

Review Article

Microbial Cellulases: A Review on Strain Development, Purification, Characterization and their Industrial Applications

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Abstract

In this advance era, the enzymes are considered as a core kernel of white biotechnology and their demand is increasing day by day. According to report published in Research and Markets (ID: 5009185), the estimated global market for industrial enzymes were USD 10.0 billion in 2019, which is continuously increasing as it is expected to reach about USD 14.7 billion by 2022. Among all enzymes, cellulases are the major group of enzymes act synergistically in breakdown of cellulose, that facilitates its conversion to various value-added products and also offer several other important applications at industrial scale. The hyper production of cellulases are required to overcome their demand of global market. Cellulases production can be enhanced by strain improvement as well as using advance fermentation technology. In this review a detail studies of strategies to enhance production of cellulases and improve their physiochemical properties for industrial application have been described.

Keywords: Enzyme; Cellulase; *Aspergillus*; Immobilization; Fermentation; Strain improvement

Abbreviations

Mpa Pressure: Mega Pascal Pressure Unit; *A. oryzae*: *Aspergillus oryzae*; *A. sojae*: *Aspergillus sojae*; CaCl₂: Calcium Chloride; NaCl: Sodium Chloride; UV: Ultraviolet Radiations; SmF: Submerged Fermentation; CMC: Carboxymethyl Cellulose

Introduction

Enzymes are the proteinaceous macromolecules and biological catalysts that are produced by all living organisms and involved in vital processes of life. They are also considered as the most promising alternative to chemical catalysts as they are highly specific and can carry out the catalysis at very minimum and milder reaction conditions [1-3]. Enzymes are employed in various processes in industries as they are non-toxic, biodegradable and can produce higher concentration of better-quality products by utilizing small number of raw materials. Enzymes are sustainable at both *in vivo* and *in vitro* conditions [4]. Various living systems are used for the extraction of industrially important enzymes including; animals (Pepsin, Trypsin), plants (Papain, Bromelain) and microorganisms (α -amylase, Glucoamylase, Cellulase) while bulk amount of enzymes can be obtained from microorganisms [5]. The demand for enzymes is increasing day by day and according to report published in Research and Markets (ID: 5009185), the estimated global market for industrial enzymes were USD 10.0 billion in 2019, which is continuously increasing so it is expected to reach about USD 14.7 billion by 2022. In this advance era, the enzymes are considered as a core kernel of biotechnology as they are the main agent to run important industrial biotechnological processes. In order to meet the increasing demands, enzymes are produced from microorganisms because they are the primary source of enzymes, having short life span, easy to culture and also desired

genetic variations to enhance production of enzymes can be carried out easily comparative to plants and animals.

The enzymes produced by microorganisms are comparatively more stable as they can work in various temperature and pH, therefore the microbial enzymes can be withstanding in industrial bioprocesses. The microorganisms can be also genetically modified or mutated to enhance the production of enzymes as well as to improve the enzymatic activity and stability [6]. The best renewable energy sources for microorganisms are lignocellulosic materials and this is also the major structural components of plants. It is composed of a complex mixture of carbohydrate polymers such as; cellulose, hemicellulose, lignin and some other components termed as extractives [7-9]. These all components of lignocellulosic biomass are interconnected with each other in different percentage and form a hetero-matrix. Mostly the composition of the main polymers of legionellosis biomass is; Cellulose (30-60 %), Hemi cellulose (20-40 %), Lignin (10-25 %) [10]. The plant genetics, environmental factors and the sources of biomass greatly influence the composition of polymers in lignocellulose materials [11]. The cellulose of biomass or plant cellulosic parts can be converted into sugars for further processing by cellulases enzyme followed by pre-treatments.

In recent few decades, the soluble sugar and other products obtained from the conversion of cellulosic materials was further used as precursor for the production of fuels, ethanol or other important biochemical products [12]. For this purpose, enzymes are playing an important role in the hydrolysis of cellulosic biomass and conversion to valuable products. Among all enzymes, cellulases are the major enzyme involved in breakdown of cellulose. Therefore, the cellulases produced through advanced biotechnological processes is further used for the depolymerisation of cellulose and also offers several

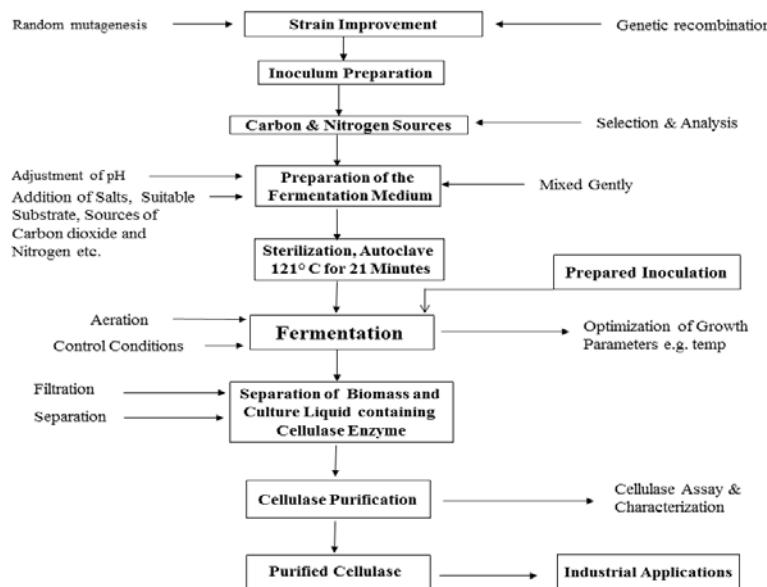


Figure 1: Flow sheet for biotechnological production of cellulase.

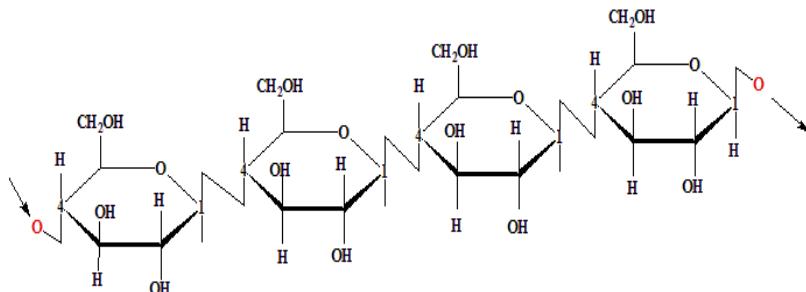


Figure 2: Diagram showing the β-1, 4 linkages of glucose units in cellulose.

other important applications at industrial scale [13,14]. Though, few reviews are available on cellulase enzyme especially focusing on their biotechnological production and strategies to improve their productions and properties. However, in this review we have reported the methods of strain improvements for optimization of cellulases enzyme production through advanced fermentation technology and their industrials applications (Figure 1).

