positive *Bacillus* strains *Firmicutes* and the Gram-negative strains *Pseudomonas*, *Rahnella* and *Buttiauxella* produce cellulase that shows highest activities in degrading the cellulosic materials (Paudel and Qin 2015). Some bacterial strains such as *Azospirillum lipoferum*, and *Bacillus subtilis* have been reported to produce bacterial laccases thereby causing depolymerization of lignin (Saritha et al. 2012). Although, the microbial degradation of lignin has been well studied in fungi and very less studied in bacteria, the scientific communities have shown their comprehensive interest in bacterial lignin degradation (Bandounas et al. 2011; Palamuru et al. 2015; De Gonzalo et al. 2016) because of recently discovered bacterial peroxidases (van Bloois et al. 2010), laccases (Chandra and Chowdhary 2015) and β-etherases (Picart et al. 2015) which can be used effectively in delignification.

5.2. Fungal pretreatment

Fungi are well known microbes for their interactive effect on decaying lignocellulosic residue by their enzymes. These fungi are widely distributed in nature, most of which produces various cellulolytic (Mandels and Reese 1960; Sukumaran et al. 2005; Ljungdahl 2008), hemicellulolytic (Ljungdahl 2008) and ligninolytic enzymes (Arantes et al. 2007; Shary et al. 2008). The

lignocellulolytic fungi include species from the ascomycetes (e.g. *Aspergillus sp., Penicillium sp., T. reesei*), basidiomycetes including white-rot fungi (e.g. *Schizophyllum sp., P. chrysosporium*), brown-rot fungi (e.g. *Fomitopsis palustris*) and few anaerobic species (e.g. *Orpinomyces sp.*) (Dashtban et al. 2009; Paudel and Qin 2015). However, the highly impermeable, resistance and recalcitrance nature of lignin; and insoluble and crystalline nature of cellulose represents a formidable challenge for enzymatic hydrolysis. The early report on *Trichoderma reesei* showed that it produces considerable amounts of xylanases and β-glucosidase with high cellulase activities (Tangnu et al. 1981). Similarly, an extensively studied soil fungus *Trichoderma longibranchiatum* is one of the promising species in solubilization of crystalline cellulose because it secrets three types of cellulases: endoglucanases (e.g. carboxymethyl cellulases), exoglucanases (e.g. cellobiohydrolases), and β-glucosidases (e.g. cellobiases). These different cellulases and substrates have their complex interactions that function in a synergistic manner (Zhou and Ingram 2000; Pérez et al. 2002; Béguin and Aubert 1994; Nidetzky et al. 1996) during hydrolysis. The lignin on the other hand has its complex intricate pathway of delignification and becoming a major hurdle to understand and selecting the efficient fungal strain. The white rot fungi (like basidiomycetes) however have its significant role in disintegration of lignin and considered as a natural lignin degrading microorganism. They depolymerize and mineralize lignin because they secrete range of ligninolytic enzymes like laccases, lignin peroxidases and manganese peroxidases (Millati et al. 2011; Bandounas et al. 2011; Guillén et al. 2005). Otjen et al. (1987) isolated 30 different wood decaying white rot fungi for lignin degradation and among these the best delignifiers reported so far were *Phellinus pini-2*, *Pholiota mutabilis*, *Phlebia brevispora-1* and *Phanerochaete chrysosporium*. However, the challenge of selecting fungal strain that effectively degrade the

lignin with simultaneous cellulose recovery is persisted, and no breakthrough yet on its commercialized application.

5.3. Other macro-organism pretreatment

Besides bacteria and fungi there are several other macroorganisms such as insects, worms, gastropods and ruminant animals which has strong ability to degrade lignocellulose. These macroorganisms are built up with some physiological mechanisms for breakdown of cellulosic biomass either by mechanical, enzymatic, gut flora and/or combination of these. These organisms have their own specific feeding/masticating mechanism for physical breakdown and different enzymatic components for efficient digestion of cellulose. There are diverse taxonomic groups of insects (more than 20 families representing 10 orders) such as termites (Isoptera), beetles (Coleoptera), wood wasps (Hymenoptera), crickets (Orthoptera), silverfish (Thysanura) etc., which are known to digest cellulosic biomass such as wood, leaf litters and forage (Sun et al. 2014). The earthworms are well known for their detritus feeding behavior. Many epizoic composting earthworms, such as *Eisenia fetida*, *Perionyx excavates, Lumbricus rubellus, etc.* can efficiently digest the organic matters (Pathma and Sakthivel 2012). The enzymatic action within the gut of earthworm accompanied by activities of microbial flora have potential in the digestion of cellulose, sugars, chitin, lignin, starch etc. (Zhang et al. 2000)(Vivas et al. 2009). Thus, the worm tea (i.e. the liquid leachate of vermicomposting) has been used as an alternative of acid pretreatment. Worm tea is considered as a microbial consortium and thus being used in biofuel production by enzymatic hydrolysis and fermentation (Siti Norfariha et al. 2013). Similarly, the microfloral consortium of gastropods and ruminant mammals also has significant role in cellulose digestion. Several studies have been carried out in microbial isolation of intestinal flora, their

application in biological pretreatment of lignocellulose and bioproducts production (Russell et al. 2009; Fondevila and Dehority 1994; Weimer et al. 2015).

7. Factors affecting in bioconversion

There are several physical factors (such as temperature, moisture, incubation time, aeration, substrate size, accessible surface area etc.), chemical factors (such as pH, composition of culture media, source of carbon, source of nitrogen, cellulose crystallinity, inorganic and organic compounds, roles of enzymes and hydrolysates, etc.) and biological factors (such as species of microorganism, consortia of microorganisms, their interaction and competition etc.). These factors affect the rate of biomass degradation and play a key role in changing physiochemical structure of lignocellulosic biomass.

7.1. Temperature

The effect of temperature on microbial growth and their enzyme activities greatly varies with the different species. It is natural to produce considerable amount of heat due to some metabolic activities of microbes during fermentation. Many bacteria and fungi can grow in large spectrum of temperature gradient. Depending on their temperature preference, microorganisms are classified into three major groups: psychrophiles (–15 to 10 °C), mesophiles (20 to 45 °C) and thermophiles (41 to 122 °C). Bacteria can grow in wider range of temperature from 4 to 60 °C. The mesophilic fungi and bacteria are the most common (Dix and Webster 1995) and most studied microbes of which their optimum temperature ranges from 25 to 40 °C. Many pathogenic bacteria prefer to grow in optimum temperature of 37 °C and on the other hand most thermophiles cannot grow

below 45°C. Similarly, some of the white rot ascomycetes grow in 39 °C whereas the basidiomycetes grow in 25 to 30 °C (Sindhu et al. 2016).

7.2. Moisture

The moisture content play a significant role in establishment of microbial growth, required for degradation of lignocellulose which greatly varies with types of substrate and microorganism involved in the pretreatment process (Sindhu et al. 2016). Many bacteria and fungi prefer to grow in optimum moisture content ranges from 40 to 70% on solid substrates (Raimbault 1998; Raghavarao et al. 2003). It has been observed that the optimum moisture of 40% and 80% were suitable for *Aspergillus niger* on rice and coffee pulp respectively (Raimbault 1998). The fungal strain, *Daedalea flavida* MTCC 145 on the other hand has highest cellulose and lignin degradation due to low particle size and high moisture content (85% moisture) in solid-state fermentation (Meehnian et al. 2016). Similar high optimum moisture level of 84% was recorded on white rot fungi *Phlebia brevispora* during pretreatment (Saha et al. 2017). Generally, the single cell microorganism requires free water for their propagation. However, very high moisture level creates anaerobiosis and very low moisture content results in delayed microbial growth (Raghavarao et al. 2003).

7.3. Incubation time

The recalcitrant nature of lignocellulose is the major limiting factor in biological pretreatment which require relatively a longer incubation time for efficient delignification than other physio-chemical methods (Sindhu et al. 2016; Zhong et al. 2011). It greatly varies with the biomass types and microorganisms involved in pretreatment process. The pretreatment of grass with *P.*

chrysosporium showed significant degradation of lignin and exposing greater amount of cellulose and hemicellulose in third week of incubation time (Liong et al. 2012). A satisfactory cellulose yield (64.3%) was obtained in 60 days' pretreatment of corn stalk with *Irpex lacteus* (Zhong et al. 2011). However, the prolonged incubation period can not only degrade the lignin but also greatly reduce the amount of polysaccharide. Thus, effective enzymatic hydrolysis for higher yield of sugars and ethanol is desirable and can be achieve by optimization of incubation time.

7.4. Substrate size and aeration

The particle size of substrate and oxygenation play a vital role in biological pretreatment of lignocellulose. The surface area of lignocellulosic biomass comprises of external surface area, depends on particle shape and size; and internal surface area, depends on capillary structure of cellulosic fibers (Maurya et al. 2015). Mechanical reduction in particle size of lignocellulosic substrate increases the surface area thus increases the hydrolytic activity of various enzymes. The larger particle size limits fungal penetration and low diffusion of air whereas very low inter-particle space in smaller substrate decrease the aeration which hinders the growth and metabolism of microorganism (Sindhu et al. 2016; Meehnian et al. 2016; Bhargav et al. 2008). Study on particle size of cotton stalk reviled that the *D. flavida* MTCC 145 have higher lignin degradation with lower cellulose loss when particle size was 5 mm (Meehnian et al. 2016). Increase in aeration not only provide enough oxygen but also support in CO_2 removal, heat dissipation and maintenance of humidity (Millati et al. 2011). Thus, appropriate substrate size and high aeration are essential for enzyme production and better hydrolytic activity.

7.5. pH

The pH of culture medium has significant role in growth and metabolic activities of microorganisms. In most of the cases the pH value is generally drop after few days of microbial incubation (Marra et al. 2015), which directly influence in production of lignolytic enzymes (Millati et al. 2011; Sindhu et al. 2016). In *Acinetobacter sp.* the pH decreased from 7.0 to less than 4.0 after 10 days of incubation (Marra et al. 2015). Most of the white rot fungi preferred slightly acidic (pH 4 to 5) environment for their better growth (Reid 1989; Agosin and Odier 1985). It has been observed that the more ligninolytic the fungus (*V. effuscata* and *Dichomitus squalens*), much lower the pH with higher enzyme activity (Agosin and Odier 1985). However, both decrease and increase in level of optimum pH during pretreatment result in low enzyme activity. The low pH inhibited the cellulases activity and in higher pH the enzymes will dissolve and lost their activity (Geiger et al. 1998).

7.6. Structural complexity

The lignocellulosic biomass has structural complexity due to cellulose crystallinity, cellulose sheathing by hemicellulose and complex phenylpropanoid unit of lignin. This structural complexity in plant cell wall results in recalcitrant biomass which is resistant to enzymatic and microbial deconstruction (Himmel et al. 2007). Cellulose has strong inclination to form inter and intra-molecular hydrogen bonds between the cellulosic chains (Mansfield et al. 1999; Rahikainen 2013) that foster its accretion into two forms of crystalline structure called Iα and Iβ (Atalla and VanderHart 1984; VanderHart and Atalla 1984). Lignin on the other hand is most recalcitrant biopolymer, insoluble in water and composed of very complex network of non-fermentable phenylpropanoid units. Nonproductive binding of cellulolytic enzymes onto lignin together with

protective covering of lignin and cellulose sheathing by hemicellulose act as a physical barrier for cellulase to reach the cellulose which inhibit the hydrolysis of lignocellulose (Mansfield et al. 1999; Rahikainen 2013). Thus, several studies have been concentrated on to remove the lignin and to decrease the cellulose crystallinity by different pretreatment methods for maximising the enzymatic digestibility. Significant amount of highly efficient lignolytic enzymes are required for their synergistic effect to yield maximum monomeric sugars from cellulose and hemicellulose fractions of lignocellulosic biomass.

7.7. Loss of polysaccharides

The major limiting factor of biological pretreatment is slow process accompanied by loss of polysaccharide (Millati et al. 2011; Narayanaswamy et al. 2013). Considerable amount of cellulose and hemicellulose are consumed during the pretreatment process. Some of the white-rot fungi such as *P. chrysosporium, C. subvermispora, Echinodontium taxodii* 2538*, Trametes ochracea, Irpex lacteus* etc. are known to degrade the lignin but also have increased risk of loss of sugars from cellulose and hemicellulose (Narayanaswamy et al. 2013). The cellulolytic enzymes secreted by white-rot fungi are used to digest the cellulose for its own growth which result in low sugar production after enzymatic saccharification (Meehnian et al. 2016). However, selection of efficient strain and optimization of culture condition can minimize the pretreatment time and sugar loss. Moreover, the technique of genetic manipulation and altering the ligninolytic or cellulolytic enzyme for efficient lignin degradation and low carbohydrate loss still need further improvement.

7.8. Microbial co-culture and adaptation

A maximum enzyme activity during pretreatment is highly desirable to everyone. But, it is not always possible to produce all the lignocellulolytic enzymatic components from a single strain of any bacterium or fungus due to their limiting levels of one or the other enzymes. Despite having complexity to grow in the same culture medium several attempts have been made on co-culture of different species to understand the microbial world of communication, their secretions, adaptation and possible application on pretreatment. The ubiquitous nature of microbes and their ability to break the recalcitrant bonding of biopolymers have better functions by balancing two or more tasks in mixed populations which could otherwise become difficult for individual strains (Brenner et al. 2008). However, finding suitable microbes for co-culture is a daunting task because of their different genetic makeup, enzymatic components and ecological niche. The ecological and adaptational factors could also play a significant role in metabolic function of microorganism. It is because the microbial communities living in natural habitat can degrade the lignocellulosic components within their intricate network of food web where the whole consortia play a vital role. Three possible pretreatment combinations for bacterial and fungal cultures could be as follow.

7.8.1. Bacterial co-culture***:*** Culture of two or more species of bacteria for efficient enzymatic hydrolysis is useful in pretreatment of lignocellulosic biomass and help in formation of biofuel and value added products. There are many bacteria belonging to *Clostridium, Cellulomonas, Bacillus, Thermomonospora, Ruminococcus and Streptomyces* that can produce various cellulases enzymes (Sun and Cheng 2002) secreted by dissimilar organisms worked together in cellulose hydrolysis (Zhou and Ingram 2000). Similarly, improved enzyme levels were also achieved by Chandra et al. (2007) when bacterial strains *Paenibacillus sp*., *A. aeurinilyticus*, and *Bacillus sp*. were cultured together that showed their high potential over the pure strains. High cellulose degradation was also observed by Kato et al. (Kato et al. 2004) in mixed culture of *C.*

straminisolvens and the three strains of aerobic isolates compared to that of the original microflora. Several attempts have been carried out in mixed culture of rumen bacteria (Russell et al. 2009; Fondevila and Dehority 1994; Weimer et al. 2015; Kato et al. 2004) for possible high enzymatic activities with coexistence and to find out their network relationship (Kato et al. 2008) so as to improve hydrolysis of lignocellulogic biomass. Moreover, the study on bacterial co-culture of *Clostridium thermocellum* with other closely related thermophilic Clostridia has shown its significant role in hydrolysis of cellulose and hemicellulose and finally converts the sugars into biofuels, the ethanol (Maki et al. 2009).

7.8.2. Fungal co-culture*:* Application of two or more species of fungi in biological pretreatment of lignocellulose has been in practiced from few decades. The fungal degradation in monoculture and co-culture is complex phenomenon and their metabolic interaction is not well understood (Salimi and Mahadevan 2013). Almost none of the fungi can produce significant amount of enzymes for hydrolysis at a same time (Dashtban et al. 2009). However, enzymes production in co-culture sometime gets better output of enzymatic composition. For example, in separate experiment on *Trichoderma reesei* and *Aspergillus phoenicis* by Wen et al. (Wen et al. 2005) showed interesting opposite level of cellulolytic enzymes sectertion: *T. reesei* produced high level cellulase, but low β-glucosidase whereas *A. phoenicis* produced low level cellulase and high β-glucosidase. On the contrary, the mixed culture of two fungi *T. reesei* with *A. phoenicis* at their optimum temperature 27 ºC and pH 5.5 resulted in a high level of total cellulase and β-glucosidase production and thus showed higher enzymatic activities (Wen et al. 2005; Madamwar and Patel 1992) probably because of high nutrient level in the substrate (Wen et al. 2005). There were multiple evidences of improved cellulolytic and hemicellulolytic activities in fungal co-culture (Salimi and Mahadevan 2013; Maheshwari et al. 1994; Ahamed and Vermette 2008; Duenas et al. 1995). Furthermore,

large amount of lignin degradation has also been reported so far by Chi et al. (2007) in co-culture of *C. subvermispora* and *P. ostreatus*, than compared to monocultures.

