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Functional Application of Thermo- Alkali- Stable Lignocellulolytic Enzymes in Kraft-Pulp Industry and Development of Fermentation Process for Production: A Review

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Abstract

For many years, microbial enzymes are commercially used as biocatalysts and efficiently catalyze various processes in industries. Biocatalysts are less corrosive to industrial processing equipment and due to their substrate specificity, they produced less toxic wastes which promotes environmental sustainability. At present, thermostable and alkali tolerant lignocellulolytic enzymes have gain enormous attention to be used as biocatalyst due their stability and robustness at high temperature and alkaline milieu. In this review, the characteristic of the several thermo–alkali-stable lignocellulolytic enzymes such as thermo–alkali-stable cellulases, thermo–alkali-stable xylanases and thermo–alkali-stable laccases as biobleaching agents in Kraft-pulp industry are described. This article discusses the characteristics of these enzymes such as their molecular weight, thermo-stability, pH tolerance, solvents compatibility and their stability towards the presence of metal ions and other chemicals. This review also discusses the development of fermentation process for the production of thermo–alkali-stable lignocellulolytic enzymes focusing on microorganisms (i.e. strain selection and strains improvement via mutation and recombinant techniques), culture medium optimization (i.e carbon, nitrogen and minerals) and other fermentation parameters (i.e. inoculums size, temperature, pH, agitation rate, aeration rate, and dissolved oxygen tension). The performances of strain producers in bioreactors and different mode of operation (i.e submerged and solid state fermentation) are also compared and discussed in this paper.

Keywords

Alkaliphilic; Biobleaching; Bioreactor; Cellulase; Fermentation; Laccase; Thermostable; Sustainable industry; Xylanase.

Introduction

Kraft-pulp industry is one of the fastest emerging markets world-wide. The world production of kraft-paper was estimated about 400 million tons in last few years [1] In Malaysia, the total production capacity in pulp and paper industry was over 1 million tones per year [1]. Woody plants are sources for Kraft-pulp-paper industry and they are composed of several structural constituent such as lignocelluloses, hemicelluloase, cellulose, xylan and lignin[1-3]. Normal pulping processes from plant rind, ramie fibers, oil-palm frond-fiber strands and kenaf uses Soda-Anthraquinone and other chemical catalyst are usually conducted at a very high temperature (150- 180°C) in NaOH (10-30%) for 30 to 100 min which eventually add cost to paper production [3,4]. The bleaching process to obtain pulp from woody plants and waste paper utilizes large amounts of chlorine and other inorganic compounds, which are not environmentally friendly and prospective toxicants, teratogens, mutagens and carcinogens [3,5]. Moreover, coupling agent such isocyanate is used to improve matrix-fiber interaction and increase thermal stability of the modified pulp-paper but its toxicity effect can cause irritation of skin and even breathing problems [3,5]. Due to these circumstances, enzymes are potential sources of biocatalysts and biobleaching agents, which have gain enormous interest due to latest discovery of enzymes, alternative to hazardous chemicals in Kraft-pulp industry [6]. Several lignocellulolytic enzymes which have been identified as important biobleaching agents are cellulases, xylanases and laccases [5-10]. These lignocellulolytic enzymes generally poses a catalytic domain, with one or more non-catalytic Carbohydrate Binding Module (CBM), accessing recalcitrant polymers [11,12,13]**.** Cellulases (E.C.3.2.1.4) such as endo-1,4-β-glucanases and exo-1,4-β-glucanases (E.C.3.2.1.91) accelerate cellulolysis process in cleaving the internal bonds of the cellulose polymer [7,8]. On the other hand, xylanases are very important to pulp and paper industries due to their ability to the breaking down of the xylan, thereby breaking down the link between the cellulose and lignin [9]. Studies also showed that xylanases also improves the brightness stability of bleached pulps. Laccases (E.C.10.3.2) are cooper containing *p*-diphenol dioxygen oxidorecductases, other enzymes important for degradation of lignin and help in grafting polymerization in pulp-paper industry [5,14]. These enzymes offer some advantages in producing high paper quality at lower temperature with increasing tensile strength of pulp-paper as compared to chemical catalysis process. However, these enzymes have been criticized due to lack of thermostability and intolerant to alkaline environment [15,16]. In industry, combination of several parameters such as high temperature, alkaline condition and time are necessary to obtain the high quality pulp and paper [3]. To date, several thermo-alkali-stable (TA) enzymes for Kraft-pulp industry has been reported. So far, the development of fermentation process for the production of TA cellulases, TA xylanases and TA laccases have been intensively studied. Some details of industrial application and fermentation techniques are rarely reviewed. Moreover, some commercially valuable knowledge has been patented by enzyme producing companies and paper manufacturers (i.e. Cartazyme, EcopulpX-200) [17,18,19]. This review describes and discusses the potential applications of TA enzymes such as TA cellulases, TA xylanases and TA laccases as biocatalyst and biobleaching agent in Kraft-pulp industry and their characteristics, as well as the development of production process via microbiology and fermentation approaches.