Lignocellulosic Biomass

Lignocellulose materials are the major structural components of plants and the best renewable energy sources for microorganisms. Lignocellulose biomass is composed by the polymerization of carbohydrate moieties such as; cellulose, hemicellulose, lignin and some other components, called as extractives and these moieties are the major constituents of plant cell wall [7-9]. The main component of lignocellulose materials is cellulose, which is the large homologous polymeric chain of D-Glucose residues connected through β -1, 4 glycosidic bonds with the repeating small subunits, cellobiose (Figure 2) [12]. Cellulose is consider the most copious and bountiful renewable sources for producing valuable products for energy sources [6], this property of cellulose make it more important to use it for the production of value-added fuels by thermochemical or biochemical processes. This has been used for the production of biofuel especially

for methane and bioethanol [15]. Beside this, the cellulose has also wide range of application and use in different industries such as food and beverage, animal feeds, detergent, agriculture, textile, pulp and paper industry. It has attracted the interest of industrial scientists, so these can be utilized for the production of enzymes such as cellulase, xylanase, α -amylase at both lab and industrial scale [4]. It has also attracted the interest of scientists as they are taking advantage of using this low cost energy source (cellulose) for the production of biological products, that would add into the economy and energy security of the country [16].

Cellulose usually occurs as fibres, densely packed with hydrogen bond and insoluble in water so it is very resistant to hydrolysis without the chemicals and mechanical degradation. Hence cellulose or other polysaccharide compounds can be converted into simple sugar or glucose units by the activity of cellulase enzymes [17]. It is also a very stable part of plant cell wall matrix and biomass, that's why plants take great benefit of it as it plays an important role to maintain the cell wall stability and integrity [18]. The structure of cellulose determines the hydrolysis pathway and compactness in cellulosic structure makes it prone to the degradation. This compact and crystalline structure of cellulose is due to inter and intra molecular hydrogen bonding. This super crystalline structure of cellulose makes

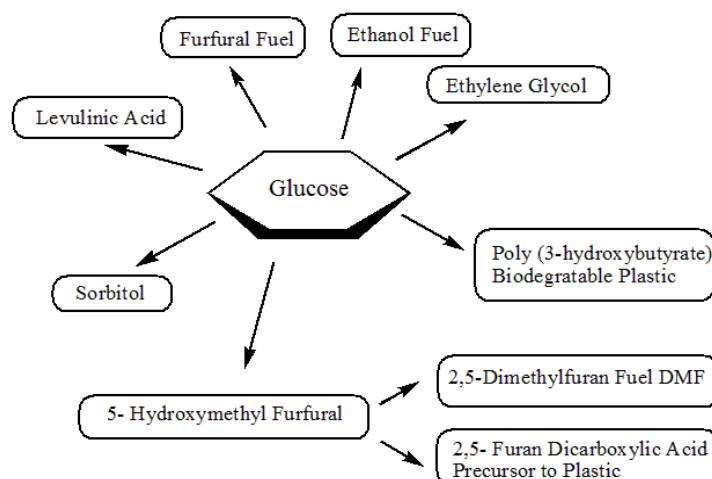


Figure 3: Fuels & chemicals derived from glucose.

it resistance to hydrolysis even at very extreme reaction conditions, therefore, it must be pre-treated to convert the cellulosic biomass or plant cellulosic parts into simple sugars. Therefore, before processing, it must be heated at 320°C at 25 MPa pressure [6]. The procedure to convert the lingo-cellulosic biomass into valuable products is facilitated with the “Biochemical conversion process”, which reduce the size of biomass by clipping. The biochemical conversion process is followed by the “Thermochemical pre-treatment step”, which make the plant cell wall more flexible and amenable for the biocatalysts attack. Then cellulase can depolymerize the cellulose into sugar very easily, and furthermore the simpler sugars can yield valuable chemicals and fuels (Figure 3) [19-21].

Factors Affecting the Hydrolysis Rate of Cellulose

The hydrolysis of cellulose is the complex and sensitive process that requires the pre-treatment and action of one enzyme in a group form. The complex, compact and rigid structure of cellulose, make it strong enough, therefore, cannot cleaved easily by enzymatic action. But cellulose is the most abundant and biologically renewable resource for the production of many important products and fuel. So it is key interest of industries to hydrolyze the cellulose into glucose or simple sugars which is the further fermented to ethanol and many other valuable products. The hydrolysis of cellulose into individual units, that is, simple sugar or glucose or easily hydrolysable shorter chains depends on some factors including; crystallinity, degree of polymerization, particle size and pore volume and availability of surface area.

Crystallinity

Crystallinity is one of the major factors which affect the procedure of cellulose hydrolysis by enzymatic process. As we know that cellulose has highly crystalline chains and tightly packed nature that make cellulose resistance to hydrolyze by enzymatic activity. Therefore, the crystallinity of cellulose is inversely proportional to the enzymatic action. The process of hydrolysis will be slow, if the cellulose has more crystalline regions.

Degree of polymerization

The longer chain length of cellulose is also recalcitrant to hydrolysis. Therefore, cellulose with smaller chains will be hydrolyzed easily and vice versa.

Particle size and pore volume

The size, shape and pore volume of cellulose also has great influence on the hydrolysis of cellulose.

Availability of surface area

Mostly cellulose or chains of cellulose are buried inside the microfibers which reduce the rate of hydrolysis due to limited access of enzyme to the glycoside bonds [22,23].

Cellulases Enzyme and Modes of Action

The cellulases are the group of enzymes that hydrolyse the β -1, 4 glycosides bond between β -D glucose units in cellulose. This group of enzymes, cellulases, are further classified into various types on the basis of their mechanisms of bond cleavage in cellulose resulting in the yield of different intermediate and end products. Moreover, the subtypes of cellulase also differ in amino acid sequence, folding and 3-D structure. The cellulase enzyme includes three major types; 1) Endoglucanase, 2) Exoglucanase or cellubiohydrolases, 3) β -Glactosidase. These enzymes are Glycoside Hydrolase (GH) which act synergistically on cellulose to breakdown into simple sugar for their application in industry e.g., textile, food, feed, pulp and paper industry [24-27]. The breaking down of cellulose molecules into simple sugars, cellobiose and other oligo-saccharides as a primary product starts with the carboxymethyl cellulase or endoglucanase, which reacts on chain of cellulose and randomly release a small fibre with non-reducing or reducing end. Afterwards, exoglucanase or cellubiohydrolases reacts on free ends of small fibres to release the cellobiose, and finally the β -glucosidase help by cleaving the cellobiose to the end product glucose (Figure 4) [28].