7.8.3. Bacterial and fungal co-culture***:*** This is a relatively new avenue of microbial co-culture of bacteria and fungi with the aim of producing continue enzymatic activities from a dynamic consortium. The main idea of these microbial consortia came from nature where different microorganisms live together, communicate each other and participate in interconnected network of food web within a microbial community. A study on four strains of white rot fungi (including *Dichomitus squalens*, *Ganoderma applanatum*, and two strains of *Pleurotus sp*.) on milled straw with addition of non-sterile soil containing soil microbes revealed that the laccase and manganese peroxidase production of *Pleurotus sp*. was not affected by soil microbiota and also showed high enzymatic activity in nonsterile soil (Lang et al. 1997). It can be compared with natural biodegradation, where the non-sterile soil contains various bacteria that interact synergistically with fungal degradation of lignocellulose result in high and fast enzymatic activities (Mikesková et al. 2012). Here in pretreatment of lignocellulosic biomass the fungi opened up the recalcitrant bonding of lignocellulose, hydrolyze the cellulose and hemicellulose into soluble saccharides, and the bacteria convert it into valued products. The study on bacterial and fungal co-culture has resulted in formation of different products like isobutanol using *Trichoderma reesei and Escherichia coli (Minty et al. 2013)* and ethanol from co-culture of *Z. mobilis* and *P. stipitis* (Fu et al. 2009). Similarly, Golias et al. (2002) observed high cellulase activity in co-culture of recombinant *K. oxytoca* P2 with *K. marxianus, S. pastorianus* or *Z. mobilis* and produced more ethanol in faster rate compared to pure culture. Since, there is higher enzyme production from bacterial and fungal co-culture and thus it is likely a better alternative for efficient breakdown of lignocellulosic residue (Kamsani et al. 2016).

8. Whole cell immobilization and industrial applications

Immobilization is a physical confinement or entrapment of cells in a distinct support/matrix with the preservation of activity (Karel et al. 1985). The immobilized cell system consist of three components: the cells, support material and interstitial space occupied by the fluid which collectively form a micro-environment (Willaert and Baron 1996). Based on physical mechanism of cell localisation and the nature of the support mechanisms, Karel et al. (1985) classified four types of immobilized cell system: attachment to a surface, entrapment within a porous matrix, containment behind a barrier and self aggregation. Now a day, the immobilization techniques have been classified as adsorption, crosslinking, covalent bonding, entrapment and encapsulation. However, the schematics presentation by Jose and Claudino (2007) gave a clear understanding on immobilization methods (Figure 4). Different supporting matrices have been used to immobilize the cells or enzymes, some of which are listed below (Table 4).

Table 4. Cell or enzyme immobilization and supporting matrices.

Types of immobilization	Supporting materials
Adsorption	Gelatin, porous glass, cotton fiber, cellulose
Crosslinking	Glutaraldehyde, diazonium salt
Covalent bonding	Titanium oxide, cellulose + cyanuric chloride, Amino group, hydroxyl group, carboxyl group, etc.
Entrapment	Agar, polyacrylamide gel, calcium alginate, aluminum alginate
Encapsulation	Polyester, alginate polylysine, nitro cellulose

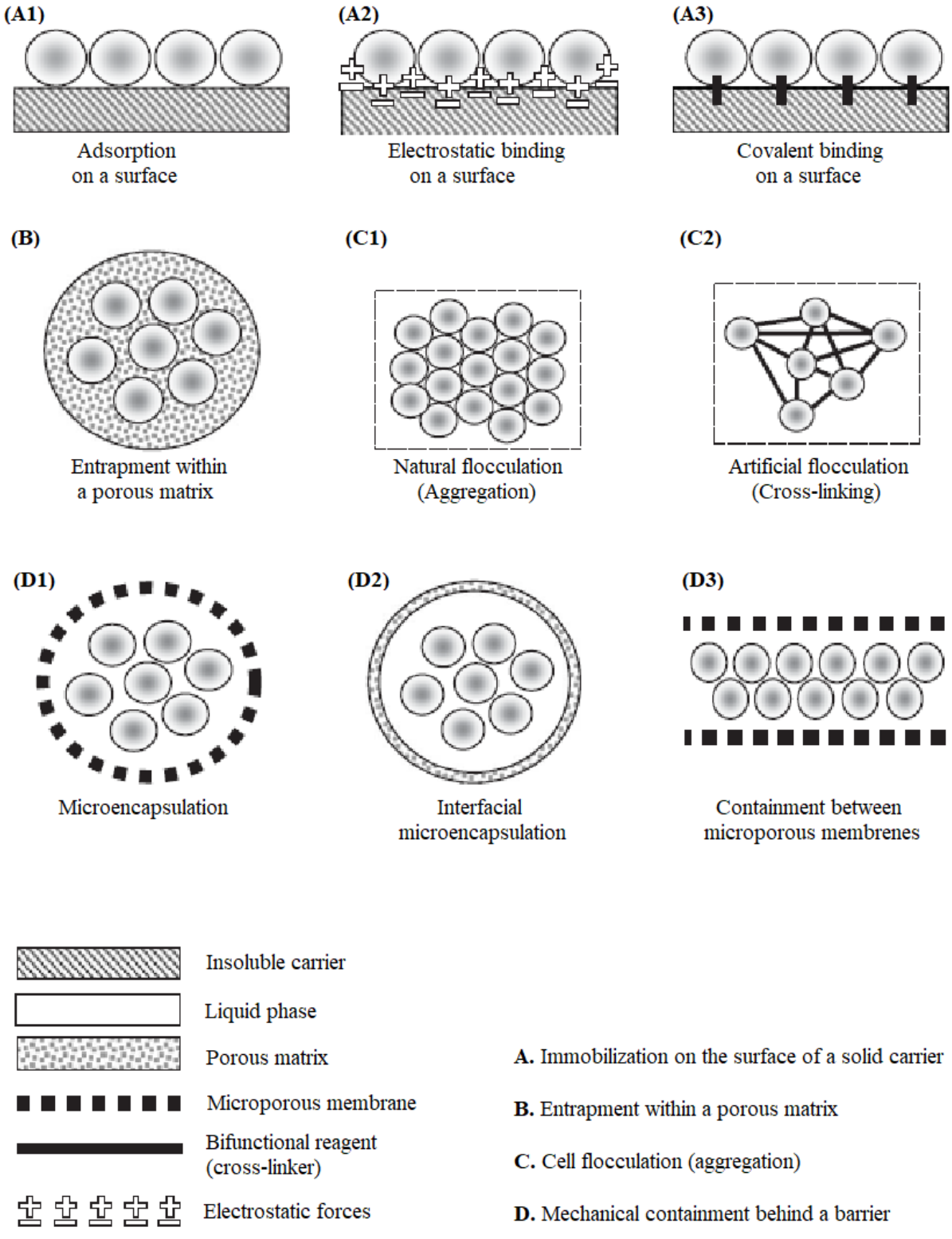

Fig 4. Schematics representation of cell immobilization methods (Jose and Claudino 2007).

The immobilization is a promising technique, has wider industrial application in different sectors including pharmaceutical, bioprocessing, biofuel, bio-refinery, food and beverage etc. (Elakkiya et al. 2007). The first scientific observation and discovery of immobilized enzymes date back to 1916 which further modified and developed to the contemporary enzyme immobilization techniques. Now, it is commercially well-established method with over 5,000 scholarly publications and patents have been made on enzyme immobilization techniques (Homaei et al. 2013). It enhances the stability of the enzyme and retain the natural catalytic activity of enzymes (Elakkiya et al. 2007; Tampion and Tampion 1987). The immobilized cells on the other hand can reuse into successive batches. The technique requires less labour input, eliminate expensive steps of isolation and purification, and thus can save the capital investment.

9. Rationale of research

Although the bacterial isolation from various sources and their characterization is a conventional technique, it is very important in terms of exploring efficient strain. In many circumstances, high enzyme yielding, robust bacterial strains, which are vigorously active at wider temperature and pH fluctuation always showed their high demand in the industries. These strains can give higher yield of enzymes for the degradation of plant biomass and various polysaccharides. Such industrial microorganisms play vital role in commercial production of enzymes such as cellulase and GI. The cellulase hydrolyze the cellulose to form glucose and the GI help in isomerization of glucose to fructose. This single step bioconversion of cellulose to form glucose and final isomerization into fructose has wider industrial application because of environmental benefit and cost-effective approach. These enzymes have huge global market due to their tremendous potential applications in food, agriculture, pulp and paper, textile and biofuels industries. It allows a progression towards

renewable fuels. However, finding efficient bacterial strains, optimization of enzyme production, selective co-culture and whole cell immobilization are the major bottle neck.

It is therefore very important to screen novel bacterial strains, optimize their enzyme production and analyze the bioconversion process. Such studies are crucial because it will not only add new bacterial repertoire with additional gene of interest but also helpful to the biofuels industries to produce various enzymes and industrial platform chemicals. Similarly, the enzymes produced by an isolated strain may be more efficient and may further improve enzymatic bioconversion. Thus, the study was mainly focused on to isolate bacteria that can produce cellulase and xylose isomerase for their possible industrial application.

10. Research objectives

The study was mainly focus on to isolate the bacterial strains capable of producing cellulase and xylose/glucose isomerase (GI) enzymes. We explored the optimum conditions for co-culture of efficient strains to produce high cellulase activities and immobilized the bacteria for improved GI activity. The low cost lignocellulosic biomass was also used as a substrate for efficient microbial degradation and enzyme production during batch fermentation. Following are the overall objectives of our research.

1. Isolation and characterization of cellulase producing bacteria.
2. Isolation and characterization of GI producing bacteria.
3. Optimization of various physio-chemical parameters to improve the enzymes production.
4. Co-culture of efficient bacterial strains for higher yield of cellulase.
5. Whole cell immobilization of bacterial strain for improved GI production.

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CHAPTER II

Cellulolytic activities of novel *Bacillus* sp. isolated from municipal sludge and gut of red wiggler worm (*Eisenia fetida*)

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Abstract

The new cellulolytic bacteria were isolated from various sources. The strain EF2 was isolated from intestine of *Eisenia fetida* and OW1-1 from municipal organic waste. The qualitative screening of six strains in carboxymethyl cellulose (CMC) agar plate showed a larger zone of clearance with Gram's iodine staining. The 16S rRNA gene sequences of the strains were uploaded into the NCBI database. Both the strains EF2 and OW1-1 were identified as a gram-positive *Bacillus* sp. Their CMCase activities reached to the significantly high of 35.307 ± 0.08 IU/ml and 29.92 ± 0.01 IU/ml, respectively, in EF2 and OW1-1 when 2.5% (w/v) of lactose was used as a carbon source at their respective optimal pH and temperature. The co-culture of *Bacillus* sp. strains EF2 and OW1-1 in contrast to their monoculture, showed 15% and 35.71% increase in CMCase activity respectively. The molecular weight of CMCase was 60 kDa in both strains. Since the strains EF2 and OW1-1showed higher CMCase activity in a wider range of temperatures and pH fluctuation, which could be a better choice for biofuel industry.

Keywords: CMCase activity, *Bacillus*, characterisation, co-culture

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1. Introduction

The biofuels such as bioethanol, biodiesel, biogas, etc. from lignocellulosic biomass are gaining popularity because it is considered as a sustainable, cost efficient, ecofriendly and showed a promising alternative to fossil fuels. The biofuels are renewable energy, has low emission of greenhouse gases and can mitigate the environmental challenges (Medipally et al. 2015; Singh Nigam and Singh 2011), thus able to make a global priority. However, the main hurdles lie in the recalcitrance of its bonding due to protective covering of lignin and crystalline nature of cellulose sheathed by hemicellulose, allowing very less surface area for enzymatic hydrolysis (Panwar et al. 2011; Sharma et al. 2017; Horn et al. 2012; Zhang et al. 2007). Moreover, the competing land uses vs demand and supply of non-food biomass (Harvey and Pilgrim 2011), requirement of multi-disciplinary teams of skilled personals, industrial infancy (FitzPatrick et al. 2010; Zhu 2015), financial investment and fuels market, etc. in many circumstances are some other bottle neck in higher yield of efficient biofuels. Although, there are several physical, and chemical methods available for disruption of lignocellulosic layers, the biological method has its financial and environmental benefits (Sharma et al. 2017).

Since, cellulose is the most abundant biopolymer found in earth, it has been highly studied in production of biofuels and bioproducts. The bioconversion of cellulosic biomass using cellulase secreted from various bacteria and fungi has its greater industrial significance. There are three major enzymatic components of cellulase: endoglucanase, exoglucanases and β-glycosidase; belonging to the glycosyl hydrolase (GH) family. The synergistic activity of these enzymes disintegrates the glyosidic bonds of cellulose. The endo- and exo-glucanases act on the chain ends of cellulose to release cellobiose and some sugar molecules whereas the β-glucosidases is essential

to break cellobiose into sugars (Maki et al. 2009; Dashtban et al. 2010). The β-glucosidase has major role in minimizing the inhibitory effect of cellodextrin and cellobiose by cleaving the final glycosidic bonds of cellobiose releasing sufficient glucose molecules which in alcoholic fermentation, produce biofuels and value-added products.

This synergism of enzymes can easily observe in the natural environment where the whole microbial consortium secret various lignocellulose degrading enzymes that works synergistically in degrading plant biomass (Hatakka and Hammel 2011) and playing a vital role in regulating the carbon cycle (Lindahl et al. 2002). Thus, it is obvious that mixed populations of microbes with individually optimized populations has ability to break the recalcitrant biopolymers by synergistic action of multiple enzymes which could otherwise become difficult for individual strains (Brenner et al. 2008). Many efforts have been made in genetic engineering (Druzhinina and Kubicek 2017; Chambergo et al. 2002; Chen et al. 2009; Ellilä et al. 2017) to produce robust microorganisms that can survive in extreme condition and continue their higher yield of cellulolytic enzymatic components. However, it is not always possible to produce significant yield of enzymes from single strain of any bacterium. Thus, the culture of two or more species of bacteria in many circumstances is beneficial for efficient enzymatic hydrolysis because they relies in metabolic cross-feeding (Pande et al. 2015; Jia et al. 2016) and produce various cellulase which synergistically work together in cellulose hydrolysis (Zhou and Ingram 2000). The strains in co-culture can adapt to the minor fluctuation in culture conditions and degrade the substrate within their intricate network where the whole consortia work together to get the improved enzyme levels in comparison to their pure strains.

The cellulose degrading microbes are found in various places including organic matter decaying sites, on the soil and in the gut of some animals such as insects, earthworm, gastropods, ruminant etc. Several studies have been conducted in isolation of efficient cellulolytic bacterial strain from different natural resources. However, the industrially important, high cellulase yielding strains vigorously active at higher temperature and pH fluctuation are still in demand. Nevertheless, the conventional technique of microbial isolation and screening for new cellulase degrading strain still has its greater significance due to likeliness of getting good strain with additional gene of interest. Thus, it is very essential and important to isolate the high cellulase degrading bacteria for their possible industrial application. This study mainly focused on isolation of cellulolytic bacteria from various sources, optimize the enzyme production from efficient bacteria, co-culture them for higher yield of cellulolytic components and compare their enzyme activity in an optimum condition.

2. Materials and methods

2.1. Media preparation

The solid agar and broth media were prepared for bacterial growth. The composition of culture media was as follow.

Luria-Bertani (LB) broth: 1.0 g peptone, 0.5 g yeast extract, 0.5 g NaCl and distill water up to 100 ml.

LB agar: 1.0 g peptone, 0.5 g yeast extract, 0.5 g NaCl, 1.5 g agar and distill water up to 100 ml.

Carboxymethyl cellulose (CMC) agar: 0.5 g CMC, 0.1 g $NaNO_3$, 0.1 g K_2HPO_4, 0.1 g KCl, 0.05 g $MgSO_4$, 0.05 g yeast extract, 1.15 g agar and distill water up to 100 ml.

Minimal salt medium: 1% (w/v) CMC and 0.5% (w/v) yeast extract in 0.1 g $NaNO_3$, 0.1 g K_2HPO_4, 0.1 g KCl, 0.05 g $MgSO_4$ and distill water up to 1000 ml.

2.2. Bacterial isolation and characterization

Samples were collected from soil, rotten wood, paper mill sludge, organic waste, wastewater and gut of earthworm (*Eisenia fetida*). After 10× serial dilution, 200 µl of each sample was inoculated in LB agar plate and incubated at 37 °C for 24 h. The bacterial colonies with morphological and physiological difference developed after 24 h were isolated and transferred into LB broth. The pure bacterial colonies were selected after repetitive streaking followed by isolation and re-culture in LB broth at 37 °C for 24 h.

The hallo (zone of clearance) was measured for qualitative comparison of relative carboxymethyl cellulase (CMCase) activity using Gram's iodine plate assay technique (Kasana et al. 2008). The DNA of some efficient bacterial strains with larger halo was extracted using bacterial genomic DNA isolation kit (Norgen Biotek Corp. ON, Canada). The universal forward primer HAD-1 (5′-GACTCCTACGGGAGGCAGCAGT) and reverse primer E1115R (5′-AGGGTTGCGCTCG TTGCGGG) were used in the reaction. The 16S rRNA was amplified using polymerase chain reaction (PCR) followed the manufacturer's instructions (FroggaBio protocol). Briefly, the PCR thermal cycle was adjusted as follows: initial denaturation at 94 °C for 3 min, 30 successive amplification cycles (denaturation: 94 °C for 30 s, annealing: 56 °C for 30 s and extension: 72 °C for 1 min) and final extension at 72 °C for 10 min. The DNA was purified using Gel/PCR DNA fragments extraction kit (Geneaid, FroggaBio). The purified 16S rRNA samples were sent to sequencing lab. The sequence results were uploaded into the National Center for Biotechnology

Information (NCBI) database and the phylogeny was evaluated by multiple alignment of sequences in phylogeny.fr, a web-based tree view software (Dereeper et al. 2008).