Application for Kraft pulp

Lignocelluloses (i.e. cellulose, xylan and lignin) are extremely complicated starting biomaterials (i.e. tight packing of linear and rigid crystalline structure), which are resistant to decompose into

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smaller units [4,16]. Several microbes such as bacteria and fungi produce complex extracellular and some intracellular enzyme such as cellulase, xylanase and laccases and they act as denim bleaching and pulp bleaching agents to convert lignocelluloses into smaller manageable units which can be further transformed into useful textile, pulp and paper products materials [4,14,20-22]. For this purpose, lignocelluloses starting materials can be degraded and depolymerized by chemical, enzymatic or combination of both techniques and processes [21]. The ability of lignocelluloses degrading enzymes from several glycosyl hydrolases (GH) families to access, penetrate and break the recalcitrant structure of cellulose in an environmentally friendly, high specificity and low-energy manner, serve a potential for purely biochemical processing of lignocellulosic biomass using enzymatic methods [14,22]. One of the most important factors limiting the wide industrial use of cellulases is the fact that these enzymes need to perform under harsh conditions, such as high temperature, alkaline and detergents milieu which can all cause protein denaturation and loss of catalytic activity [2,14,21-22]. Under such environment, the majority of the existing enzymes perform very poorly. Therefore, new and improved TA lignocellulolytic enzymes (TA cellulase, TA xylanase and TA laccase) with ability to retain their catalytic activity in such industrial environments were identified and studied [23,24]. It has been reported that the purified CMCase from *B. licheniformis* AMF-07 retained its activity in the alkaline environment from 33% to 122% [25]. The purified CMCase from *B. halodurans* CAS1 retained its activity in the presence of some commercial detergents in alkaline environment from 64.67% to 85.33% [26]. CMCase from *B. licheniformis* AMF-07 was highly stable towards temperature at 70°C and pH 9.0 could make this enzyme as a good candidate for Kraft-pulp application [17]. Studies also showed that the action of cellulases treatment on kraft pulp decreased defibrillation and fibre roughness and improve brightness as well as physical properties of pulp [23-24,27]. The xylanase from *Bacillus sp, Staphylococcus sp*. SG-13, *Bacillus sp*. NCIM 59 and *Aspergillus sp.,* showed their significant application for biobleaching of kraft pulp at 60.0°C, pH 9.0 as indicated by an increased brightness, chromophores and sugars release and reduction of kappa number [24,28]. For instance, TA xylanase from Bacillus strain Ag strain Ag efficiently catalyzes bleaching process of pulp after 2 h treatment [28]. The addition of EDTA and hydrogen peroxide help in reducing the kappa number by up to 75.3% and increased the brightness by up to 82 ISO units [28]. An additive such as hypochlorite also help in biobleaching of kraft pulp with significant reduction in the kappa number by 30%, enhanced the brightness up to 11% [24]. On the other hand, in Kraft pulp alkali extraction process by xylanase enzymes of *Aspergillus sp* increased brightness up to 45.0 ISO units (3 folds) and reduced kappa number to 5.0% from 18.6% [24]. Recently, study showed that xylanase treatment suppresses light- and heat-induced yellowing of pulp [1]. In kraft-plup industry, laccases biocatalyze attachment of functional phenolic compounds onto cellulosic fibers, to improve paper quality against microbial degradation and increase tensile pulp strength in various environmental conditions [24,28,29,30].