Cellulases are the world's third largest group of enzymes and have been considered as one of the most important targets for both academic and industrial research for more than 30 years. The structure of fungal cellulases are comparatively simpler than

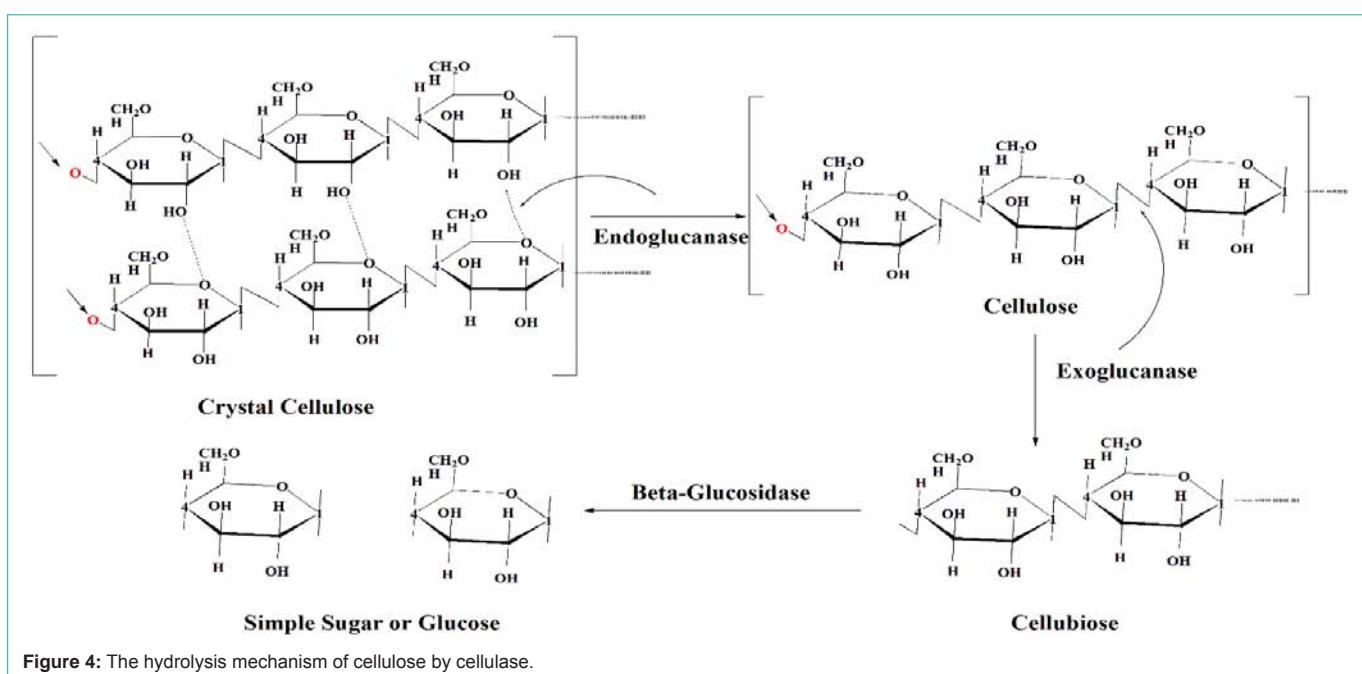


Figure 4: The hydrolysis mechanism of cellulose by cellulase.

Table 1: Cellulase Producing Microorganisms.

	Microbes	Ref	Microbes	Ref
Fungi	<i>Aspergillus niger</i>	[136]	<i>Trichoderma Longibrachiatum</i>	[137]
	<i>Trichoderma Harzianum</i>	[137]	<i>Rhizopus sp.</i>	[138]
	<i>Aspergillus ustus</i>	[139]	<i>Aspergillus terreus</i>	[140]
	<i>Penicillium decumbens</i>	[141]	<i>Penicillium funiculosum</i>	[142]
	<i>Trichoderma reesei</i>	[143]	<i>Aspergillus Fumigatus</i>	[144]
	<i>Aspergillus unguis</i>	[145]	<i>Aspergillus tubingenesi</i>	[146]
	<i>Aspergillus heteromorphu</i>	[147]	<i>Fomitopsis sp</i>	[148]
Bacteria	<i>Streptomyces Sp. G12</i>	[149]	<i>B. subtilis</i>	[150]
	<i>Anoxybacillus flavithermus</i>	[150]	<i>Bacillus stratosphericus</i>	[151]
	<i>Vibrio parahaemolyticus</i>	[152]	<i>Bacillus pumilus EB3</i>	[153]
	<i>Bacillus mojavensis</i>	[154]	<i>Bacillus Amyloliquefaciens</i>	[155]
	<i>Branhamella sp.</i>	[156]	<i>Microbulbifer hydrolyticus</i>	[157]

that of bacteria known as cellulosome as it contains cellulosome-cohesin containing enzymes for scaffolding and dockerin while fungal cellulases contains two domains; catalytic domain or catalytic modules and cellulose binding domain composed of 35 amino-acids and both domains are linked by short poly-linker which are rich in threonine and serine residues [29]. On the basis of three dimensional structure and amino-acid sequences, the catalytic domain is classified into different Glycoside Hydrolase (GH) families and a proton donor and nucleophile residues are present at the catalytic site that are involved in the catalysis of cellulose through acid-base mechanism [10]. Glycoside Hydrolase (GH) families 5-9, 12, 44, 45, 48, 51 and 74 are specific for endoglucanase [30]. In a study it was reported that the catalytic domain also known as “Concanavalin-like domain”, is structurally same in both bacterial and fungal cellulases, binds through a short sequence to Cellulose Binding Domain (CBD) or cellulose binding module (CBM). The CBD is mostly found in bacterial enzymes and it also contains cellulosome, having both types

of cellulases, that is; 1) Cellulases bind with CBD, 2) Cellulase without CBD [4]. The bacteria, fungi, protozoan, plants, and animal are the producers of cellulase (Table 1) [31,32].

Strain Development

Cellulases production can be enhanced by strain improvement through genetically modifying the microbial strains *via* advanced techniques, which has gained much importance [3]. Commercial production of cellulases is enhanced by genetic modifications and random mutagenesis of the parental strains [33]. Moreover, the molecular cloning of cellulases genes or increasing copy number also have great impact on degradation of cellulosic material and production of high quality value-added products [10].