2.3. Preparation of seed culture and size of inoculum

The seed culture for quantitative assay was prepared from stock culture in agar plate by transferring strains into the tube containing 5 ml LB broth using inoculation loop. The LB broth seed culture was incubated in shaking incubator at 37 °C and 200 rpm for 24 h. The proportion of inoculum size was maintained at 1:50 ratio where 1 ml LB seed culture was transferred into a 250 ml Erlenmeyer flask containing 50 ml culture medium in each batch fermentation.

2.4. Qualitative cellulase assay

The overnight cultured bacterial strains in LB broth (5 µl) was inoculated at the centre of CMC agar plate (containing 0.5 g CMC, 0.1 g $NaNO_3$, 0.1 g K_2HPO_4, 0.1 g KCl, 0.05 g $MgSO_4$, 0.05 g yeast extract, 1.15 g agar and distill water up to 100 ml) and kept in incubator at 37 °C for 48 h. Qualitative screening of cellulolytic bacterial strain was performed with Gram's iodine test (Kasana et al. 2008). The CMC plate was filled with 3% (w/v) Gram's iodine solution and observed the zone of clearance after 2 min. The cellulase produced from bacteria degrade the cellulosic content of agar plate into some monosaccharides and disaccharides which give zone of clearance (halo) with the iodine solution (Gohel et al. 2014). The halo measurement for control was compared with *E. coli* (-ve control) and cellulase from *T. reesei* (+ve control). The halo size was recorded and the strains with larger halo were further analysed to quantify their cellulase activities.

2.5. Quantitative cellulase assay

1 ml of overnight cultured LB broth seed culture was transferred into a 250 ml flask. Each flask containing 50 ml minimal salt medium with 1% CMC as a substrate was used for bacterial growth and enzyme production, keeping a constant agitation of 200 rpm throughout the experiment. The physiochemical parameters (including temperature, pH, carbon and nitrogen sources) were optimized by considering one parameter at a time. The culture broth of 1 ml was harvested and centrifuged at 12,000 × g for 3 min. The supernatant was collected for extracellular crude enzyme and analyzed the reducing sugars yield by 3,5- dinitro-salicylic acid (DNS) method (Miller 1959) with some modification. Briefly, the reaction mixture containing aliquot (50 µl) of crude enzyme from supernatant and 50 µl of 0.5 M citrate buffer (pH 6.0) with 0.5% CMC were transferred into a 1 ml microcentrifuge tube. The reaction mixture was incubated in hot water bath at 55 °C for 30 min. The DNS solution of 200 µl was added to the reaction mixture and the tube was kept in boiling water bath for 5 min. The reducing sugars liberated in reaction mixture were estimated by using glucose standard curve. The absorbance was measured in room temperature at OD 540 nm in a micro-plate reader spectrophotometer (BioTek, USA). The bacterial growth was measured in term of biomass (absorbance at OD 600) whereas the enzyme activity was express in IU/ml. One unit of CMCase enzyme corresponds to the release of 1 µM of reducing sugar equivalent per minute from substrate (CMC).

2.6. Effect of temperature and incubation period on enzyme production

The 250 ml flask containing 50 ml of minimal salt medium (with 1% CMC) and 1 ml overnight cultured bacterial strain (seed culture) were incubated in shaking (200 rpm) incubator at 30, 35, 40, 45 and 50 °C for four days. The effect of temperature in cellulase production was quantified by DNS method by harvesting 1 ml of culture solution each day.

2.7. Effect of pH on enzyme production

The flasks containing 50 ml of minimal salt medium (with 1% CMC) and 1 ml overnight cultured bacterial strain (seed culture) were incubated in shaking (200 rpm) incubator in the pH ranges from 5 to 9 at an optimum degree of temperature. The effect of pH in cellulase production was evaluated at their optimum temperature and incubation time.

2.8. Effect of carbon sources on enzyme production

The effect of different carbon sources including CMC, D-glucose, D-fructose, D-sorbitol and D-lactose were evaluated with the estimation of cellulase activity by DNS method at their optimum temperature, pH and incubation time.

2.9. Effect of nitrogen sources on enzyme production

The effect of different nitrogen sources including peptone, urea, yeast extract and $(NH_4)_2SO_4$ were evaluated with the estimation of cellulase activity at their optimum temperature, pH and incubation time.

2.10. Effect of co-culture on enzyme production

The seed culture of both bacterial strains, EF2 and OW1-1 were prepared separately in LB broth. A total volume of 1 ml seed culture was maintained by transferring 500 µl of each overnight cultured strain into a 250 ml flask containing minimal salt medium with 1% (w/v) CMC. The enzyme activity was estimated in optimum condition by harvesting 1 ml of sample from the culture medium.

2.11. SDS-Polyacrylamide gel electrophoresis

The cellulase enzyme was separated in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The hydrolytic activity of cellulase was observed in zymogram. The presence of protein bands and hydrolysis bands in the gel was compared with standard protein marker (Bio Basic, Canada) to estimate the molecular weight of cellulase. The crude enzyme samples from strain EF2 and OW1-1 were run along with protein ladder (Bio Basic, Canada) in 10% of acrylamide gel. A constant supply of 120 V was maintained until the sample crossed the stacking gel, while the 160 V was maintained in the separating gel. The gel was cut into two parts, one of which was used to detect the protein while the other half used to perform the zymogram. One half of gel was immerged in Coomassie Brilliant Blue for 45 min and de-stained with decolouring buffer until the bands were prominent. Whereas the other half of gel was used to observe the hydrolytic activity after washed with 1% (v/v) Triton X-100. The gel was immerged in 0.5 M citrate buffer (pH 6.0) with 0.5% CMC and incubated in hot water bath (55 ºC) for 30 min followed by stained in 0.1 % Congo red for 30 min. 1 M sodium chloride solution was used to distain the gel and was treated with 4% (w/v) acetic acid solution to make a prominent hydrolytic band in zymogram analysis. The bands observed were compared with standard protein marker.

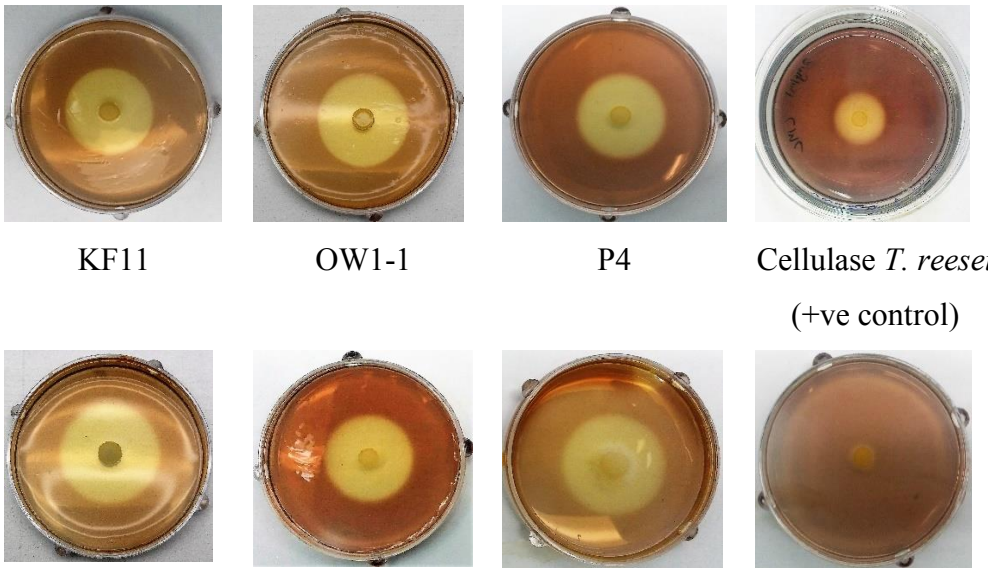
2.12. Statistical analysis

All the experimental data were obtained in the form of triplicates and results were expressed in terms of mean ± standard deviation (SD). The mean values of triplicates were analysed by one way analysis of variance (ANOVA) followed by two tailed T-test using corresponding confidence level of 95% (i.e. *P* value at less than 0.05). The multiple comparison among the different variables were made by the post-hoc Bonferroni correction of T-test values at less then *P*/n level of significance.

3. Results and discussion

3.1. Qualitative CMCase activity assay

Out of 26 isolates tested in Gram's iodine, 25 strains showed their positive results in plate assay with various sizes of halo. Only six of the strains with larger halos (figure 1) in CMC plates were selected for further characterization. The halo sizes were recorded (diameter shown in figure 2) and compared with *E. coli* and cellulase (dilution- 2 mg/ml) from *T. reesei* ATCC 26921 (Sigma Aldrich - C2730, Canada) for negative and positive controls respectively. The strains EF2 isolated from intestine of red wiggler worm (*Eisenia fetida*) and OW1-1 isolated from municipal organic waste sludge showed larger halo sizes and enzymatic index. Moreover, the strains EF2 and OW1-1 also showed a proximity in their phylogenic relationship (figure 2) thus, were selected for their further quantification of CMCase activity.



Enzymatic index:

KF11:	3.26
OW1-1:	3.46
P4:	2.97
P5-2:	3.16
EF1:	3.25
EF2:	3.26
T. reesei:	2.25
E. coli:	0

Fig 1. Plates having zone of clearance (halo) in Gram's iodine test for six cellulase producing isolates and their enzymatic index values were compared with *E. coli* as a negative control and cellulase (2 mg/ml) from *T. reesei* ATCC 26921 as a positive control.

3.2. DNA extraction and characterization of bacterial strains

Bacterial DNA of six strains with higher cellulase activity in the plate assay were isolated using bacterial genomic DNA isolation kit (Norgen Biotek Corp) and amplified its 16S rRNA using PCR (FroggaBio) following their protocols. The amplified DNA fragments were run in 1% agarose gel for validation of 16S rRNA. It showed the clear bands of about 800 bp. It was purified using Geneaid PCR/Gel purification kit and sent to Euroffins Genomics for sequencing. The nucleotide sequences were BLAST in NCBI database for possible identification of bacterial genus. The strains identified were gram +ve bacteria, five of them were *Bacillus* sp. and one of *Lysinibacillus* sp. with 97 to 99% homology. Both the strains EF2 and OW1-1 were identified as *Bacillus* sp. There sequences were submitted into the NCBI to get the accession numbers of each strain. The GenBank accession numbers of *Bacillus* sp. strains EF2 and OW1-1 were obtained as MG827113 and MG827116 respectively. The web-based phylogeny.fr software was used for making tree view (figure 2) of phylogeny which showed the strains EF2 and OW1-1 have proximity in phylogeny, and thus could be suitable for co-culture.

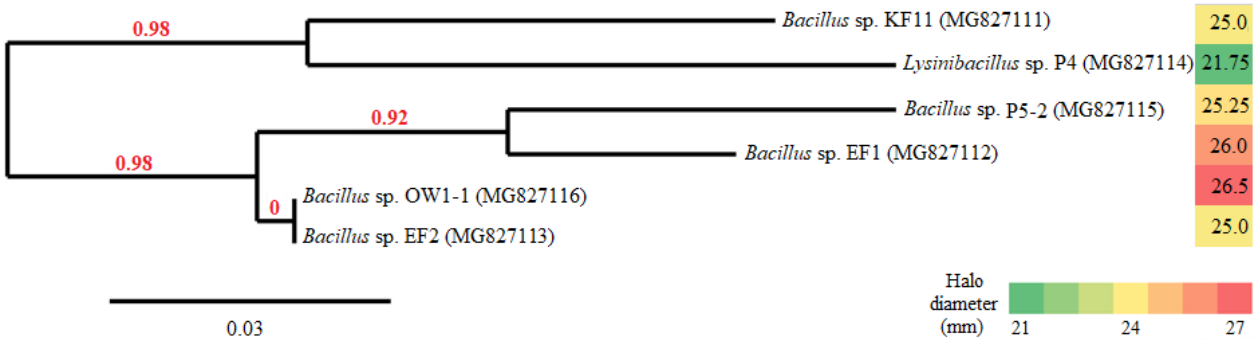


Fig 2. Phylogeny of bacterial strains made from multiple alignments of sequences in phylogeny.fr software. The red coloured number represents a branch support values, the accession numbers are given in the brackets. Colour scale on the right shows the size of halo (mm) in CMC agar plates.

3.3. Effect of incubation time on enzyme production

The strains EF2 and OW1-1 were separately cultured in 250 ml conical flask containing 50 ml minimal salt medium for four days. The bacterial culture of 1 ml was harvested every 12 h for four days to analyze the biomass and enzyme activity. The bacterial growth was measured in terms of biomass at OD 600 nm and the reducing sugars formed was estimated in OD 540 nm. The higher CMCase production was observed in 24 h which gradually decreased with increases in incubation time perhaps due to the nutrient depletion leading to physiological stress and thereby resulting in inactivation of enzymes secretory machinery (Nochur et al. 1993; Gautam et al. 2011). The activity from 24 h of incubation was significantly different than other incubation times with the $P<0.05$ for both strains. It was in harmony with the earlier finding where rapid increase in production of endoglucanase was recorded from 6 h, which reached a maximum at 24 h and steadily decrease thereafter (Seo et al. 2013).

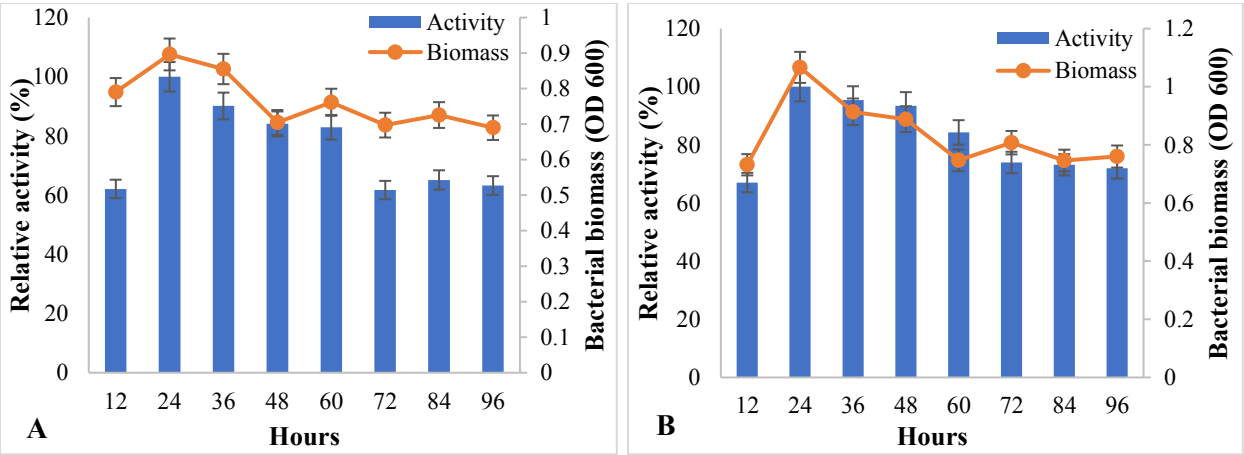


Fig 3. Effect of incubation time in biomass and CMCase activity by strain EF2 (A) and OW1-1 (B)

3.4. Effect of temperature and pH on enzyme production

The enzyme production was greatly influenced by temperature and pH. The different bacteria have their own optimum temperature. The strains were cultured in minimal salt medium at 30, 35, 40,

45 and 50 °C temperature. The strains have an ability to digest cellulose in wider range of temperature from 35 to 45 °C. These strains are mesophilic bacteria which preferred moderate temperature of 40 °C for maximum CMCase production. The mesophilic *Bacillus subtilis* and *Bacillus circulans* gave maximum yield of cellulase at 40 °C (Ray et al. 2007). Some other bacteria such as *Bacillus, Cellulomonas* and *Micrococcus* sp. showed their higher endoglucanase activity in 40 °C (Immanuel et al. 2006). The relative CMCase activity of EF2 and OW1-1 was calculated in comparison to their maximum observed activity. The CMCase production was high in 40 °C and its activity was significantly different than other temperatures with the $P<0.05$ for both strains. The activity is decreased with change in temperature perhaps due to inhibition of cellulase multienzyme complex system.

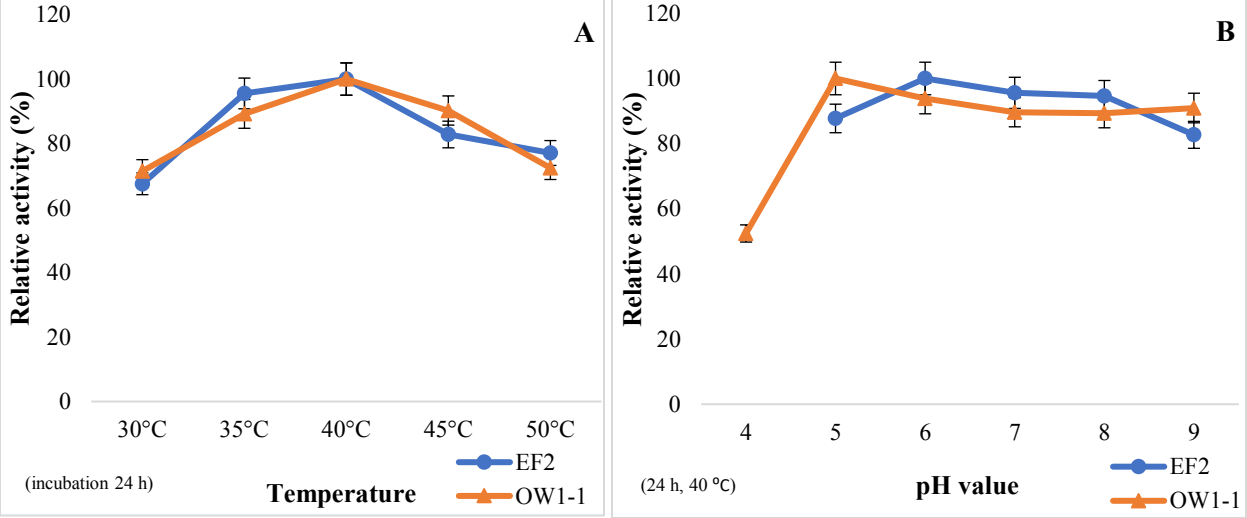


Fig 4. Effect of temperature (A) and pH (B) in CMCase activity by strains EF2 and OW1-1.