TA Enzymes characteristic

In general, TA enzymes molecules are normally have single polypeptide chain and may appear as monomeric molecules. Cellulases were also found in Bacillus species where size of the TA cellulase from *B. pumilus* S124A and *B. licheniformis* AMF-07 were 40 kDa and 37 kDa, respectively [25,31]. The TA cellulase from *B. licheniformis* AMF-07 remained 100% of its catalytic activity at temperature up to 70°C, and started to undergo denaturation where enzyme activity was substantially reduced after heat treatment at temperature greater than 80°C. The thermostability of the purified TA cellulase AMF-07 at 70°C, was similar to that of TA cellulase of *Bacillus sp*. SMIA-2 and comparatively higher than TA cellulase of *B. subtilis* 4-1 (60°C) and *B. pumilus* S124A (50°C) [31-32]. Thermo-stable enzymes of such cellulases are advantageous for Kraft-pulp applications, because higher processing temperatures can be employed, with consequent faster reaction rates, improved hydrolysis of cellulosic substrates, and reduced incidence of microbial contamination from mesophilic microorganisms. TA cellulase from *B. licheniformis* AMF-07 and *B. pumilus* S124A was found to be stable in the broad range of pH (6.0–10.0) where enzymes activity was retained to about 68% to 75% [31]. However, CMCellulase by *B. pumilus* S124A was optimally active at pH 6 [31]. TA cellulase from *B. licheniformis* AMF-07 was not only tolerant at alkaline pH but also halo tolerant in high concentration of NaCl (20-30%) [25]. TA cellulase (CMCase) activity from *B.licheniformis* AMF-07 was greatly enhanced in the presence of Ca^{+2} and Cu^{+2} metal ions as compared to other metal ions such as Mn^{+2} and Zn^{+2} and surfactant such as Triton-100. However, TA cellulase AMF-07 activity was greatly inhibited by Co^{+2}, Hg^{2+}, K^{+}, and hydrogen peroxide (H_2O_2) [25]. The inhibition by Hg^{2+} ions is not just related to binding the thiol groups but may be the result of interactions with tryptophan residue or the carboxyl group of amino acids in the enzyme [15,26]. The activity was greatly inhibited by EDTA, indicated that the cellulase purified from this study was a metal containing enzyme [27]. Moreover, Cellulase AMF-07 had high catalytic activity in the presence of organic solvents such as cyclohexane (134%) and chloroform (120%) [25]. On the other hand, cellulase from *Bacillus vallismortis* RG-07 had molecular mass of 80 kDa as demonstrated by SDS-PAGE analysis which among few cellulases with high molecular weight [9,25,27]. Even though optimal temperature and pH for TA cellulase RG-07 catalytic activity was at 65°C and 7.0 respectively, its relative catalytic activity could be retained for up to 95% at 95°C and 75% at pH 9.0 [7]. TA cellulase RG-07 catalytic activity was also stimulated when come in contact with several hydrophobic solvents such as n-dodecane, n-decane, n-butanol, xylene, toluene, isooctane, n-haxane and cyclohexane but greatly inhibited by metal ion such as Hg^{2+} [9]. However, catalytic activity of endoglucanase Mut43 could be enhanced in the presence of Mg2+ and Ca2+ at certain concentration [33]. TA cellulase (endo-1,4-beta-glucanase) from Bacillus KSM-S237 had a molecular mass of approximately 86 kDa with optimal pH at 8.6-9.0 and displayed maximum activity at 45°C [27,34]. The TA endo-1,4-beta-glucanase was stable up to 50°C and more than 30% of the initial activity was maintained at 100°C and pH 9.0 for 10 min incubation time. TA laccase from Bacillus subtilis cjp3 also very stable at temperature ranged from 20-80°C, pH 9.0 up to 10 h [35]. On the other hand, TA xylanase from a thermophilic *Anoxybacillus sp*. Ip-C, was about 45 kDa, and had optimum catalytic activity up to 90% at pH 9.0 and 70°C for 96 hrs [9,20]. Metal ions such as Ca^{+2}, Fe^{+2} and Mg^{+2} highly enhanced the Ip-C enzyme catalytic to about 122.45, 119.06 and 118.98% respectively; whereas SDS and Hg^{+2} completely inhibit its activity [20]. The catalytic activity of some TA enzyme such as cellulase RG-07, xylanase *Anoxybacillus sp*. Ip-C and cellulase Cel5R was inhibited by thiol reagents like Hg^{2+} could suggest that cysteines which are presence in their molecules structure might play important role in catalysis [10]. However, structure-biochemical characterization has demonstrated that free cysteine residues have stabilizing effect and play a role both in thermo stability and catalytic activity to the protein even though they are not part of the catalytic active site [10]. Several characteristic of TA cellulases, TA xylanases