Strain improvement is a promising approach for enhancing the production of cellulases [34]. The wild type or normal strains may possess some specific characteristics and found potential applications

Table 2: Strain improvement through mutagenic treatments for enhancing cellulase production.

Microbial Strain		Mutagenic Treatment	Effect on Cellulase Production (Folds, Percentage)	Ref
	<i>Trichoderma viride</i> TL-124	UV Radiations and N-methyl-N'-nitro-N-Nitro Guanidine (NTG)	2.61 folds	[158]
	<i>Penicillium janthinellum</i> EU1	UV Radiations and Ethyl-Methyl Sulphonate	2 folds	[159]
	<i>Aspergillus</i> sp. SUI4-M15	Co60 γ -rays, UV Radiations and NTG	2.2 folds	[160]
	<i>Trichoderma reesei</i> SCB18	Genetic Engineering (Deletion of <i>cre1</i> gene and cloning of <i>bgI</i> A from <i>A. niger</i>)	29.80%	[161]
	<i>Aspergillus terrus</i> M-17	γ -rays, UV Radiations and Ethyl-Methyl Sulphonate	1.97 folds	[162]
Fungal	<i>Trichoderma reesei</i> - Δ P70	Deletion of Protease genes (tre81070, tre120998 and tre 123234)	6 folds	[163]
Strains	<i>Penicillium decumbens</i> Mutant JU-A10-T	Genetic Engineering (Deletion of <i>creA</i> and <i>amyR</i> genes)	9 folds	[164]
	<i>Trichoderma citrinoviride</i>	Ethyl-Methyl Sulphonate and Ethidium Bromide	2.10 folds	[165]
	<i>Trichoderma reesei</i> Mutants (TA-32, TB-87, TE-6)	Agrobacterium mediated T-DNA Insertional Mutagenesis	38%, 51% & 31% respectively	[166]
	<i>Cellulomonas</i> sp. TSU-03 (M23)	UV Radiations and N-methyl-N'-nitro-N-nitro guanidine	1.30 folds	[167]
Bacterial	<i>Acidothermus cellulolyticus</i>	Single Active-Site Cleft Mutation (from tyrosyl residue to glycyl residue)	40%	[168]
Strains	<i>Acidothermus cellulolyticus</i>	Designed Mutation at active site	40%	[169]
	<i>Streptomyces griseoaurantiacus</i> (SG _{UV30} and SG _{Uvs})	UV Mutagenesis	57.40%	[170]
	<i>Bacillus</i> sp. Hcb-21	Chemical Mutagenesis (EMS, MMS, ENU, MMU & 5-bromouracil)	10 folds	[171]
	<i>Bacillus</i> N3	Physical and Chemical (Nitrous acid) Mutagenesis	1.7 folds	[172]
	<i>Acremonium cellulolyticus</i>	UV Radiations and N-methyl-N'-nitro-N-Nitro Guanidine (NTG)	1.4 folds	[173]

Table 3: Optimized parameters for microbial cellulase production through fermentation.

Microbial	Fermentation	Optimized Parameters					Ref
		pH	Temp	C Source		N Source	
<i>Pleurotus sapidas</i>	Submerged	5	25°C	Fructose		Beef Extract	[174]
<i>Aspergillus niger</i>	Both Solid State and Submerged	6	30°C	Lactose		Peptone	[175]
<i>P1</i> fungal Isolate	Solid State	6	30°C	Lactose		Peptone	[176]
<i>Aspergillus</i> sp. B11	Solid State	7	37°C	Banana Leaves		Tryptone	[177]
<i>Aspergillus flavus</i> AT-2	Solid State	5.3	35°C	Wheat Straw		(NH ₄) ₂ SO ₄	[178]
<i>Enhydrobacter</i> sp. ACCA2	Submerged	6.5	30°C	CMC		Peptone and (NH ₄) ₂ SO ₄	[179]
<i>Bacillus subtilis</i> K-18	Submerged	7	30°C	2% Potato peel		1% yeast Extract	[180]
<i>Streptomyces</i> sp.	Submerged	7-7.5	26°C	Meat Extract and Tryptone		Glucose and Cellobiose	[181]
<i>Mirococcus</i> sp.	Submerged	5	25°C	Yeast Extract and Tryptone		CMC	[182]

in industries. In order to further exploit the potential of wild type microorganisms, strain improvement is required which can modify or eliminate specific trait [35]. The commercial production can be enhanced by Recombinant DNA technology [11], Site directed Mutagenesis [2], Recombination Protoplast fusion [36] and Random Mutagenesis as explained in (Table 2) [37]. Immobilization of cellulase on any solid support is also one of method to improve the efficiency of catalytic activities of enzymes for the industrial applications.

Genetic Recombination

Strain improvement through genetically modifying the microbial strains *via* different molecular techniques, which has gained much importance [38].

Recombinant DNA Technology

The Recombinant DNA technology is applied to introduce novel characteristics in an organism by manipulating their genome through

inserting or removing the genes or by modification of regulatory regions. The strain improvement through this technology involves the introduction of genes through vectors and desirable traits from different sources [39]. The specificity and yield of cellulase can be enhanced by recombinant DNA technology to insert the cellulases producing gene in the targeted microbial strains [40]. The cellulase production by *Trichoderma reesei* (filamentous fungi) was enhanced by recombinant DNA technology [41]. Similarly, the *A. oryzae* A4 specie isolated from soil was mutagenized through Recombinant DNA technology by inserting four cellulase genes (*cel A*, *cel B*, *cel C* and *Cel D*) comprised the cellulase expression cassette. The increased secretion of cellulase will facilitate the degradation of cellulosic biomass and the conversion of end products [42].

Site Directed Mutagenesis

In site directed mutagenesis, the gene responsible for specific characteristic is manipulated through molecular tools that will