The effect of pH on CMCase production was measured in optimum 40 °C temperature and 24 h incubation time. The pH was adjusted to 4, 5, 6, 7, 8 and 9 using 2 N KOH and 0.2 N HCl. The bacterial strains EF2 and OW1-1 have better CMCase production in the broader pH ranges from 5 to 7 however the highest activity observed in pH 6 and pH 5 respectively. Similar optimum pH of

5.7 to 6.1 was reported in *Clostridium thermocellum* (Johnson et al. 1982). The activity gradually decreased with further change in pH values. The optimum activity was significantly different than other pH values with the $P<0.05$ for both strains. The bacteria such as *Micrococcus* sp. SAMRC-UFH3 (Mmango-Kaseke et al. 2016), *Clostridium straminisolvens* strain CSK1 (Jungang et al. 2017) decreased their activity with changes in optimum pH. However, some of the industrially important *Bacillus* sp. give better enzyme activity at wider pH ranges (Kim et al. 2005; Samiullah et al. 2009; Gaur and Tiwari 2015). For these bacteria, minor fluctuation in acidic environment does not necessarily affect their cellulolytic capacity.

3.5. Effect of nitrogen on enzyme production

The CMCase production has been greatly affected by various sources of nitrogen. We tested the effect of 0.5% w/v of each $(NH_4)_2SO_4$, peptone, urea, yeast extract in enzyme production at their respective optimum pH (as pH 6 for EF2 and pH 5 for OW1-1), temperature of 40 °C and 24 h incubation time. The result showed significantly higher yield of CMCase when yeast extract was used as a source of nitrogen. It was followed by peptone, $(NH_4)_2SO_4$ and the least activity observed in urea in both strains. The optimum CMCase activity was significantly different with the $P<0.05$ for both strains. The yeast extract and peptone are organic nitrogen sources which can results in better cellulase production (Enari and Markkanen 1977; Yang et al. 2014). However, the lower activity observed in inorganic nitrogenous sources (such as urea and ammonium sulphate) might be due to the inhibitory effect of medium acidification resulted from metabolism of inorganic nitrogen which in turn affects cellulase production (Yang et al. 2014).

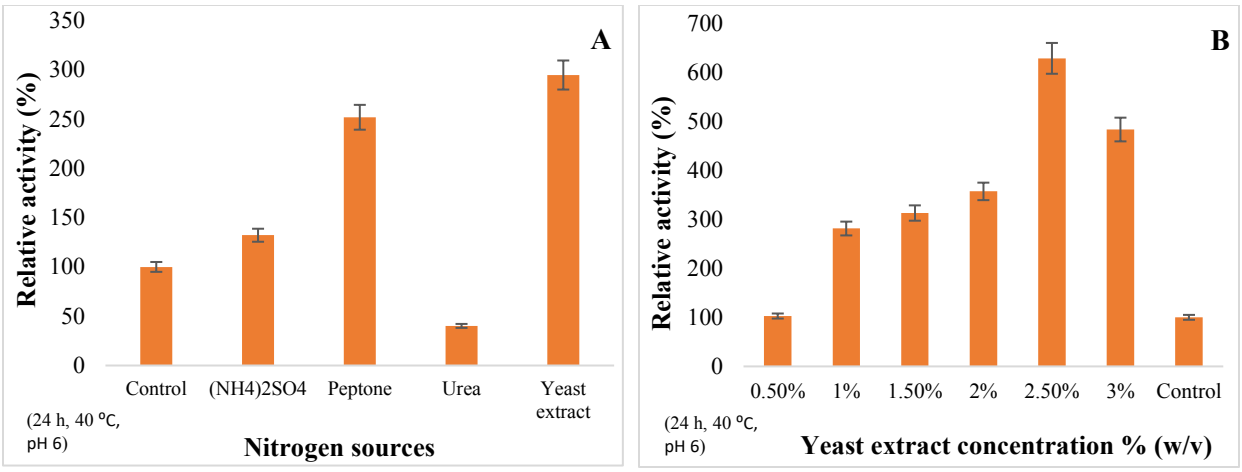


Fig 5. Effect of nitrogen sources (A) and yeast extract concentration (B) on CMCase activity by strain EF2.

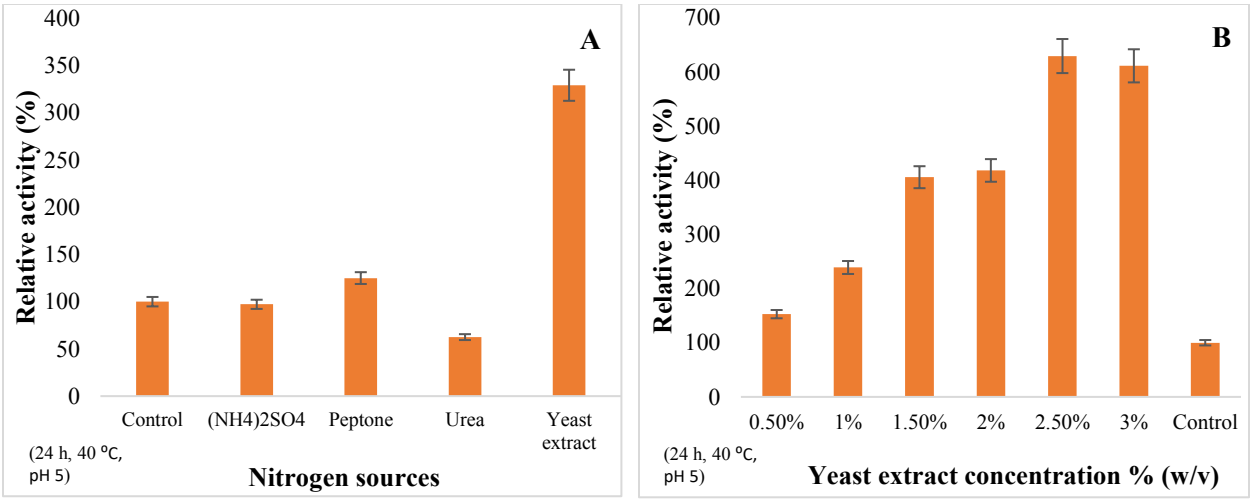


Fig 6. Effect of nitrogen sources (A) and yeast extract concentration (B) on CMCase activity by strain OW1-1.

The gradual increase in yeast extract concentration in minimal salt medium increases the enzyme production until it reaches to the optimum 2.5 % (w/v) in both strains. The results of ANOVA (with the $P>0.05$) showed that the optimum activity was not significantly different than other concentrations of yeast extract for both strains. High CMCase activity was observed in *Bacillus*

sp. C1AC55.07 when 2% yeast extract was used (Dias et al. 2014). Since, nitrogen is one of the major constituents of proteins, the bacteria are sensitive to the types and concentration of nitrogen sources which are specific to the species. Thus, the bacterial growth and enzyme production for dissimilar species differ dramatically with respect to their optimum condition. The highest CMCase activity in yeast extract among both strains EF2 and OW1-1 may be due to the presence of some other supplemental elements such as vitamin, trace nutrients, sulfur, etc. in yeast extract which are suitable for bacterial growth (Grant and Pramer 1962).

3.6. Effect of carbon on enzyme production

The strains EF2 and OW1-1 can utilize various carbon sources for CMCase production. Both strains were grown separately in minimal salt medium containing 1% (w/v) of each CMC, glucose, fructose, lactose and sorbitol in their respective optimum culture condition. The presence of lactose in culture medium yield a significantly higher CMCase production then other carbon sources. The CMCase activity of 13.742 ± 0.09 IU/ml and 12.812 ± 0.07 IU/ml were recorded in EF2 and OW1-1 respectively. This CMCase production was gradually increased with increase in lactose concentration and attained its maximal production at 2.5 % (w/v) concentration in the medium. The highest CMCase activity of 35.307 ± 0.08 IU/ml and 29.92 ± 0.01 IU/ml were recorded from EF2 and OW1-1 respectively when 2.5 % (w/v) lactose was used with their optimum culture condition (figures 7 and 8). The results of ANOVA with the *P*-values of 0.0019 and 0.0016 respectively for EF2 and OW1-1 (i.e. $P<0.05$) showed that the optimum enzyme activity was significantly different than other concentrations of lactose in both strains. Similar higher CMCase activity in lactose as a carbon source was observed in *Microbacterium* sp. (Sadhu et al. 2011), *Bacillus* sp. strain K1 (Paudel and Qin 2015), *Aspergillus hortai* (El-Hadi et al. 2014). It might be

due to the lactose-induced mechanism of cellulase production (Karaffa et al. 2006) which could be helpful to improve its industrial application (Sadhu et al. 2011).

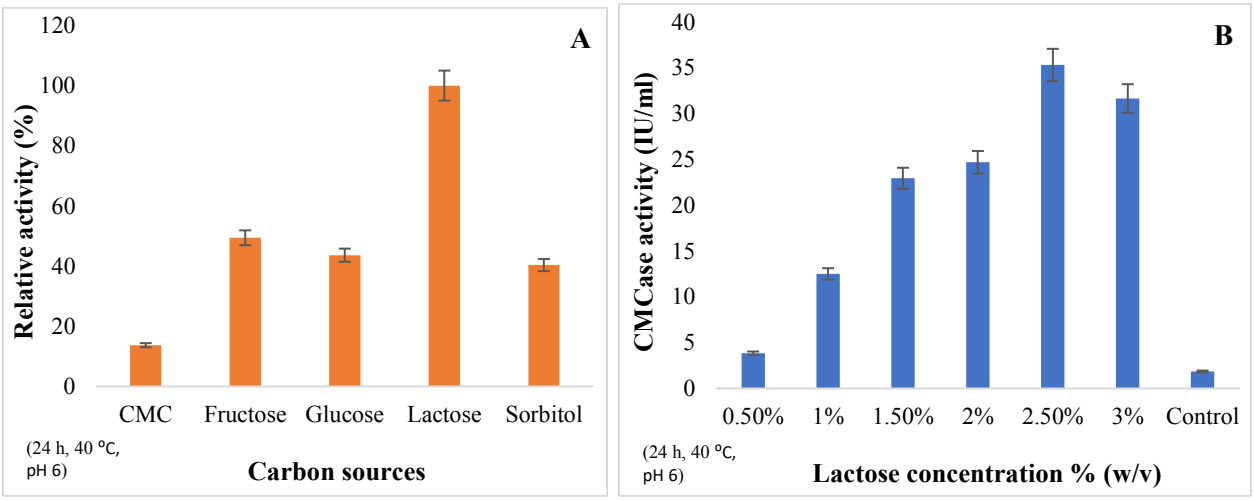


Fig 7. Effect of carbon sources (A) and lactose concentration (B) on CMCase activity by strain EF2

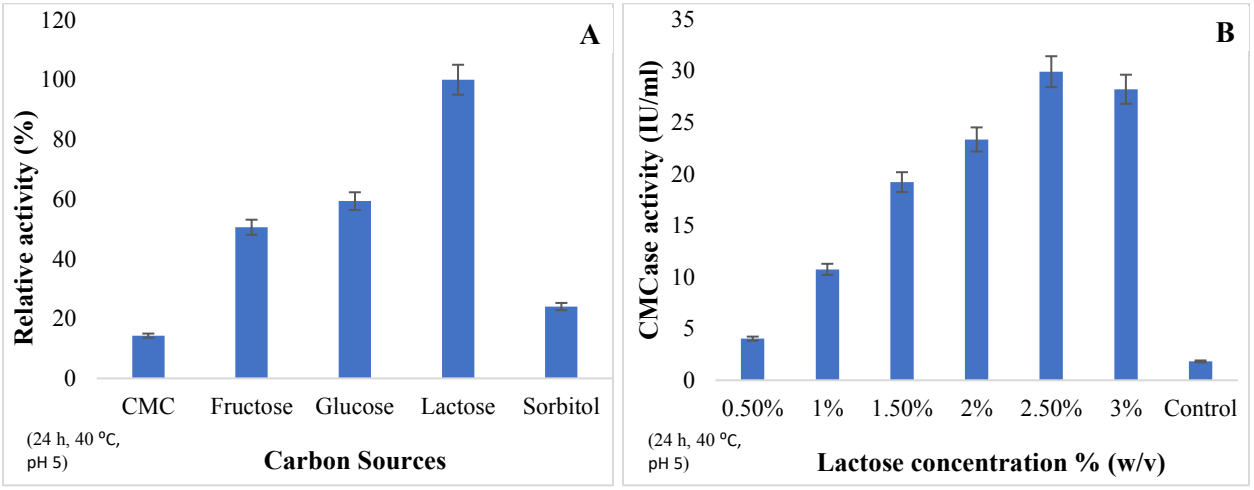


Fig 8. Effect of carbon sources (A) and lactose concentration (B) on CMCase activity by strain OW1-1

3.7. Effect of co-culture on enzyme production

The monoculture of strains EF2 and OW1-1 have shown their higher cellulase production at 40 °C but at dissimilar optimum pH 6 and pH 5 respectively. Thus, the pH was optimized by co-culturing the strains in 50 ml minimal salt medium containing 1% CMC with pH 5, 6, 7, 8 and 9 at an optimum 40 °C temperature. The higher CMCase activity of 1.925 ± 0.005 IU/ml was recorded when co-culture was provided with 1% CMC as a carbon source at pH 6. The activity was significantly different (P<0.05) than other pH values. It was a minimal increased in value in comparison to their pure mono-culture. However, the CMCase activities of co-culture (i.e. 40.605 ± 0.04 IU/ml) was 15% and 35.71% higher than monoculture of pure strains EF2 (i.e. 35.307 ± 0.08 IU/ml) and OW1-1 (i.e. 29.92 ± 0.01 IU/ml) respectively in optimum lactose concentration. Similarly, the CMCase activity in co-culture was significantly higher than pure strain OW1-1 when optimum yeast extract concentration was used. Generally, the microbial consortia are better adapted to minor fluctuation in pH and temperature (Poszytek et al. 2016). Several white rot fungi (such as *Fusarium* sp., *Phanerochaete chrysosporium*, *Ceriporiopsis Pleurotus ostreatus, subvermispora*, etc.) and some bacterial strains of the genus *Clostridium* are commonly practiced in co-culture (Poszytek et al. 2016). An efficient lignocellulolytic enzymes complex has been reported by constructing composite microbial system from mesophilic bacteria belonging to the genera *Clostridium, Bacteroides, Alcaligenes, Pseudomonas*, etc. (Guo et al. 2010). The consortium showed the high performance in degradation of lignocellulosic biomass due to synergistic enzymes at optimum temperature and pH of 40 °C and 6.0 respectively (Guo et al. 2010). The engineered microbial consortia are usually adapted to environmental fluctuation and known to perform complex functions that are difficult to individual populations (Brenner et al. 2008). However, there are several other limiting factors such as variation in substrate utilization, nutritional requirement, dissimilar requirement of ionic concentration in culture medium, different

genetic makeup, varied enzymatic components, diverse ecological niche and adaptational factors etc. could play a vital role in metabolic functions of microorganisms thereby affected in enzyme production. Thus, the maximum increase in yield of enzymatic components can be achieved after careful consideration of as much factors as possible. This co-culture opened up a metabolic pathway study for possible discovery of underlining cellular machinery of strains, their metabolic cross feeding mechanism and synergistic effect of multienzymes complex.

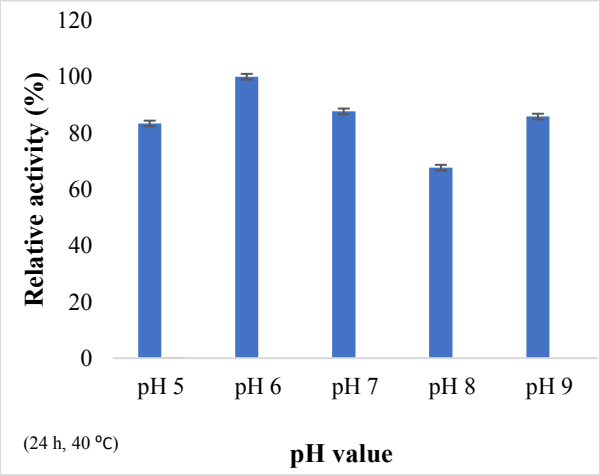


Fig 9. Effect of pH in CMCase activity by co-culture of strain EF2 and OW1-1.