Table 1: Characteristics of several thermo–alkali-stable lignocellulolytic enzymes.

	TA enzymes	MW	T-opt (°C)	pH	Stimulants	Inhibitors	Chemicals with no effect	Reference
Cellulases	Avicelase SMIA-2	-	70	7.5	-	TritonX-100, H_2O_2	SDS, RENEX-95	[21]
	CMCase SMIA-2	-	70	8.0	-	-	protease	[21]
	Cellulase AMF-07	37	70	9.0	NaCl, C_6H_{12}, chloroform	Co^{+2}, Hg^{2+}, K^+, H_2O_2	-	[17]
	Cellulase *S124A*	40	40-70	4-8	$CoCl_2$	$HgCl_2$, EDTA, PMSF	-	[20]
	Cellulase 4-1	-	20-90	5-10	-	-	iodoacetate, EDTA, $C_{12}H_8N_2$, PCMB	[26]
	Cellubiohydrolase and endoglucanase SWU-27	-	80	10	-	-	-	[43]
	Cellulase KSM-S237	86	45	8.6-9.0	-	-	-	[18]
	Cellulase RG-07	80	65-95	7-9	Ca^{2+}, $C_{12}H_{26}$, C_8H_{18}, $C_{10}H_{22}$,xylene,toluene, hexane,butanol,C_6H_{12}, Tween-60,C_2H_6OS, NaClO	Hg^{2+}	-	[6]
	Cellulase ITI-378	49	90	6-8	-	-	-	[30,31]
	Cellulase HTA426	40	50-70	4-8	$CaCl_2$, NaCl, KCl, SDS, Triton X-100, Tween-80	$ZnSO_4$, $CuSO_4$	-	[24]
	Endoglucanase *Mut43*	30	80	8.0	Mg^{+2} and Ca^{+2}	Proteinase, SDS, organic solvents	-	[22]
Xylanases	Xylanase ARMATI	-	60	9	-	-	-	[29]
	Xylanase Ip-C	45	70	9	Ca^{+2}, Fe^{+2} and Mg^{+2}	SDS and Hg^{+2}	-	[7]
	Xylanase Ag	-	60	9	-	-	-	[19]
	Xylanase XynHB	30	60	8.6	-	-	-	[15]
	xylanase J18	-	70	7-8	-	-	-	[44]
Laccasess	Laccase SN4	-	90	8	-	-	-	[13,14]
	Laccase WT	180	55	5-8.0	Na^+ and Ni^{2+}	L- Cysteine	NaCl, NaN_3	[9]

Note: MW, molecular weight (kDa); T-opt, optimal or favourable temperature for enzyme activity; C_6H_{12}, Cyclohexane; H_2O_2, hydrogen peroxide; NaClO, Sodium hypochloride; C_2H_6OS, mercaptoethanol; $C_{12}H_8N_2$, 1-10-phenanthroline; $C_{12}H_{26}$, dodecane; SDS, sodium dodecyl sulfate; PCMB, 4-chloromercuribenzoic acid; RENEX-95, detergent; C_8H_{18}, isooctane; $C_{10}H_{22}$, decane; PMSF, phenylmethane sulfonyl fluoride; EDTA, Ethylenediaminetetraacetic acid

Table 2: Source microorganisms of thermo–alkali-stable lignocellulolytic enzymes.