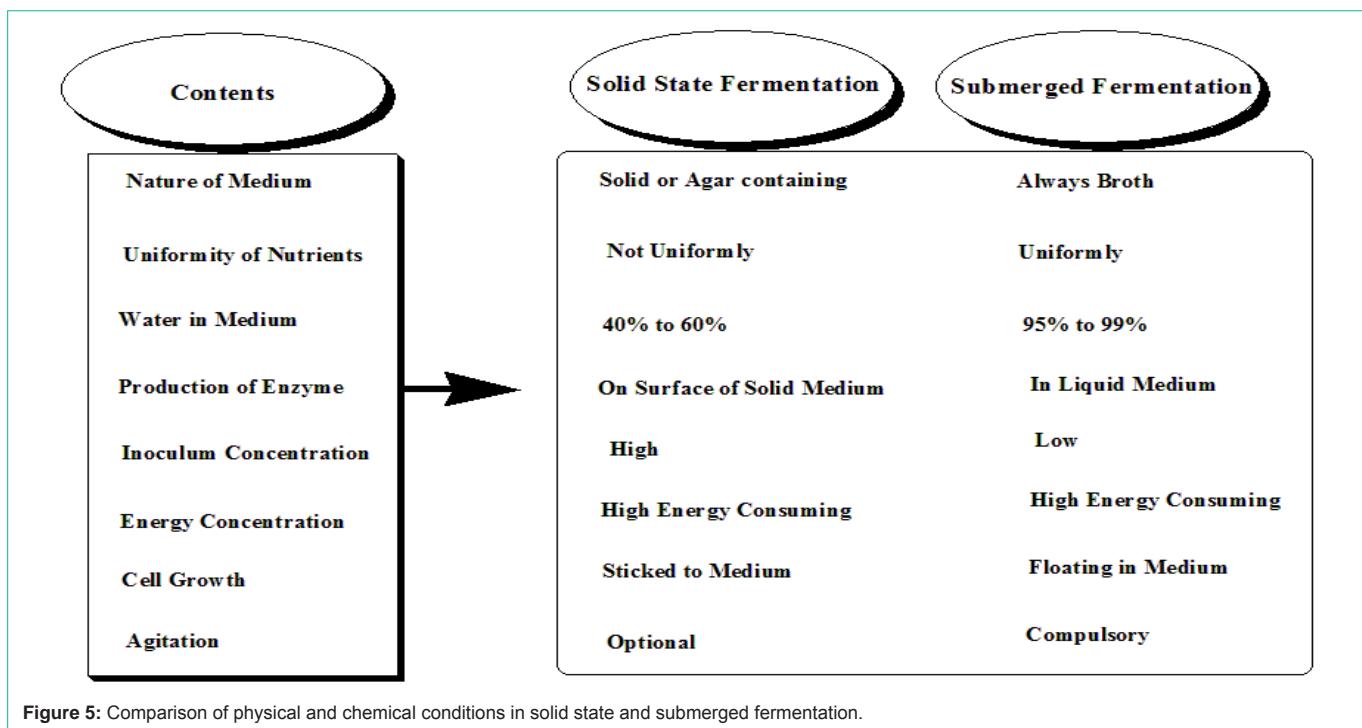


Figure 5: Comparison of physical and chemical conditions in solid state and submerged fermentation.

alternatively alter the function of that specific gene [43]. In this type of mutagenesis, the mutants are further improved through engineering, consequently the properties of enzymes such as temperature and pH profile, activity and thermo-stability will be enhanced. For the engineering of enzymes in directed evolution, the knowledge about the structure and function of enzymes, the enzyme-substrate interaction and catalysis is required [44]. Zhang et al. [45] improved the endoglucanases isolated from *Thermotoga maritima* through site directed mutagenesis. Similarly, Zhang et al. [46] also improved the β -galactosidase produced from *Aspergillus candidus* in order to reduce the galactose inhibition during hydrolysis of lactose.

Protoplast Fusion

Protoplast fusion is a recent technique for the strain improvement through which the desirable characteristics such as disease resistance, rapid growth, drought, heat and cold resistances, increasing protein quantities and for enhancing the industrially important products by fusion of protoplasts of two genetically different strains. Protoplast fusion is mostly used for the engineering of industrially important microorganisms and filamentous fungi [47]. This technique is non-specific recombination method and involves the breakdown of cell wall, protoplast regeneration and finally fusion of intragenic, intragenic and intraspecific protoplasts through chemicals. Moreover, electric current can also be used for producing potential mutants having specialized characteristics and also gain importance in applied genetics [48,49]. The interspecific protoplast fusion between different fungal strains that have been already reported includes *A. oryzae* and *A. sojae*, *A. fumigatus* and *A. nidulans*, *A. falipes* and *Aspergillus* sp, *Pencillium chrysogenum* and *Penicillium notatum*. The protoplasts of *Trichoderma harzianum* and *Aspergillus oryzae* were fused for the enhanced production of chitanases [50]. The β -galactosidase production was improved by fusing the protoplasts of *A. niger* and

A. oryzae and the fusion was facilitated by polyethylene glycol, CaCl_2 and NaCl [51].

Random Mutagenesis

The production of industrially important metabolites/enzymes can be shifted to desirable concentration and some new products can be produced by changing the pathways through mutagenizing the microbial strains. Some mutations can have detrimental effects and usually eliminated during screening while the potent mutants are selected during strain and process development by fermentation scientists. For random mutagenesis, the selected microbial strain is treated with mutagenic substance or radiations and after a desired log kill the survivors are selected and screened. The potent chemical mutagens for introducing random mutations includes; EMS (Ethyl methyl sulphonate), NTG (Nitrosoguanidine), HA (hydroxyl amine, MMS (Methylmethane sulphonate) while gamma radiations and ultra violet radiations are classified as effective mutagenic radiations [52]. Random mutagenesis or classical strain improvement is usually employed in food industries and involves the introduction of random mutations in the genome through ionizing radiations or different other mutagens, screening of mutants and selection of mutant possessing the desirable characteristic for the further use [35].

The strain improvement and selection of superior mutated strain for particular enzyme is the most painstaking effort. Vu et al. [38] reported the fungal strain improvement through repeated and sequential mutations for CMCase hyper-production by treatment with gamma-rays (γ -rays) of Co [60], UV and NTG. Ghonemy et al. [12] reported that the cellulase production from *A. oryzae* can be enhanced by multi-step mutations strategy such that in first mutation the fungus is treated with ultraviolet radiations (UV) and chemical mutagen N-methyl-N-Nitro-N-Nitrosoguanidine (NTG) and in second mutation step the fungal strain is treated with

ultraviolet radiations and ethidium bromide (Et-Br). Reddy et al. [53] mutagenized the *A. niger* by irradiating with UV and then treated with EMS (ethyl Methyl Sulphonate) for the enhanced production of cellulases. The improved strains can reduce the cost of the processes with increased productivity and may also possess some specialized desirable characteristics [54].

Production of Cellulase through Fermentation Technology

Cellulases enzyme can be produced from number of microorganisms such as yeast, fungi and bacteria. It can be produced in large quantity through fermentation technology [55]. To overcome the increasing demand for industrially important cellulases, the global production can be improved by improving the fermentation technology and augmentation of downstream processing for the recovery of purified cellulases in large amount [56]. The industrial production of cellulases can be carried out in both solid state fermentation and submerged fermentation [57]. Furthermore, according to Mahmoud et al the industrial production can be carried out in Submerged Fermentation (SmF), Solid State Fermentation (SSF) and Biofilm Fermentation (BF) (Figure 5) [58]. The cellulase activity can be determined by the standard protocol presented by Siddiqui et al [59]. One-unit cellulase activity was defined as the amount of enzyme required to release 1 μ mol of reducing sugar (glucose) per min from soluble cellulose at optimized conditions.