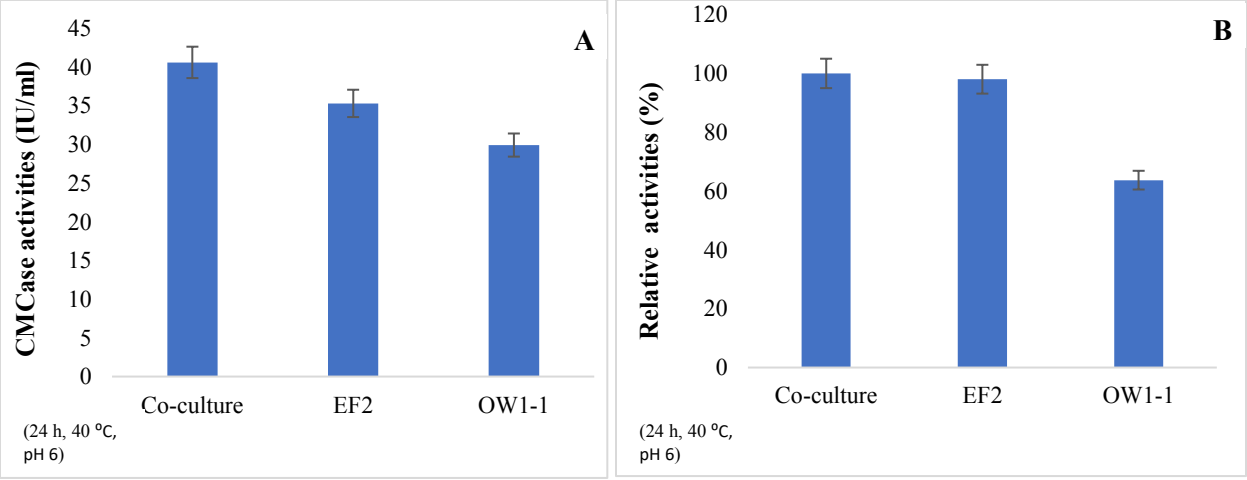


Fig 10. CMCase activities of mono and co-culture in optimum concentration of 2.5% (w/v) of lactose (A) and 2.5% (w/v) of yeast extract (B).

3.8. SDS-PAGE and zymogram

The protein bands of CMCase was observed in 10% acrylamide gel. The hydrolytic band of CMCase clearly indicated the cellulolytic activity in zymogram. It was run under the same conditions of SDP-PAGE which correspond to ~60 kDa in both strains EF2 and OW1-1 (figure 11). A relatively a higher molecular weight of 80 kDa has been reported in *Bacillus vallismortis* RG-07 (Gaur and Tiwari 2015), and 83 kDa and 50 kDa CMCase in wild type strain of *Aspergillus niger* (Coral et al. 2002). However, the CMCase with molecular weight of 55 kDa was observed in *Bacillus* sp. C1AC5507 (Padilha et al. 2015). Similarly, the cellulase bands in the range of 30-65 kDa in *Bacillus pumilus* EB3 (Ariffin et al. 2006) and 60 kDa in *A. niger* (Baraldo Junior et al. 2014) have been estimated from SDS-PAGE .

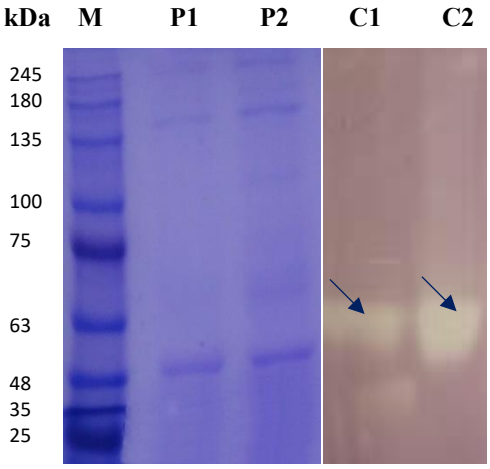


Fig 11. SDS-PAGE and zymogram of crude CMCase from strain EF2 and OW1-1. (M protein marker, P1 and P2 protein bands of EF2 and OW1-1 respectively, C1 and C2 CMCase hydrolytic bands of EF2 and OW1-1 respectively in zymogram). The molecular wt. of CMCase was estimated about 60 kDa in both strains.

3.9. Statistical analysis

The comparison of enzyme activity among different independent variables (including incubation time, temperature, pH, lactose concentration and yeast extract concentration) were first checked for its significance with ANOVA followed by post-hoc Bonferroni correction of T-test values. The ANOVA result with the *P* value of 0.000012 and 0.000002 (i.e. $P<0.05$) showed that there is significant different among the variables with their optimum enzyme activity in *Bacillus* sp. strain EF2 and OW1-1 respectively (figure 12). The post-hoc Bonferroni correction of two tail T-test comparison demonstrated that the pH has significantly higher (i.e. $P/n<0.01$) activity than incubation time in strain EF2. Similar, significantly high (i.e. $P/n<0.01$) enzyme activity in lactose concentration than all other variables showed that the optimum lactose concentration contribute as a major carbon source induced for maximum cellulase activity in both *Bacillus* sp. strain EF2 and OW1-1.

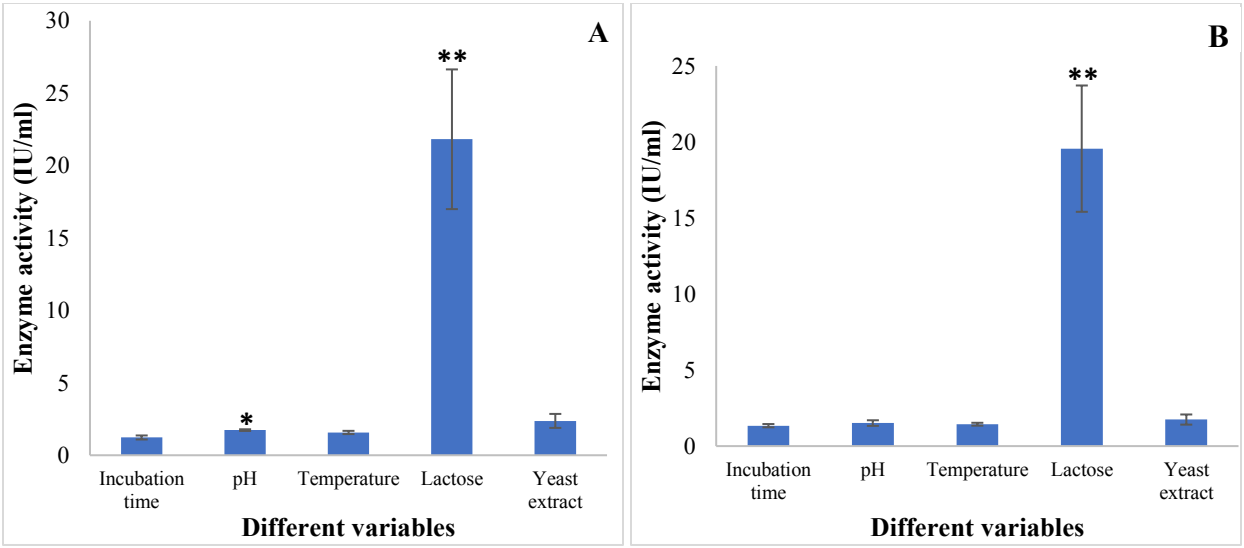


Fig 12. ANOVA was significant ($P<0.05$) in multiple comparison of independent variables and the post-hoc Bonferroni correction of T-test showed significantly different between pH and incubation time (*) in *Bacillus* sp. strain EF2, and lactose concentration and all other variables (**) at $P<0.01$ (i.e. P/n) level of significance in *Bacillus* sp. strain EF2 (A) and strain OW1-1 (B).

4. Conclusion

Some of the efficient cellulolytic bacteria can degrade the crystalline cellulose and release monomeric sugar molecules. These sugars can be converted to biofuels and value-added products after alcoholic fermentation. Thus, the isolation of such efficient bacteria is a fundamental key step of biofuel industries. Six cellulolytic bacteria were isolated from different sources including soil, rotten wood, organic waste, paper mill sludge and gut of earthworm. The 16S rRNA sequence identified the strains were of gram +ve bacteria belonging to genus *Bacillus* and *Lysinibacillus*. Two efficient *Bacillus* sp. strains EF2 and OW1-1 were selected for comparative enzyme activity assay in monoculture and co-culture. The presence of yeast extract and lactose in the culture medium induced the higher enzyme activity. The monoculture of strains EF2 and OW1-1 showed significantly increased ($P<0.05$ level of significance) CMCase activity of 35.307 ± 0.08 IU/ml and 29.92 ± 0.01 IU/ml with lactose in the culture medium at 40 ºC and optimum pH 6 and pH 5 respectively. The co-culture of these *Bacillus* sp. produced 15% and 35.71% higher CMCase activity than monoculture of EF2 and OW1-1 respectively. According to the hydrolytic activity shown in zymogram the molecular weight of CMCase was 60 kDa. The strains showed greater enzyme activity in broad range of temperatures (from 35 to 45 °C) in acidic pH which suggest that the *Bacillus sp.* strains EF2 and OW1-1 could be the potential cellulolytic candidates for biofuel industry.

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CHAPTER III

Bacterial immobilization for low cost conversion of glucose to fructose

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Abstract

The bacterial strain HK2 was isolated from forest soil nearby Kingfisher Lake. The qualitative screening of strain in xylose agar plate treated with 2,3,5-triphenyltetrazolium showed a zone of clearance, which clearly indicated that the strain has potential to produce xylose/glucose isomerase (GI) activities. The 16S rRNA gene sequence of strain HK2 was uploaded into the NCBI GenBank Database and identified as a gram-negative *Serratia marcescens*. The quantitative enzyme activities assay of the strain revealed that *S. marcescens* HK2 preferred the temperature of 35 to 40 °C and pH of 8 to 9 for efficient enzymes production. The GI activity was high when 1.5% xylose as a carbon source and 1:3 ratio of peptone and yeast extract were used in the culture medium. The SDS-PAGE analysis of crude enzyme revealed that the molecular weight of GI was about 63 kDa. This study discovered a novel finding in GI production that *S. marcescens* HK2 can utilize the low cost agricultural residue for production of GI and the whole cell immobilization can further minimize the cost of down streaming processing. Thus, *S. marcescens* HK2 could be a promising candidate for foods and biofuels industries.

Keywords: Glucose isomerase, characterization, immobilization

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1. Introduction

Glucose and fructose are six-carbon sugars having same molecular formula but different chemical structures, called isomers. The xylose/glucose isomerase (GI) catalyzes the isomerization of glucose and xylose to fructose and xylulose respectively (Bhosale et al. 1996). The isomerization of glucose to fructose is gaining its popularity in industries because fructose is the sweetest of all naturally occurring carbohydrates, thus being widely used in production of high-fructose corn syrup (HFCS), also added into the foods and drinks to enhance the taste. The GI is a most important commercial enzyme after amylase and proteases. The GI enzyme has great market demand in food industry due to a significant role in the production of HFCS (Bhosale et al. 1996; Lipnizki 2017). It has been estimated that the global market of GI worth $1 billion with annual production of 100,000 tons of enzyme (Sathya and Ushadevi 2014). Out of worldwide annual production of 17 million tons of starch-based sweeteners, about 16 million tons correspond to the production of HFCS derived from the corn (Lipnizki 2017) where the GI paly a pioneering role in conversion since 1965 (Deshpande and Rao 2008).

Recently, fructose is discovered as a more reactive feedstock than glucose for the production of 5-hydroxymethylfurfural (5-HMF), a high-value platform chemical to produce carbon neutral, high efficiency, furan based biofuels and other value-added bioproducts such as levulinic acid, furan dicarboxylic acid (FDCA) and bio-based polyesters such as polyethylene furanoate (PEF)- a green substitute for polyethylene terephthalate (PET), polyamides, polyurethanes, etc. (Thombal and Jadhav 2014; Yong et al. 2008; Melo et al. 2014; Chheda et al. 2007). Several studies have been conducted on catalytic compounds, $CrCl_2$ or $CrCl_3$ for conversion of glucose to 5-HMF involving fructose as a reaction intermediate (Li et al. 2009; Yuan et al. 2011; Zhao et al. 2007). Remarkably,

Su et al. (Su et al. 2009) studied in two metal chlorides: $CuCl_2$ and $CrCl_2$, dissolved in 1-ethyl-3-methylimidazolium chloride resulted in 55.4 ± 4.0% HMF yield from single step conversion from cellulose to HMF (Su et al. 2009). Although, the single step catalytic conversion of glucose to HMF is highly desirable for industrial applications, the HMF yield was still low due to the chemical equilibrium limitation of isomerization step involved in the process. The financial and environmental concerns associated with isomerization is another limitation of catalytic conversion.

Thus, there is an increasing interest on use of bacteria or fungi for biological conversion of glucose to fructose. These bacteria or fungi produce GI enzyme which help in isomerization of glucose to fructose (Suekane and Iizuka 1981; Bandlish et al. 2002). The production of GI was first reported from *Pseudomonas hydrophila* (Marshall et al. 1957) however, some other bacteria such as: *E. coli, Aerobacter* sp.*, Pseudomonas* sp.*, Sarcina* sp.*, Arthrobacter* sp.*, Streptomyces murinus* etc. have been reported for their ability of biological conversion of glucose to fructose (Suekane and Iizuka 1981). Some of the recent GI production was recorded form *Bacillus licheniformis* (Nwokoro 2015), *Thermus thermophilus* (Lönn et al. 2002), *Anoxybacillus gonensis* (Yanmiş et al. 2014) *Orpinomyces sp*. (Madhavan et al. 2009) etc. However, most of the commercial GI is produced from *Streptomyces* or *Actinoplanes* species (Hua and Yang 2016) and the enzyme immobilization has been widely practiced for industrial applications to minimize the production cost but maximize its recovery and reusability (Bhosale et al. 1996).

Immobilization is a physical confinement or entrapment of cells in a distinct support/matrix. There are different immobilization techniques that include: adsorption, crosslinking, covalent bonding, entrapment and encapsulation. It is a promising technique, has wider application in biotechnology,

pharmaceutical, environmental, bioprocessing, biofuel, bio-refinery, biodiesel, food and beverage industries, pulp and paper, wastewater treatment, biodegradation and bioremediation etc. (Elakkiya et al. 2007). There are several advantages of using immobilized bacterial cells for biological conversion. It is cost effective technique as it enhances the stability of the enzyme, retain the natural catalytic activity of enzymes, eliminate expensive steps of isolation and purification, can reuse the immobilized cells, less labour input and thus saving in capital investment (Guisan 2006; Elakkiya et al. 2007; Tampion and Tampion 1987). Although several studies have been conducted on enzyme immobilization for conversion of glucose to fructose which is an established practice in industrial level, very little information is available on bacterial immobilization for this bioconversion. The biological conversion method using microorganisms is comparatively slower process and cannot easily control but it is cost effective technique, requires low energy input, no chemicals and environment friendly (Wan and Li 2012; Shi et al. 2011). Thus, the study was focused on isolation and characterization of GI producing bacteria, optimize the enzyme activity and immobilize the strain for low cost bioconversion of glucose to fructose.

2. Materials and Methods

2.1. Media preparation

The solid agar and broth media were prepared for bacterial growth. The composition of culture media was as follow.

Luria-Bertani (LB) broth: 1.0 g peptone, 0.5 g yeast extract, 0.5 g NaCl and distill water up to 100 ml.

Xylose agar plate: 1.5 g xylose, 0.1 g $NaNO_3$, 0.1 g K_2HPO_4, 0.1 g KCl, 0.05 g $MgSO_4$, 1.15 g agar and distill water up to 100 ml.

Culture medium: 1% (w/v) xylose, 0.15 g peptone, 0.15 g yeast extract, 0.1 g K_2HPO_4, 0.01 g $MnCl_2.4H_2O$, 0.1 g $MgSO_4.7H_2O$, diltill water up to 100 ml.

2.2. Bacterial isolation and characterization

The soil sample was kept in 10 % xylose solution for 5 days followed by streaking in xylose agar plate. The plate was incubated at 37 °*C* for 24 hr. The bacterial colonies with different morphological and physiological properties were isolated, and loop transferred in 5 ml of LB broth for overnight incubation at 37 °*C* in a shaking incubator at a rotating speed of 200 rpm. The pure bacterial colonies were selected after successive streaking and re-cultured in nutrient media. The bacterial DNA was extracted after confirmation of GI activity from qualitative assay. The 16S rRNA was amplified using polymerase chain reaction (PCR). The PCR thermal cycle was adjusted as follows: initial denaturation at 94 °*C* for 3 min, 30 successive amplification cycles (denaturation: 94 °C for 30 seconds, annealing: 58 °C for 30 seconds and extension: 72 °C for 1.3 min) and final extension at 72 °C for 10 min. The amplified 16S rRNA was run through 1% agarose gel-electrophoresis and purified using PCR/Gel fragments extraction kit (Geneaid, FroggaBio). The pure 16s rRNA was sent to Eurofins Genomics, ON, Canada for sequencing and finally characterized by using basic local alignment search tool (BLAST) of national center for biotechnology information (NCBI) data base.

2.3. Bacterial seed culture and inoculum size

The LB broth seed culture for quantitative assay was prepared from agar plate stock culture by loop transferring of strain into 5 ml LB broth. The seed culture was incubated at 37 °C and 200 rpm for 24 h. The seed culture of 1 ml was transferred into a 250 ml Erlenmeyer flask containing 50 ml culture medium (i.e. 1:50 ratio of inoculum size) in each batch fermentation.