Type	Microorganisms	Origin	References
Thermophile	*Bacillus licheniformis* AMF-07	Gorooh hot spring	[17]
	Geobacillus sp. HTA426	Hot spring district	[24]
	Aneurinibacillus thermoaerophilus WBS2	Indian hot spring	[25]
	Anoxybacillus sp. Ip-C	hot spring of Ladakh	[7]
	Paecilomyces themophila J18	Soil samples	[44]
	Rhodothermus marinus	alkaline submarine hot springs	[30,31]
	Aspergillus terreus 10138	Wadi El-Natrun soda lakes in northern Egypt	[39]
Mesophile	*Bacillus* sp. SMIA-2	Soil sample from Campos dos Goytacazes city	[21]
	B. subtilis 4-1	Traditional Korean fermented soybean paste	[26]
	K. pneumoniae SWU-27	Garbage Dump	[43]
	Bacillus vallismortis RG-07	Soil sample	[6]
	Bacillus tequilensis strain ARMATI	Feces soil samples from poultry farm	[29]
	Bacillus strains (Ag12, Ag13, Ag20, Ag32)	Acı-Göl Lake	[19]
	Micrococcus sp.	Decaying plant biomass (sawdust)	[34]
	B. pumilus S124A	Soil sample	[20]
	Bacillus sp. strain WT	Urmia lake, a hypersaline lake in Iran	[9]

and TA laccases related to its optimal catalytic temperature, pH and stability in organic solvent and metal ions are summarized in Table 1.

Development of TA enzymes fermentation

Microorganisms: The major source of TA cellulases, TA xylanases and TA laccases was belonged to *Bacillus sp*, a member of the genus Firmicutes (Table 2). Examples of TA cellulases from *Bacillus sp.*, are such as *Aneurinibacillus thermoaerophilus* WBS2, *Bacillus sp.* (i.e. strain KSM-S237, SMIA-2), *B. licheniformis* AMF-07, *B. vallismortis* RG-07, *Anoxybacillus sp., B. subtilis* (i.e. 4-1, LM01, LM04), B. *pumilus* S124A and *Geobacillus sp.* HTA426. These industrially important *Bacillus sp.*, were mainly isolated from a variety of natural sources, such as soil, long-term garbage dumps, decayed plant materials, faeces soil samples from poultry farm, hot springs, organic matter, traditional soybean paste and feces of ruminants and compost [7,25,27,31,36-40]. Other sources of TA cellulases were originated from *Trichoderma reesei, Klebsiella pneumoniae* SWU-27 *Thermomonospora sp*. (T-EG) and even from thermophilic eubacteria such as *R. marinus* [41]. Other TA lignocellulolytic enzyme such as TA xylanase was also derived from *Bacillus sp*. (i.e strains Ag12, Ag13, Ag20 and Ag32), *B. tequilensis* (i.e strain ARMATI) and *Anoxybacillus sp*. Ip-C and even from *Staphylococcus sp*. SG-13 [9,28,42]. They were isolated from hot spring and soil composite [9,28,42]. On the other hand, TA laccases were obtained from bacteria and fungi such as *Bacillus tequilensis* SN4, Pycnoporus sanguineus, *Trametes trogii* LK13 and *Bacillus sp* WT [21,22]. Source of lignocellulolytic enzymes can also be obtained from actinomycetes, white-rot fungi and some microorganisms isolated from *Bulbitermes Sp*. Termite Gut [43-47]. In many cases, extremophilic organisms (thermophile, alkalophile) are a very rich source for such TA enzymes, as they have evolved to thrive in such extreme environments.