Solid State Fermentation

The fermentation in which microorganism are grown in lower amount of water for the production of industrially important products is known as solid state fermentation or SSF and was used for thousands of years in Western and Eastern countries [60]. The most attractive and promising technique for production of cellulases is solid state fermentation because it provides natural habitats to the fungi with minimum amount of water consequently results in the more fungal growth with higher enzyme yield. Moreover, it is preferred because of comparatively simpler technique, with lower substrate and energy utilization, and production of least amount of waste products [61]. Therefore, the down-stream processing will be comparatively easy. Cellulases produced by various microbial strains supplemented with different substrates in solid state fermentation is presented in (Table 3).

Submerged Fermentation

The other type of fermentation, which is utilized for the production of industrially important enzymes involve the culturing of microorganism in submerged or liquid media containing high amount of water named as Submerged Fermentation (Smf) [60]. However, submerged fermentation is advantageous because of simple instrumentation and easy recovery of product after fermentation including separation of biomass. The parameters such as scaling up, aeration, mixing, and water-soluble molecules content, temperature, pH and dissolve oxygen can be comparatively controlled easily [62]. The enzymes can be conventionally produced through submerged fermentation and is advantageous because of control and monitoring of environmental factors and easy handling [63]. Gamarra et al. [64] reported that the large scale cellulase production from filamentous fungal strains can be carried out on different carbon sources through

both submerged and solid state fermentation. It is also reported that the properties of enzymes produced by same fungal strain may vary when grown in different fermentation processes such as the properties of cellulases produced by same filamentous fungi in solid state fermentation may vary from that in submerged fermentation [15].

Biofilm Fermentation

Similarly, the biofilm fermentation is the advanced forms of solid-state fermentation, in which microbial cells are physiologically adhere to one another. The biofilm fermentation process can be used for the production of cellulases, different metabolites and some other metabolites along with its major application in waste water treatment. However, this type of fermentation process is most common for bacterial strain rather than fungal strain [65]. By attachment with any solid surface, the physiology of fungal cells changes due to differential expression of genes. However, the physiology of filamentous fungal cells grow in biofilm fermentation differs from that in submerged fermentation. The biofilm formation of filamentous fungal cells is usually easy as they can naturally attached to any solid substrate. For the immobilization of fungal cells for the production of enzymes, commonly surface binding and entrapment techniques are used [66].

Factors Affecting Cellulase Production

The established approach for enhancing of the enzyme productivity and cost reduction at industrial level involves the designing of experiments for the optimization of physical and factors [67]. The production of cellulase including endoglucanase can be influenced by both physical parameters such as temperature, pH and moisture and chemical factors such as carbon, nitrogen and minerals. Among all chemical factors, carbon is the most important source that usually acts as a substrate and depends on the yield of enzyme. The substrate must be easily available and cost effective. The selection of appropriate and cheap carbon source, nutrient media, culture and optimization of conditions are required for the maximum production of Endoglucanase [68]. The optimization of physical (temperature and pH) and Chemical (Carbon and Nitrogen sources) for cellulase production by bacterial and fungal strains is reported in (Table 3).

Hoa and Hung [69] optimized the chemical requirements (substrate, carbon, nitrogen and trace elements), and physical conditions (temperature, pH, moisture content and incubation time) for the production of Endoglucanases from *Aspergillus oryzae*. It is reported that different parameters such as temperature, pH and inoculum size were optimized for the cellulase and pectinase enzymes produced by *Aspergillus niger* on corn cob (substrate). For the maximum production of cellulases from fungal strains, bagasse powder can also be used as a substrate. The physical (temperature, pH) and chemical conditions (different substrates and other nutrients) for novel fungal strain *Aspergillus tubingensis* isolated from rotten tomato was optimized through response surface methodology [70,71]. Goyal et al. [72] also investigated the effects of temperature, pH, incubation time, different substrates in variable concentrations (carbon source) and nitrogen sources on the maximum production of CM Cases from *A. niger*.

Purification and Characterization of Cellulases

Due to wide variety of applications, the cellulases must be

Table 4: Purification of microbial cellulase.

Microbial Strain		Ammonium Sulphate Saturation (%)	Purification Method	Purification Folds	Recovery (%)	Ref
Fungal	<i>Penicillium decumbens</i>	80%	Ion-exchange Chromatography	4.3	42%	[1]
	<i>Schizophyllum Commune</i>	60%	Ion-exchange Chromatography & Gel-Filtration Chromatography	4 & 3.7	41-72.8 %	[2]
	<i>Aspergillus terreus</i>	80-95 %	DEAD-Cellulase Chromatography Ultra Gel Column Chromatography	270	22.11%	[3]
	<i>Aspergillus terreus</i>	-----	Ion-exchange Chromatography	7.6	82.20%	[4]
Bacterial	<i>Trichoderma longibrachiatum</i>	80%	Ion-exchange Chromatography & Gel-Filtration	7.85 & 9.69	78% & 68.8%	[5]
	<i>Pseudomonas Fluorescens</i> (Wild type)	90%	Ion-exchange Chromatography & Gel-Filtration	24 & 52	44% & 19%	[6]
	<i>Bacillus Vallismortis</i>	80%	Ion-exchange Chromatography & Gel-Filtration	11.6 & 39.1	75.4% & 28.8%	[7]
	<i>Pseudomonas</i> sp.	40%	Anion-exchange Chromatography	14.52	33.40%	[8]
	<i>Paenibacillus</i> sp.	80%	DEAD-cellulose Column Chromatography & CM-cellulose Column Chromatography	5.9 & 9.7	78.9% & 35.7%	[9]

purified and characterized as this will removed contamination as well as enhance their activity for specific function. In a study conducted by Elshafei et al. [73] the endoglucanase from *Aspergillus terreus* was purified through acetone fractionation and Sepharose-4B chromatographic column. Nehad et al. [74] reported that for better understanding the mechanism of hydrolysis of cellulose into end products, the cellulases purified and characterized after isolation through different techniques. The crude enzyme can be partially purified through ammonium sulphate saturation and then further purified through ion-exchange chromatography and high-pressure liquid chromatography. Nguyen and Quyen purified the endoglucanase from *Aspergillus oryzae* by 65% ammonium sulphate saturation followed by dialysis and further purified by Sephadex G-100 column washed with 50mM potassium phosphate buffer of pH 7.5 [75].