2.4. Qualitative enzyme assay

The qualitative screening for xylose isomerase was carried out using the strain cultured in LB broth. 5 µl of overnight cultured strain was inoculated in a D-xylose agar plate and was incubated at 30 ºC for 48 h. The pate was treated with 2,3,5-triphenyltetrazolium in alkaline medium (NaOH) using plate assay technique (Sapunova et al. 2004) followed by observation of zone of clearance.

2.5. Quantitative enzyme assay

The seed culture of 1 ml was taken as a standard inoculum size for each 250 ml Erlenmeyer flask containing 50 ml culture medium. Each 100 ml of culture medium contained 1% (w/v) xylose, 0.15 g peptone, 0.15 g yeast extract, 0.1 g K_2HPO_4, 0.01 g $MnCl_2.4H_2O$, 0.1 g $MgSO_4.7H_2O$. The bacterial production of extracellular GI was optimized by changing various physiochemical parameters of culture medium by considering one parameter at a time. The quantitative enzyme activity was analyzed by harvesting 1 ml of culture medium each day for 5 days. The extracellular enzyme was obtained by centrifugation (1200 ×g for 3 min) of culture solution each day and an aliquoted of supernatant (200 µl) was transferred to a reaction mixture containing 0.5 M D-glucose solution 100 µl, 0.2 M K-Na-Phosphate buffer (pH 7.8) 75 µl, 0.1M $MgSO_4 \cdot 7H_2O$ 25 µl. The reaction mixture was kept into the hot water bath (70 °C, 1 h) for isomerization. The enzyme activity assay of GI was carried out with some modifications on Cysteine-Carbazole method (Sapunova et al. 2004)(Tsumura and Sato 1965). Briefly, the reaction was terminated using 10 µl of 0.2 N HCl by keeping into ice cold water for 5 min. It was followed by addition of 50 µl of 1.5% cysteine hydrochloride solution, 50 µl of 0.12% alcoholic solution of carbazole (prepared in 100% ethanol) and 1 ml of 70% H_2SO_4. The solution was vigorously mixed and kept in 50 ºC hot water bath for 20 min to allow the purple colour development of fructose formed in the mixture after isomerization reaction. The measurement of fructose was performed in triplicates using 96

wells plate and spectrophotometer (BioTek, USA) at OD 540 nm. Whereas the bacterial growth in term of biomass was measured at OD 600 nm.

2.6. Effect of pH on enzyme production

Different pH ranges from 5 to 10 was analyzed for optimum enzyme production at 37 °C temperature. The flask containing 50 ml of minimal salt medium (containing 1% xylose) with 1 ml seed culture was incubated in shacking (200 rpm) incubator for five days and analyzed the enzyme activity. The further GI activities were evaluated at their optimum pH value.

2.7. Effect of temperature on enzyme production

The temperature of 25, 30, 35, 40, and 45 °C were selected for optimization of enzyme production. The LB seed culture (1 ml) was transferred into each flask containing 50 ml of minimal salt medium at optimum pH in a shacking (200 rpm) incubator for five days. The effect of temperature in isomerization was evaluated at 50, 60, 70 and 80 °C by keeping the reaction mixture for 1 h. The optimum temperature for enzyme production and isomerization reaction were estimated by standard assay procedure.

2.8. Effect of carbon sources on enzyme production

The carbon sources such as glucose, xylose and various lignocellulosic biomass were selected at their different concentrations in the production medium at their optimum temperature, pH and incubation time. The GI activity was estimated in its standard assay condition.

2.9. Effect of nitrogen sources on enzyme production

The effect of different nitrogen sources including peptone, urea, yeast extract and $(NH_4)_2SO_4$ were selected at different concentration in the production medium at their optimum temperature, pH and incubation time. The GI activity was estimated in its standard assay condition.

2.10. Effect of bacterial immobilization on enzyme production

The bacterial strain was immobilized in calcium alginate beads with some modification in entrapment method (Kierstan and Bucke 1977; Mukhopadhyay and Majumdar 1996). Briefly, the sodium alginate of 0.25 gm was mixed in 5 ml distill water and the LB broth seed culture of equal volume (5 ml) was thoroughly mixed into it. The mixture was collected into a disposable syringe and dropped from 15 cm height into $CaCl_2$ solution (1.5 gm of 0.2 M $CaCl_2$ in 100 ml distill water) with continuous steering to form small beads. The beads were leave (20 min) into $CaCl_2$ solution to become harden. The strain HK2 entrapped into calcium alginate beads were washed with autoclaved distilled water and used for enzyme production in 50 ml minimal salt medium in its optimum culture condition. The concentration of alginate gel was optimized using different concentrations- 1, 1.5, 2, 2.5, 3, 3.5% of sodium alginate. The inoculum size of immobilized strain was equivalent with the seed culture inoculum size of free strain as used in all other experiments. All the procedures were performed by using autoclaved apparatus and chemical reagents in a laminar air flow cabinet to minimize the contamination.

$2Na(Alginate) + Ca^{++} \rightarrow Ca(Alginate)_2 + 2\ Na^{+}$

2.11. SDS-Polyacrylamide gel electrophoresis

The GI enzyme was separated in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The crude enzyme from strain HK2 was run along with protein marker in 10% of

acrylamide gel. A constant supply of 120 V was maintained throughout the experiment. The gel was immerged in Coomassie brilliant blue for 45 min and de-stained with hot water until the bands were prominent. The presence of protein bands in the gel was compared with standard protein marker (Bio Basic, Canada) to estimate the molecular weight.

3. Results and Discussion

3.1. Qualitative screening of GI activity assay

Strains were isolated from soil after serial dilution and successive streaking. The plate assay technique was performed for qualitative screening. The strain was incubated at 37 °C for 48 h followed by treatment with 2,3,5-triphenyltetrazolium in alkaline medium (NaOH). The strain HK2 showed positive result (figure 1a) with a zone of clearance in plate assay thus, was selected for their further quantification of GI activity.

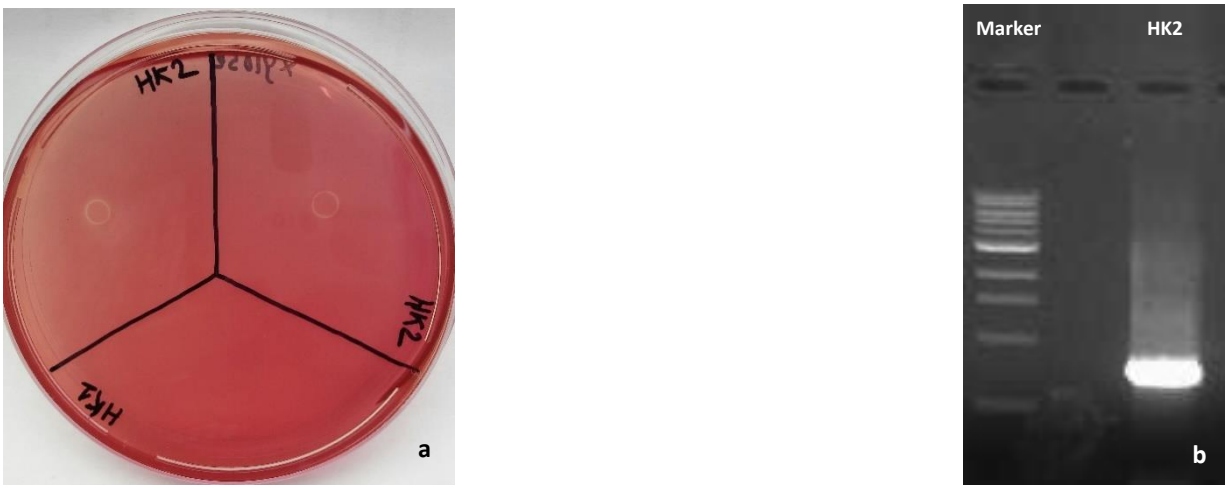

Fig 1. Plate having zone of clearance/halo in 2,3,5-triphenyltetrazolium test showing positive GI activity (a) and validation of 16S rRNA band in 1% agarose gel (b).

3.2. Bacterial isolation and characterization

Bacterial DNA of strain HK2 was isolated using bacterial genomic DNA isolation kit (Norgen Biotek Corp) and amplified its 16S rRNA using PCR (FroggaBio), universal forward primers HAD-1 (5′-GACTCCTACGGGAGGCAGCAGT) and reverse primer E1115R (5′-AGGGTTGCGCTCGTTGCGGG) following their protocols. The amplified DNA sample was run in 1% agarose gel-electrophoresis and validated the presence of 16S rRNA (figure 1b). The nucleotide sequence of 1115 bp obtained from genomic lab was submitted in NCBI data base and identified the strain HK2 as gram -ve bacteria of genus Serratia marcescens with 100% homology. The *Serratia marcescens* strain HK2 produced a characteristic red coloured pigment called prodigiosin. This pigmented S. marcescens is considered as less infectious than non-pigmented strains (Carbonell et al. 2000), thus reducing the risk of infection in careful laboratory handling.

3.3. Quantitative GI activity assay

The enzyme activity of bacterial strain *Serratia marcescens* HK2 was further quantified by Cysteine-Carbazole method. The minimal salt medium with 1% xylose was used for bacterial growth and enzyme production. The extracellular crude GI enzyme (200 µl) from supernatant was used in the reaction mixture containing 100 µl of 0.5 M glucose, 75 µl of 0.2 M Na-K Phosphate buffer (pH 7.8) and 25 µl of 0.1 M MgSO4.7H2O throughout the experiment. The reaction mixture was allowed for isomerization reaction by keeping in hot water bath at optimum temperature for 1 h. The fructose formed after isomerization was allowed for colour development following Cysteine-Carbazole method. The purple colour developed after 20 min of hot water (50 °C) bath represents the presence of fructose in the reaction mixture (Dische and Borenfreund

1951)(Nakamura 1968). Finally, the absorbance was measured at OD 540 nm in a micro-plate reader spectrophotometer (BioTek, USA) and fructose was estimated using fructose standard curve (figure 3). One unit of GI enzyme corresponds to the formation of 1 µM of fructose equivalent per minute from substrate (glucose) and the GI activity was quantified in U/ml.

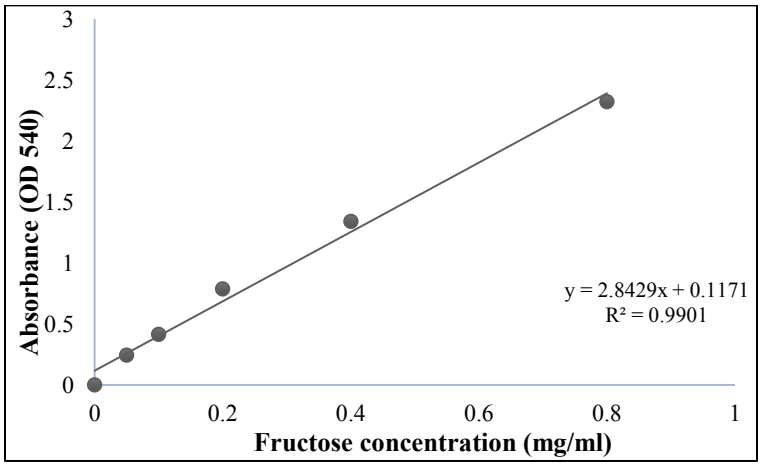

Fig 2. Standard curve of fructose

3.4. Effect of pH and incubation time on enzyme production

The enzyme production has been greatly influenced by various pH ranges and incubation time. Thus, the pH of minimal salt medium was adjusted to 5, 6, 7, 8, 9 and 10 using 2 N KOH and 2 N HCl. The effect of pH on enzyme production was measured in 37 °C by harvesting 1 ml of culture broth each day for 5 days. It has been studied that the optimum glucose isomerase production was achieved in alkaline medium with pH range of 7 to 8.5 (Mishra and Debnath 2002) and incubation period of 24 to 96 h (Habeeb et al. 2016). Chou et al. (1976) also reported the pH range of 7 to 8 for optimum xylose isomerase production. Our study on *Serratia marcescens* strains HK2 agreed with similar earlier findings. It showed higher GI activity when grown in pH 8 at 72 h of incubation. The enzyme production was gradually increased with increasing pH, reached the highest at pH 8 and decreased in pH 9 and 10 after 72 h of incubation. The optimum GI activity was significantly different in pH 8 than other pH values with the $P<0.05$. Similar, maximum GI

activity from *Neurospora crassa* was recorded at pH 8 in 72 h of incubation (Rawat et al. 1996). In another finding, the GI production in *Streptomyces* sp. CH7 was high in 72 h when grown in 1% oat-spelt xylan (Chanitnun and Pinphanichakarn 2012) and in *Streptomyces* sp. HM5 the GI production was high when grown in 1.5% xylose with an initial pH 7.5 in 72 h of incubation (Muhyaddin et al. 2008).

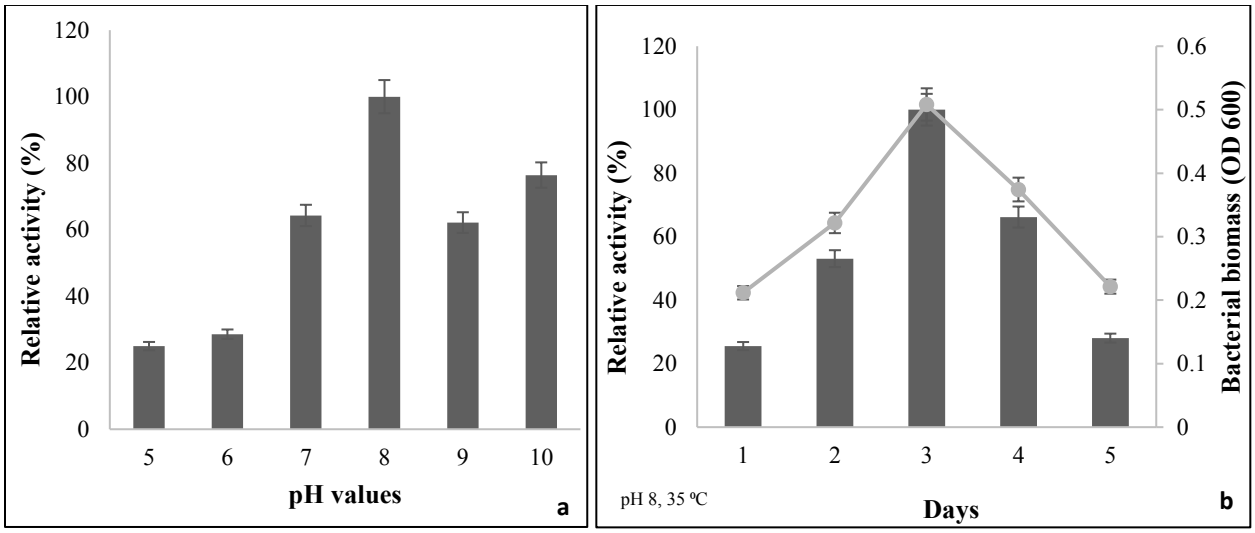

Fig 3. Effect of different pH (a) and incubation time (b) in enzyme production by strain HK2.

3.5. Effect of temperature on enzyme production and GI activity

Different species of bacteria have their own preferred temperature for high enzyme production. The enzyme itself has highest activity at its optimum temperature which is greatly influence by minor fluctuation in temperature. The strain HK2 was grown in minimal salt medium at different temperature of 25, 30, 35, 40 and 45 °C for 5 days. The optimum enzyme activity was determined by incubation of reaction mixture in hot water bath at 50, 60 70, 80 °C for 1 h followed by measurement of GI activity. The strain HK2 produced an extracellular GI in wider range of temperature from 35 to 45 °C. Similar higher GI production at 37 °C has been reported in *Enterobacter agglomerans* isolated from garden soil (Nobel et al. 2011). The commercial GI

producers are usually the mesophiles strains isolated from various sources (Tayseer et al. 2012). The mesophilic strain *Serratia marcescens* HK2 also preferred 40 °C for maximum GI production however the optimum temperature of 70 °C for significantly high (P<0.05) GI activity. This result was in harmony with some species of *Streptomyces* which have been recorded for their optimal GI activity at 70°C (Manhas and Bala 2004; Dhungel et al. 2009). Although, some species of *Bacillus* have been reported with optimum GI activity at temperature of 50 °C (El-Shora et al. 2016; Nwokoro 2015), most of the microbial GI are known to produce maximal activity in relatively higher temperature range from 60 to 80 °C (Walsh 2002) more likely because the equilibrium of isomerization of glucose to fructose is driven by higher temperature (Jia et al. 2017).

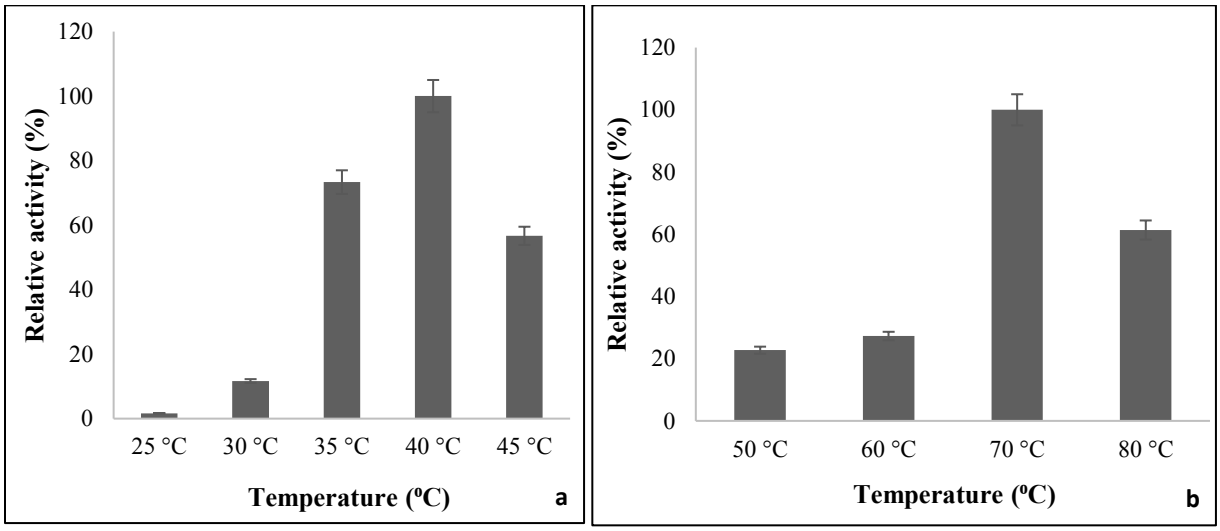

Fig 4. Effect of temperature in enzyme production (a) and optimum GI activity at different temperature (b) by strain HK2.

3.6. Effect of nitrogen on enzyme production

The sources of nitrogen affect the enzyme production. The experiment was conducted on various nitrogen sources including $(NH_4)_2SO_4$, peptone, urea and yeast extract to observe their effect in enzyme production. Although peptone and yeast extract showed least GI activity when used

separately in the medium, the activity was greatly increased when both used in a same culture medium (figure 5a). The GI production of HK2 in mixed peptone and yeast extract was significantly different (P<0.05) than other nitrogen sources. The yeast extract and peptone are organic nitrogen sources which supported in higher GI production than inorganic nitrogen (Nwokoro 2015; Deshmukh et al. 1994; Givry and Duchiro 2008). It may be due to the presence of suitable supplemental growth elements such as vitamin, trace nutrients, sulfur, etc. in yeast extract (Grant and Pramer 1962). Similarly, the different proportion of peptone and yeast extract have greater impact in enzyme activity. The highest GI activity was observed in 1:3 ratio of peptone and yeast extract (figure 5b) which showed that a relatively increasing yeast extract concentration in the medium resulted in higher GI activity (Givry and Duchiro 2008). On the contrary the presence of inorganic nitrogen such as urea in the culture medium did not support in bacterial growth or enzyme production (Deshmukh et al. 1994). The least activity observed in urea and $(NH_4)_2SO_4$ might be due to the acidification of medium resulted from metabolism of inorganic nitrogen which in turn affects enzyme production.

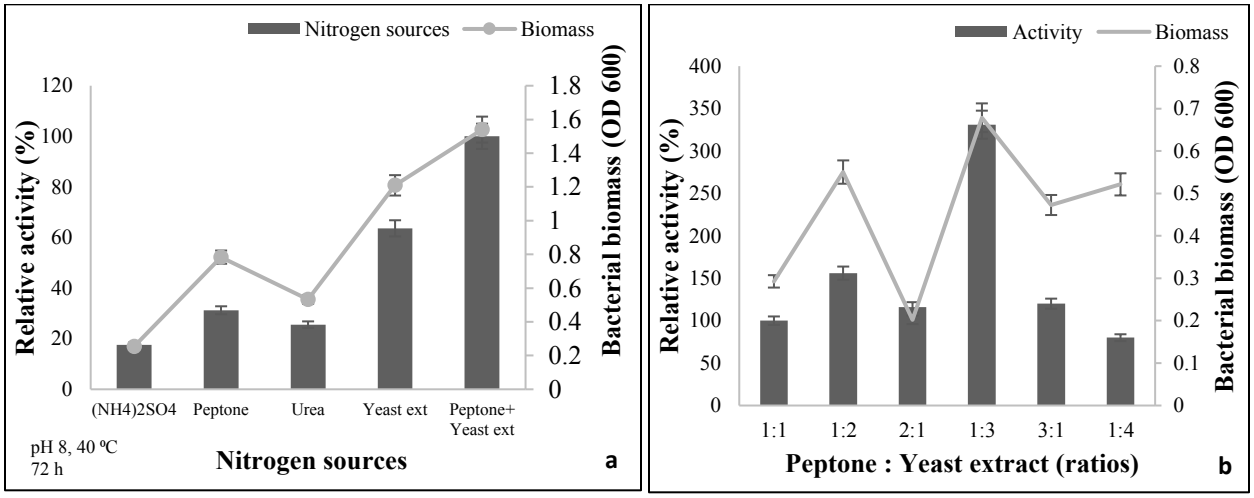

Fig 5. Effect of different nitrogen sources (a), and proportion of peptone and yeast extract (b) in GI production.

3.7. Effect of carbon on enzyme production

The strains HK2 can utilize glucose, xylose or various lignocellulosic biomasses as a carbon sources for GI production. The strain was grown separately in minimal salt medium containing 1% (w/v) of carbon sources. The presence of different carbon sources in culture medium have significant impact in enzyme production. It was observed that the strain HK2 produced GI when grown in either glucose or xylose (figure 6a). Although the GI activity (1.88 ± 0.004 U/ml) was higher in 48 h with the glucose as a carbon source, the activity (3.256 ± 0.003 U/ml) was significantly high (i.e. $P<0.05$) in 72 h when xylose was used as a carbon source. The observed GI activity was higher than GI activity in *Streptomyces* sp. SB-P1 (1.3 U/mL) (Bhasin and Modi 2012), *Streptomyces* sp. (0.14-0.73 U/ml) (Lobanok et al. 1997), *Pseudomonas* sp. 0.7 U/ml, and *Bacillus* sp. 0.3 U/ml (Tayseer et al. 2012) where xylose broth medium was used. We observed nearly 2 folds higher GI activity in the medium containing xylose than compared to the medium containing glucose as a carbon source. The GI activity in the xylose medium was increased because the GI production in most of the bacteria is induced by xylose (Bhasin and Modi 2012) (Sayyed et al. 2010). This result was in harmony with GI activity in *Streptomyces* sp. SB-P1 (Bhasin and Modi 2012), *Streptomyces* sp. CH7 (Chanitnun and Pinphanichakarn 2012), *Saccharomyces cerevisiae* (Karhumaa et al. 2005) and *Bacillus thermoantarcticus* (Lama et al. 2001) where the activity was high in the presence of xylose as a carbon source.

The concentration of glucose or xylose in the culture medium also effect the enzyme production. Thus, both carbon sources were optimized separately to estimate the GI activity. The activity gradually increased with increase in carbon concentration from 0.5 % (w/v) and attained the maximum GI activity at 1.5% (w/v) in both glucose or xylose then after it decreased (figures 8,

and 9). The higher GI activity of 3.515 ± 0.019 U/ml and 4.384 ± 0.002 U/ml observed in optimum concentration of glucose and xylose respectively. However, Habeeb et al. (Habeeb et al. 2016) recorded higher GI activity of 13.6 U/ml from *Streptomyces* sp. SH10 in its optimum production medium containing 1.5% xylose as a carbon source.

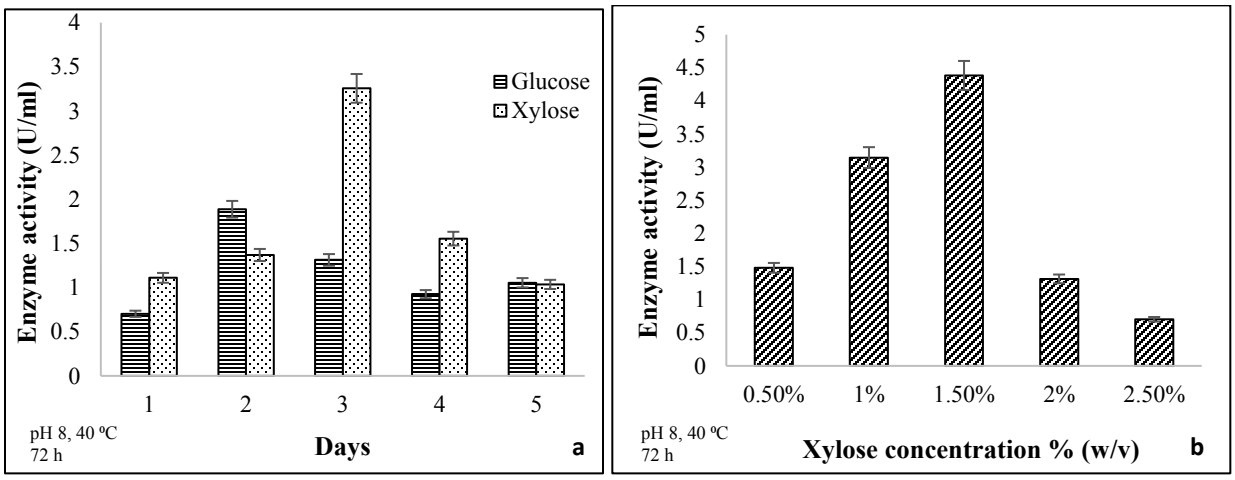

Fig 6. Effect of carbon sources (a) and xylose concentration (b) in GI production by strain HK2.

Various lignocellulosic biomasses including barley straw, corn stover, wheat bran, *Miscanthus* and wood dust were tested discretely in GI production medium. The biomass of 1% (w/v) was used as a sole carbon source at pH 8 and 40 °C followed by measurement of GI activity in standard assay condition. However, 1 ml of 2% glucose was introduced into the culture medium maintaining total volume of 50 ml to initiate the bacterial growth. The HK2 strain was found to utilize various lignocellulosic biomass in GI production. The higher GI activity was observed in barley straw as sole carbon source (figure 7a). It showed continues increase in activity with increased in barley concentration until it reached to 2% (w/v) which subsequently decreased above this concentration (figure 7b). Several studies have been conducted in GI production by using cheap carbon sources. In an earlier study, the extracellular GI (1.5 U/ml) was produced from *Streptomyces flavogriseus* when grown on straw hemicellulose in about 72 h (Chen et al. 1979). Chanitnun and

Pinphanichakarn (Chanitnun and Pinphanichakarn 2012) found the corn husk at 2.5% (optimum concentration) resulted in higher GI production from *Streptomyces* sp. CH7. Similarly, the agricultural residues such as corn cob and wheat husk have been recorded for high GI yield from *Streptomyces* sp. SB-P1 (Bhasin and Modi 2012). Our finding agreed with previous suggestion that the application of barley straw, a cheap agro-residue, can act as a good substitute of expensive glucose or xylose in the production medium. Use of such agricultural residues in industrial production line not only minimize the overall economics of enzyme production but also help to overcome the environmental issues associated with agricultural waste disposal.

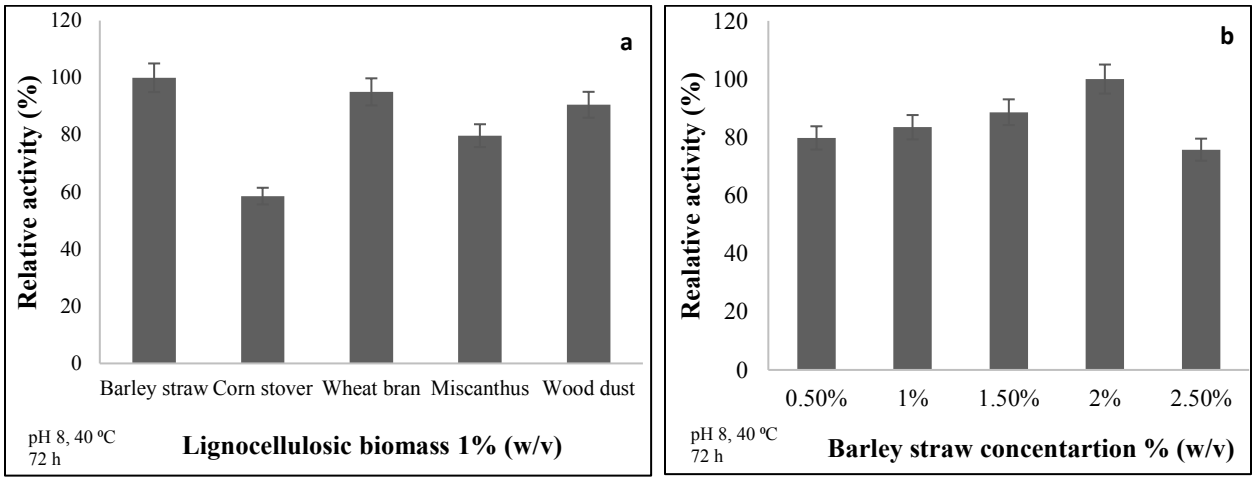

Fig 7. Effect of different lignocellulosic biomass (a) and barley straw concentrations (b) in GI production by strain HK2.

3.8. Bacterial immobilization and enzyme production

The seed culture (1 ml) of strain HK2 was immobilized and transferred to the 250 ml flask for enzyme production. The GI activity was measured for 5 days in standard assay condition. The immobilized strain HK2 produce higher GI activity of 3.348 ± 0.02 U/ml in 48 h when 1% xylose was used in culture medium. The improvement in GI activity was also recorded when 2% barley straw used as a carbon source in 5th days of incubation in optimum condition. There was an

improved GI activity in immobilized strain than its free strain. Similar improved GI activity have been reported in whole cell immobilization of *Streptomyces phaeochromogenes* (Vieth et al. 1973; Kumakura et al. 1979) and *Streptomyces kanamyceticus* (Mukhopadhyay and Majumdar 1996). As the alginate beads offered increase in porosity and retaining property (Kierstan and Bucke 1977), the immobilized strain HK2 entrapped into the calcium alginate beads can receive nutrients or inducements from culture media into the microenvironment so as to improve the enzyme production. The encapsulation or entrapment of cells provide higher cell density and cellular interaction which create favorable microenvironment and increased productivities (Looby and Griffiths 1990; Pilkington 2005). Studies on whole cell immobilization have reported the improved activity than free cells (Jobanputra et al. 2011; Jung et al. 2008) because the immobilized cells were less sensitive to minor temperature and pH fluctuations (Jobanputra et al. 2011).

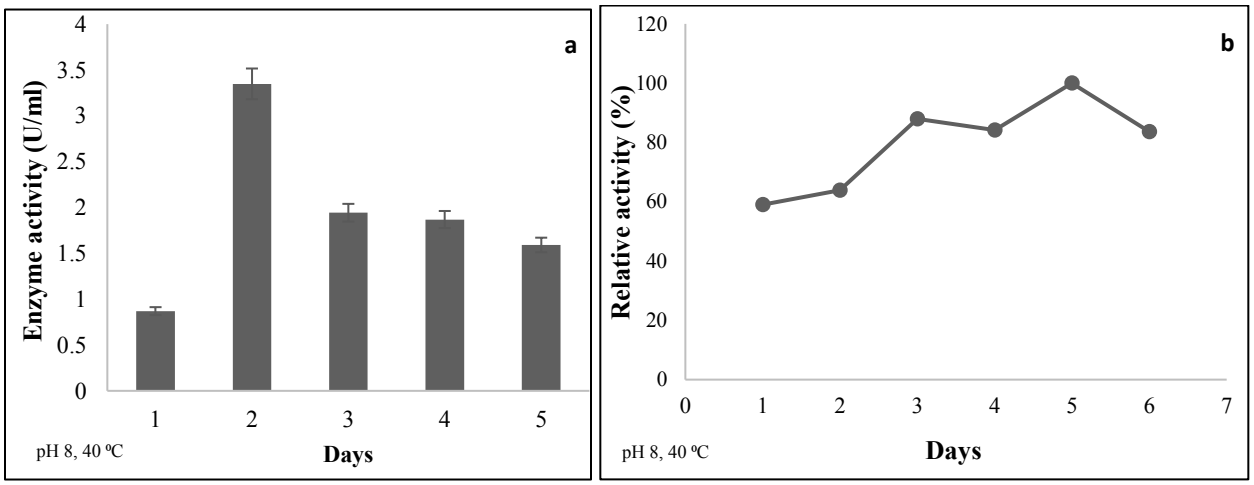

Fig 8. Effect of immobilized strain HK2 in calcium alginate beads and cultured in 1% xylose (a), and 2% barley straw (b) as a sole carbon source.

3.9. Effect of alginate concentration on enzyme production

The porosity, gel strength, size of alginate beads and cell activity depends on the concentration of sodium alginate and $CaCl_2$ thereby affect the enzyme production efficiency of immobilized strains

(Zhu 2007). The strain HK2 was immobilized in six different concentrations-1, 1.5, 2, 2.5, 3, 3.5% of sodium alginate and tested for enzyme production using same nutrient broth. The beads of 1% alginate were very soft, gradually lost its binding affinity and observed disruption into smaller fragments after 48 h of incubation with low enzyme yield. Whereas the immobilized strain in 3% of sodium alginate beads significantly increased the GI activity (7.12 ± 0.021 U/ml) in 48 h The disruption of beads in 1% alginate and increased polymethylgalacturonase production in 3% alginate has been observed in immobilized *Aspergillus niger* 26 (Angelova et al. 1998). Similar increased enzyme production have been reported from immobilized strains in 3% sodium alginate (Bernardi et al. 2017; Ellaiah et al. 2004). However, GI production and its activity in reutilization were reduced as reported by (Bernardi et al. 2017; Ellaiah et al. 2004) in contrast to increased enzyme production from successive second cycle onwards (Angelova et al. 1998).