The β -glucosidase produced by *A. oryzae* and *A. niger* fusants was characterized and purified by ammonium sulphate precipitation and ion-exchange and gel filtration chromatography using G-150 sephadex gel and the specific activity of enzyme was enhanced after purification. [51]. Mostly enzyme has their specific/optimum parameters at which it works properly and maximum. Above or below the optimum parameters such as pH, temperature, the activity of enzyme declines. The purification methods of cellulase produced by different microbial strains and the folds and recovery obtained after purification is explained in (Table 4).

Immobilization of Cellulase Enzyme

An alternative to the soluble enzymes is the use of immobilized enzyme on a supporting material. Using suitable matrix for the purpose of enzyme immobilization was discovered in 1916. Various types of materials, ranging from natural to synthetic including synthetic polymers, inert, inorganic polymers and nanoparticles, are being used for immobilization. The attachments of enzymes to these supports enhance their stability/adoptability, selectivity, manoeuvrability and product recovery [4,76,77]. Furthermore, the recyclability of the immobilized enzymes reduces the cost of the production process [78-80]. Immobilized enzymes can be employed in chemical, food and pharmaceutical, textile industries and found potential applications in pharmacology, agricultural sciences, cosmetology, bio medicines as a biosensor for the diagnosis of different diseases such as diabetes and as drug delivery system [81]. In industrial bioprocess large

concentration of cellulases loading is required for the degradation of polymeric Cellulose. Thus, the degradation of cellulose into monomeric product can improve by immobilizing the cellulases on solid matrices which results in the improvement in thermal stability, reusability and efficiency. Therefore, cellulase immobilization is considered as very cost effective. The two most important factors that can affect the immobilization process include immobilization methods and immobilized solid matrices [82]. Recently in 2018, Benkun Qi and co-workers immobilized cellulase on MOFs (UIO-66-NH) and reported that the process has improved the catalytic activity and stability of cellulase mainly in the harsh conditions of temperature and pH. Due to improved catalytic activity the cellulases can be reused in multiple catalytic reactions as compared to soluble cellulases [83].

Industrial Application of Cellulase

Cellulases are the major industrial enzymes that play a key role in agriculture and Biotechnological industry. Cellulases enzymes are produced widely in lab and industrial scale for the different applications in food industry, improvement in animal feeds, fermentation technology, textile industry, detergents, cosmetics, pulp and paper industry, brewing and wine making, biomass refining, and laundry (Table 5) [84,85]. Cellulases have been used in detergent and soap industry to remove stains and in production of biofuel for transport by hydrolysis of agricultural waste to bioethanol [38,86].

Pulp and Paper Industry

The use of enzymes in pulp and paper industry for the recycling of pulp so this is the key interest to overcome the limitations of other ways of recycling of pulp. Due to the limitations of other methods, the interest of using cellulases for the applications in pulp and paper has been increased since 1980s [87]. The cellulases are successfully used for the removal of ink and polluted particles without any damage to the strength and brightness of the paper. This enzyme also has been successfully used for the pulping, bleaching, bioremediation of waste that are produced as a raw material during processing and have also great application in enhancement of fibres [88-91]. The biomechanical pulping, grinding, and refining are the energy saving (20-40 %) process and lead to the improvement of sheet strength and brightness [91,92-98]. Cellulases treatment cleans the surface of fibres by removing the fines and peel off. The advantages of using cellulases

Table 5: Industrial applications of cellulase.

Industry	Process	Ref
Pulp and paper industry	Recycling of pulp	[87]
	Removal of ink and polluted particles; Bioremediation of waste	[88-91]
	Improvement of sheet strength and brightness; Cleans the surface of fibres; Improve the strength properties: Brightness: Cleanliness: Fairness: Deinking; Reduce the chlorine requirement	[92-98,91]
Bio-fuels production	Production of biofuels and ethanol	[101]
	Production of second and third generation biofuels	[99]
Textile industry	Wet processing	[103,104]
	Bio stoning of jeans; Biopolishing, Removal of dyes: Improvement in softness	[106,105]
	De-fibrillation of Lyocell; Bio-carbonization and Wool Scouring	[111,88,112-116]
Laundry and detergent industry	Used in detergents for the removal of dirt and for enhancing the glossier appearance, brightness and smoothness of the fabrics	[118]
Food and feed industry	Improves the methods of stabilization, extraction of fruit and vegetable juices; Found productive by improving the colour and texture	[88,120,121]
	Decreases viscosity, improves flavour properties; Reduces the bitterness of citrus fruits;	[88,183,184,104,185]
	Improve the animal feed by providing extra supplementary support for better digestion and also cause gain in the weight	[88,104, 124]
Agricultural industries	Degradation of the cell wall of the plant pathogens and keep plant safe from dangerous diseases; Improves roots, crop yield, germination of seed, plant growth	[88,107]
Treatment of diseases	Can be used for the diagnosis and treatment of many diseases like Phytozoa, Blinding keratitis and Granulomatous Amoebic Encephalitis	[129,128]
Waste management	By utilizing cellulosic wastes, it converts them into valuable products; Cellulosic waste can also be used as a Carbon source in fermentation process	[130]
Flavonoid's extraction	Used in the isolation of carotenoids and other flavonoids	[131]
	These flavonoids have applications in pharmaceutical, cosmetics and nutraceutical industries due to their anti-cancerous, anti-oxidant, anti-mutagenic, anti-aging and anti-inflammatory properties; Also used in the treatment of many diseases such as Alzheimer's disease, Cardiovascular disease, Atherosclerosis and many age-related diseases.	[132,133]
Research purposes	Useful for studying single cell proteins: In plant studies such as metabolic and genomics; Virology; For the isolation of protoplast	[71]

enzymes in pulp and paper industry are, to limit the alkali usage and reduces the fine particles, improve the strength properties, the brightness, cleanliness, fairness, deinking and the main advantage of application of cellulases in pulp and paper industry is that it reduce the chlorine requirement which control the environmental impact [91,92-98].

Bio-Fuels Production

Cellulases have also found its potential application in the production of biofuels and ethanol. At industrial level the cellulases hydrolyse the fermentable sugars in the cellulosic and lignocellulose biomass and convert them into biofuels [99] by the action of cellulases enzyme system composed of endoglucanase, β -glucosidase and exoglucanase [100]. The demand for renewable energy sources is increasing day by day due to the depletion of naturally available biofuels. Therefore, the thirst for the cellulases production at commercial scale is also increased because of its application in the production of second and third generation biofuels [101]. However if the ethanol becomes the transportation fuel, then cellulases will be the major required industrial enzyme in the world market due to its potential role in the ethanol production by degradation of lignocellulose biomass [102].