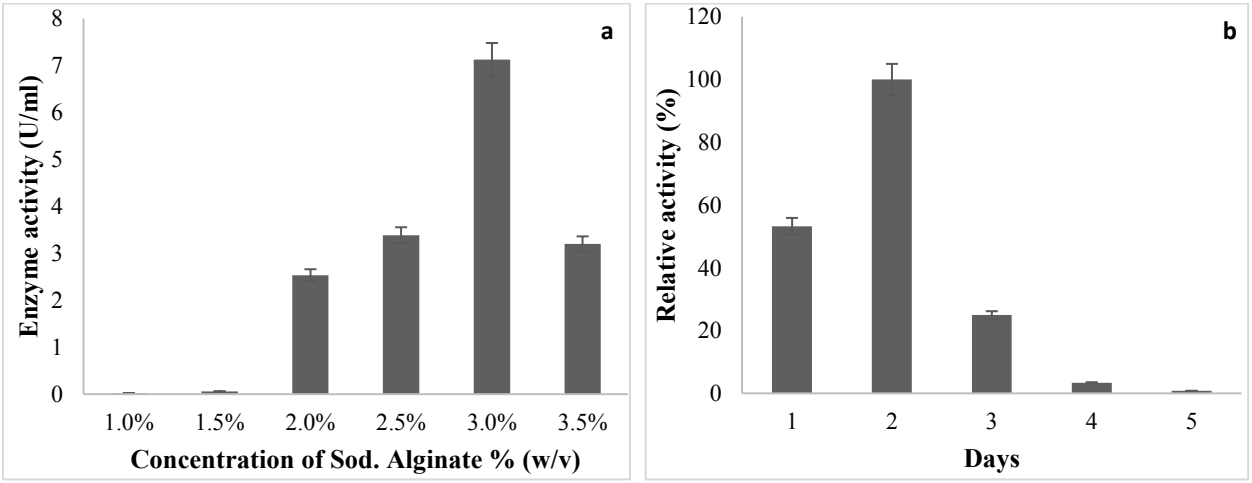

Fig 9. Effect of sodium alginate concentration (a) and GI activity of immobilized strain in 3% alginate (b).

3.10. SDS-PAGE

The protein band of GI was observed in 10% acrylamide gel. Multiple bands were observed in the gel due to crude extraction and production of some other proteins in the medium. However, the

band which correspond to 63 kDa confirm the presence of GI enzyme. A relatively similar molecular weight of 60 kDa has been reported from *Bacillus megaterium* BPTK5 (Mukesh Kumar et al. 2012) and *Enterobacter agglomerans* (Nobel et al. 2011). Some also reported the GI of 49 kDa from *Geobacillus thermodenitrificans* TH21 (Konak et al. 2014) and 43 kDa from *Streptomyces chibaensis* J 59 (Joo et al. 2005).

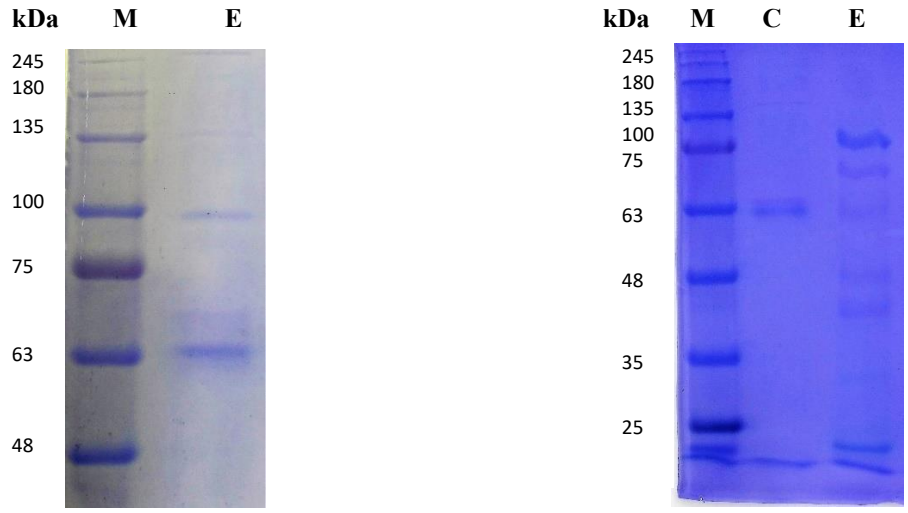

Fig 10. SDS-PAGE of crude GI from strain HK2. (**M** protein marker, **C** protein bands of crude enzyme, **E** protein bands after denaturation by boiling). Molecular weight of GI was estimated about 63 kDa in HK2.

4. Conclusion

The strain HK2 isolated from soil sample was identified as *Serratia marcescens* from partial sequence of 16S rRNA with 100% homology. Several bacterial species of *Arthrobacter, Streptomyces, Bacillus* etc. were reported in production of GI however, to the best of our knowledge this study was first recorded GI activity from *S. marcescens*. Thus, besides having an important contribution in secondary metabolite production from *S. marcescens*, it opened up new

scope in food and biofuel industries. The strain can effectively utilize the barley straw, a cheap agriculture residue as an alternative to expensive xylose as a carbon source for GI production. Additionally, the whole cell immobilization of strain can further facilitate the purification steps and economize the downstream processing.

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CHAPTER IV

Discussion and future recommendations

1. Discussion

The increase in human population and industrialization resulted in soaring up of global energy demand. It is expected to increase of 48% global energy demand over the 28 years period- from 2012 to 2040 where the renewable energy account an increase of 2.9% per year (Diefenderfer et al. 2040). The lignocellulosic biomass is one of the renewable resource that can be utilize in formation of biofuels. Cellulose is the most abundant natural biopolymer, composed of D-glucose subunits linked by β-1, 4 glycosidic bonds. The crystallinity of cellulose can be degraded into monomeric sugar units by synergistic action of hydrolytic enzymes collectively called as cellulase. It consists of 1,4-β-endoglucanase, 1,4-β-exoglucanase or cellobiohydrolase (CBH), and 1,4-β-glucosidase, belonging to glycosyl hydrolase (GH) family (Henrissat and Davies 1997). Among the 128 GH families, the CBH can be found in GH families 5, 6, 7, 9, 48, and 74 (Annamalai et al. 2016). Two major types of cellobiohydrolase are CBHI and CBHII which effectively degrade the crystalline cellulose, presumably by peeling the microcrystalline structure of cellulose chain; whereas endoglucanase typically acts on more soluble amorphous region of cellulose, showing high degree of synergism and thus releasing the sugar molecules (Maki et al. 2009; Dashtban et al. 2010). However, the selection of proper microbial consortia with diverse enzymatic composition and efficient hydrolytic activity is a challenging task. Thus, several attempts have been made to explore industrially efficient, vigorously active bacteria, which act on greater temperature and pH fluctuation. Thus, the concept of consolidated bioprocessing (CBP) has been introduced as a single step process of simultaneous saccharification and fermentation for bioconversion of lignocellulosic biomass to biofuels using single microorganism or microbial

consortium (van Zyl et al. 2007). Many bacteria and fungi have lignocellulose degrading capability however the anaerobic, thermophilic and cellulolytic bacteria are mainly used in CBP for manufacture of second generation biofuels (Singh et al. 2017).

The ubiquitous nature of bacteria and their ability to produce various enzymes and secondary metabolites have established themselves as a best candidate in wide array of disciplines such as foods and beverages, medicine and pharmaceutical, biofuels and bioproducts, ecology and environment etc. They play a significant role in biogeochemical cycles of various elements including carbon, oxygen, nitrogen, sulfur, phosphorus, iron, etc. Moreover, some of the autotrophic bacteria are the primary producers in some ecosystem. The bacteria can adapt themselves to different environmental conditions and can easily grow in the laboratory for numerous scientific experiments. Nevertheless, the increasing scholarly interest on using microorganisms such as bacteria and fungi have reflected its brighter optimistic side of bioconversion. However, the cumulative capital investment of pretreatment facility is major financial concern among biorefinery and biofuel industries. Thus, the conventional technique of microbial isolation and screening for bioconversion has its greater significance due to the likeliness of getting high yielding strain with additional gene of interest which can economize the overall enzyme loading cost. For this reason it is very essential and important to isolate the efficient bacterial strains for their possible industrial application. Thus, the study was conducted to isolate cellulase and xylose isomerase producing bacteria from various sources and optimize the enzyme activities by altering various physiochemical parameters.

We isolated 24 cellulase producing bacterial strains from various sources including forest soil, rotten woods, municipal organic sludge, wastewater and intestine of red wriggler worm. The qualitative screening of strains using plate assay techniques was performed in standard CMC agar plates and observed the positive results with a zone of clearance in Gram's iodine test. These strains possess higher potential in production of extracellular enzymes showed high degree of cellulolytic activity. Two efficient cellulolytic strains EF2 and OW1-1 were selected for further characterization using 16S rRNA gene sequences. The strains EF2 and OW1-1 were identified as gram (+ve) *Bacillus* sp. and their cellulase activities were further quantified using DNS method. Both strains preferred 2.5% of yeast extract as nitrogen source and 2.5% of lactose as a carbon source for significant increase in enzyme production than other sources of nitrogen and carbon. The Carboxymethyl cellulase (CMCase) activities of strain EF2 was 35.307 ± 0.08 IU/ml at pH 6 and strain OW1-1 was 29.92 ± 0.01 IU/ml at pH 5 when 2.5% (w/v) of lactose was used in cultured medium at optimal 40 °C temperature in both strains. The CMCase activities were higher than activity reported by several other researchers (Zeng et al. 2016; Miklaszewska et al. 2016; Ghosh et al. 1998). Similar increase in CMCase activity were observed by (Paudel and Qin 2015; El-Hadi et al. 2014) when they used lactose as a carbon source. The supplemental growth elements such as vitamin, trace nutrients, sulfur, etc. provided in the yeast extract and lactose induced gene expression for CMCase production could be the possible reasons for higher CMCase activity. It has been observed that the CMCase activity was induced by application of lactose in the medium (Bischof et al. 2013) and it is considered as an inexpensive soluble substrate suitable for CMCase production (Lo et al. 2010). The SDS-PAGE and zymogram analysis showed the molecular weight of CMCase was 60 kDa in both strains which was comparable with other similar findings (Baraldo Junior et al. 2014; Padilha et al. 2015; Zeng et al. 2016). Further, the study on co-culture of two

Bacillus sp. strains EF2 and OW1-1 were conducted in their optimum culture condition. It is always recommended to consider the optimal acceptable ranges of various physiochemical parameters like pH, temperature and substrate of individual microbes to set up their co-culture (Bader et al. 2010). The strains preferred similar culture conditions for optimum enzyme yield which can avoids competition for substrates between the species (Maki et al. 2009). The co-culture of *Bacillus* sp. strains EF2 and OW1-1 in contrast to their monoculture, showed 15% and 35.71% increase in CMCase activity respectively. The phylogenetic relation of strains showed that EF2 and OW1-1 are closely related *Bacillus* sp. which may have similar genetic makeup and the synergistic action of cellulase enzymatic components could be the possible reason for improved CMCase activity. Generally, minor fluctuation in pH and temperature do not necessarily affect the enzyme production in microbial consortia (Poszytek et al. 2016). It can be concluded that the strains EF2 and OW1-1 are good candidate for co-culture and maximum CMCase production.

Similarly, the strain HK2 showed GI activity on peptone-xylose agar plate with a hallo when treated with 2,3,5-triphenyltetrazolium in alkaline medium (NaOH). The strain was further characterized using 16S rRNA sequence and identified as a gram (-ve) *Serratia marcescens* with 100% homology. The quantification of GI activity was performed by cystine carbazole method using spectrophotometer. The strain HK2 preferred the temperature of 40 °C and pH 8 for optimum enzyme production. The two-fold increase in GI activity was observed when 1.5% xylose and 1:3 ratio of peptone and yeast extract were used in the culture medium. The GI activities of strain HK2 was 4.384 ± 0.002 U/ml in optimum culture condition. Peptone and yeast extract are the organic nitrogen suitable for bacterial growth (Givry and Duchiro 2008) and the xylose in the culture medium induced the GI production (Bhasin and Modi 2012). Further, the strain was immobilized

in calcium alginate beads and allowed for GI production in optimum condition. The GI activity was significantly high when 3% sodium alginate was used for immobilization. The improved enzyme activities of immobilized microbes have been reported in several other studies (Vieth et al. 1973; Kumakura et al. 1979; Mukhopadhyay and Majumdar 1996). The fine porous structures of the beads not only allow a nutrient supply but also the entrapped bacterial strains were provided with higher cell density and better cellular interaction within microenvironment of beads (Looby and Griffiths 1990) which make them better adapted to minor fluctuation of temperature and pH in the environment. The SDS-PAGE and zymogram revealed that the molecular weight of GI from *S. marcescens* HK2 was 63 kDa. The strain *S. marcescens* HK2 can utilize low cost agricultural residue for production of GI and the bacterial immobilization can further minimize the cost of down streaming processing. To the best of our knowledge the GI activity of *S. marcescens* HK2 was first discovered in this study. This novel finding could be a matter of interest among the novice researcher and some food and biofuels industries.

2. Future recommendations

The enzyme loading, its digestibility, production of sugars, energy consumption, quality of biofuels and bioproducts etc. are some important parameters and techno-economic bottlenecks that demands the commercial potential of pretreatment facility. The quality and price of bioproducts depend on types of biomass and process conditions used in the manufacturing plant (Kumar and Murthy 2011). Thus, pretreatment is essential step to solubilize the biomass which offer higher cost of enzymes and other chemicals during bioconversion. More study should focus on to explore the suitable combination of microbial consortium which can adapt to major fluctuation in pH, temperature and give a higher yield of enzymatic components to disintegrate the plant cell wall.

Different populations have their differences in physiological capabilities, cellular structures, and ecological niches, which can surpass its value in share adaptation (Cohan and Koeppel 2008). The genetic makeup of organism allows them in pre-existing adaptation to either invading a new environment or advancement of adaptation characters in its existing niche. The molecular ecology of microbes based on 16S rRNA gene sequence represents a perfect molecule to study their diversity, phylogeny, evolution, and adaptation (Grice et al. 2009). However, the progress in adaptation of organisms in new environment and its evolution is determined by their population size, its survival, spread, and/or transmission of an organism within a specific ecological niche (Preston et al. 1998). Thus, the systematic laboratory experiments on ecological aspect and molecular level are essential to understand the underlying mechanisms of adaptation of microbes in different ecological niches.

The thermophilic bacteria such as *Clostridium thermocellum* (Demain et al. 2005; Taylor et al. 2009; Akinosho et al. 2014) and *Clostridium phytofermentans* (Jin et al. 2011)(Jain et al. 2013) are well studied CBP bacteria (Weimer 2013). Some other anaerobic, thermophilic bacteria such as *Thermoanaerobacter sp.* (spp Qiang He et al. 2011; Svetlitchnyi et al. 2013) and *Caldicellulosiruptor sp.* (Svetlitchnyi et al. 2013; Chung et al. 2014) have been studied in CBP for production of biofuels. The future research should be focused on CBP using microbial consortium for biofuel and value-added product formation.

Further studies on gene cloning of EF2 and OW1-1 can be done for industrial scale production of cellulase enzymatic complex. The application of gene editing technique such as CRISPR-Cas9 (Liu et al. 2017) and its metabolic engineering (Lin et al. 2014) could result in robust, vigorously

active, highly productive strains. Such mutant strains can effectively take part in bioconversion of lignocellulosic biomass to bioethanol and value added bioproducts formation.

The catalytic activity of GI enzyme from *S. marcescens* HK2 is comparable to other known efficient strains. Since, the GI activity of strain was first reported in this study, it opens up new avenue of research in possible gene cloning of strain to maximize the production of enzyme. The enzyme characterization could be a next step major research project which could be applicable to different foods and biofuel industries.

The strain HK2 can utilize the cheap agricultural residue for GI production. Thus, the future research should focus on to explore the regulatory mechanism of enzyme secretion which could be helpful to understand the pathway on different substrates. More study in optimization of different physiochemical parameters such as aeriation, agitation, metal ions, enzyme stability, etc. should be conducted to maximize the enzyme production in industrial scale.

The bioconversion of lignocellulosic biomass/organic matters and its fermentation can produce biofuels and other value added bioproducts important to biofuel industries. Such end products analysis should be conducted using various analytical tools such as GC-MS, HPLC, FTIR, etc.

The *S. marcescens* HK2 can produce prodigiosin, a red pigment as a ssecondary metabolite. It is an expensive natural dye worth about USD 5000×10^5/kg (Venil et al. 2013). The prodigiosin is known for its antimalarial (Castro 1967), antibacterial, antioxidant, antifungal, immunosuppressant and anticancer properties, etc. (Venil et al. 2013; Darshan and Manonmani

2015). More studies on different aspects of such a valued pigment will surely attract the researchers to work on the biosynthesis of pigments, its application in biomedical science and pharmaceutical industries.

3. References

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