Textile Industry

Cellulases enzymes have wide applications in textile for wet processing [103,104], bio-stoning of jeans, bio-polishing, removal of dyes, improvement in softness, removal of fuzz from surface of

fibres [105], reduce formation of pill, enhancing colour of fibres without need of chemical coating [106], improve brightness, bio-finishing [107] bio-stonewashing, bio-scouring [108,109], defibrillation of Lyocell [110], bio-carbonization and Wool Scouring [108]. Cellulases enzymes are used for bio-stoning which limited the abrasives washing. Blue jeans or denim garments have a great value in market from a few years but washing these garments from chemical treatment effects their shining and down their market. The alternative of washing from chemicals is enzymes processing from bio stoning which is also called bio stone washing. The use of cellulases for bio stone washing removes the dyes from the surface of fabrics and makes them to give look beautiful and shaded without damaging their strength [88,111-116]. The major advantages of using cellulase enzymes in textile industry are to improve shine and appearance. This process is permanent, environment friendly, highly productive, does not require intensive work, shorter time process, no heavy machinery required, fabrics give beautiful look and also the enzyme treatment is economical in comparison of other chemical treatment [88,117,116].

Laundry and Detergent Industry

Cellulases in combination with lipases and proteases are used in detergents and used for the removal of dirt and for enhancing the glossier appearance, brightness and smoothness of the fabrics [118]. The cellulases produced from *Bacillus* were also used in detergent and found that it restores their colour, improves softness and damage was negligible. Nowadays liquid laundry detergents have high market value and they improve the stability. Its components are

anionic surfactants, protease, cellulases, boric acid and citric acid etc. Cellulases degrades the cellulose elements/molecules and remove the roughness from the surface and give smoother look [119,104].

Food and Feed Industry

Cellulases have a key role in food biotechnology industry to improve the methods of stabilization, extraction of fruit and vegetable juices. The cellulases enzyme with the combination of other important enzymes are used in food processing industry to improve the extraction and clarification methods of vegetable and fruit juices and they also play role to increase the yield of juice without too much capital investment [88,120,121]. The cellulases have potential to improve the animal feed and their performance [122]. The nutritional value of agriculture silage and grain feed has improved by treating with cellulases and xylanase. Cellulases enzyme also remove anti-nutritional element factors and using of cellulases enzyme into animal feed also provide extra supplementary support for better digestion [123]. The supplementation of enzymes including cellulases with animal feed cause to gain weight and become healthy. The digestion and absorption of feed materials are improved by addition of enzyme including cellulases into feed, then it make the digestive process very proper and it help them to be healthy by gaining weight [88,104,124].

Agricultural Industries

Cellulases alone or in combination with pectinase, hemicelluloses have remarkable and diverse application in agriculture industries. These enzyme control diseases that are associated with plants and enhance yield of crops [88,107]. Cellulases with combination of related enzymes degrade the cell wall of the pathogens that cause disease to plant thus it keeps plant safe from dangerous diseases. β -Glucosidase is the key enzyme which play a great role in degrading the cell wall of plant pathogens [125]. Cellulolytic fungi also play role to improve roots, crop yield, germination of seed, plant growth. Cellulases improve soil quality thus it limited the use of mineral fertilizer [126,127].

Treatment of Diseases

Fungal and bacterial cellulases are used in the treatment of diseases such as Phytobezoars (caused by ingestion of plant that is indigestible and blocked gastrointestinal tract), blinding keratitis and granulomatous amoebic encephalitis (caused by *Acanthamoeba*) by degrading the cell wall of plants and biofilms [128]. The immobilized cellulases can be used as a bioreactor for enzyme related (Enzyme deficient diseases), use as a sensor for bioremediation and analytical flow system and can be used for the diagnosis and treatment of many diseases in the medical field directly or indirectly. The cellulases either individually or in enzyme cocktail form can be used in the treatment of many pathogenic diseases by disrupting the cellular membrane [129].

Waste Management

Cellulases also play key role in the waste management by utilizing forests, agricultural and industrial cellulosic wastes and converted into valuable products. The cellulosic waste can be used as a carbon source in fermentation process and consequently produce food and feed products, enzymes, organic acids, biofuels etc [130].

Flavonoids Extraction

Cellulases are also used in the isolation of carotenoids and other flavonoids from flowers used as colouring agents in the food industries [131]. Flavonoids are low molecular weight secondary metabolites produced in plants, vegetables, flowers and also present in some beverages and present in variable structures. The flavonoids also found potential applications in pharmaceutical, cosmetics and nutraceutical industries due to their anti-cancerous, anti-oxidant, anti-mutagenic, anti-aging and anti-inflammatory properties. Flavonoids are also efficient in inducing enzyme systems in humans that are involved in providing protection against bacterial and viral infections. Moreover, these biological active molecules are also used in the treatment of many diseases such as Alzheimer's disease, cardiovascular disease, atherosclerosis and many age related diseases [132,133]. Haung et al. [134] reported the extraction of flavonoids from medicinal plant *Illicium verum* by cellulase-ultrasonic assisted technology. Chen et al. [135] also reported that cellulases involve in the modification of flavonoids by trans-glycosylation as well as also assisted in the extraction of the modified pigments from the plants (*Ginkgo biloba*) by degrading the cell wall.

Research Purposes

Through the advancements in molecular biology cellulases also play key role in academic and industrial research. Cellulases are useful for studying single cell proteins and in plant studies such as metabolic and genomics, virology and in the development of hybrids and different varieties of plants by protoplast fusion. For the isolation of protoplast, the cellulases are used for the degradation of cellulose in plant cell wall [71].

Conclusion

Cellulases production from microorganism is gaining attention worldwide due to its increasing demand. Therefore, extensive research has been done in screening potent hyper producers of cellulases and already conquered the world enzyme global market to great extent. Cellulases can be produced from microbial strains through solid state and submerged fermentation processes at industrial scale by optimizing temperature, pH and nutritional requirements of growth media. Furthermore, microbial strains can be improved through mutagenic treatments (chemical and physical mutagens) for enhancing the production of cellulases. The stability and recyclability of cellulases can be enhanced through immobilization on solid supports. Cellulases has immense potential applications in various industries such as food and feed, laundry and detergents, paper and pulp, textile, biofuels and agriculture industries. Moreover, it is also employed for research purposes and in the treatment of diseases. Further advancements in biotechnology and microbiology are still needed to unlock the potential capabilities of cellulases.

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