Strain improvement

The improvement of TA lignocellulolytic enzymes by strain producer through mutation and recombinant has been reported. A mutation strain XynHBN188A was developed via site-directed mutagenesis of xynHB where it has been demonstrated that mutant strain has an increased thermo-stability up to 1.5-fold as compared to wild strain at 60°C for 30 min [23]. Another examples showed that the mutant XynATM1 harboring a Glycoside Hydrolase Family 11 (GH11) catalytic module without non -catalytic carbohydrate -binding modules (CBM) showed an improved thermal stability compared to XynA17 [23]. In recombinant strain construction, DNA isolated from respective source was sequenced, bioinformatic analysis to identify sequences encoding for putative cellulolytic enzymes [48]. Several studies have reported the role of different host strains produce high level of exogenous cellulases, xylanases and laccases, like *Bacillus, Trichoderma reesei, Pichia pastoris, Saccharomyces cerevisiae and Escherichia coli* [23,48-53]. For instance, glycoside hydrolase family 5 (GH5) cellulase, CelDZ1 gene was cloned and overexpressed in *Escherichia coli* BL21(DE3) with pET-CelDZ1α [53-55]. However, *P. pastoris* is known for its properties of efficient enzyme secretion, cellulase free, and fast growth with high cell density in simple media suitable for specific recombinant cellulase production or cellulase-free xylanase synthesis [23,51]. For example, the mutant GH11 TA xylanase gene xynHB from *B. pumilus* HBP8 was cloned and expressed in *P. pastoris* [23]. The xylanase activity in the supernatant of the recombinant *P. pastoris* expressing mutant xynHB was effective with high catalytic activity in alkaline condition (pH 8.6) at high temperature (60°C) [23,51]. The recombinant strains producing high-yield of TA cellulase and TA xylanase are potentially used for industrial application with less downstream processing and purification, thus TA enzymes could be produced at ease and cost efficient. In addition, studies have showed that thermo-stability of xylanases was improved via the introduction of disulphide bridges in their 3D conformation structure which enhance the protein positive charges and lower the configuration entropy of unfolding (i.e. hydrogen bond system). For instance, thermo-stability of xylanase (20 kDa) of *B.circulans* was significantly increased by the introduction of both intra and intermolecular disulfide bridges via site-directed mutagenesis [54]**.** On the other hand, recombinant TA laccase from gene of *Streptomyces griseorubens* JSD-1 was also successfully expressed in *E. Coli* Trans B(DE3) and its expression was induced by isopropyl β-D-1-thiogalactopyranoside (IPTG) [55]. Study also reported that E .coli harboring laccase gene from B. subtilis cjp3 expressed a high laccase activity (7320 U/L) at an optimized experimental condition in shake flask [54]. On the other hand, laccase gene from *Bacillus licheniformis* LS04, mutant LS04 and Trametes versicolor was expressed in *P. pastoris* where experimental conditions were further optimized in shake flask to obtain a high expression of laccase (227.9 U/L to 12, 344 U/L) [50, 55-59].

Inoculums size

Seed cultures were normally prepared using 6 to 16 h old inoculums (i.e for bacterial culture) at respective fermentation medium and condition. Size of inoculums are varied and depends on type of microorganisms to be inoculated into the fermentation medium although ranged of 1 to 5% (w/v) have been reported sufficient for maximum activity and yield of TA lignocellulolytic enzymes. TA glucanases was optimally produced by *B. licheniformis* AMF-07 at 5% (v/v) inoculums size [25]. The inoculums size of 1.25% (v/v) of *Bacillus sp*. was used for maximum TA xylanase production [20]. Furthermore, inocolum size of 2% (v/v) has been reported for TA enzymes of *B. subtilis* 4-1, *Aneurinibacillus thermoaerophilus* WBS2 and *Micrococcus sp*. It is essential to determine the suitable and the optimum inoculums size for optimal number of active microbial cells needed for TA enzymes production. Large amount of inoculums size can cause overproduction of microbial mass and numbers of microbial cells, which later cause inefficient mass transfer, low oxygen level and eventually, affect overall production of TA enzymes. Moreover, high inoculums size was likely unsuitable in scale up process and large-scale production in bioreactor. Significance study of inoculums size to other fermentation parameter and yield of TA enzymes was usually determined using software like response surface methodology (RSM) and artificial neural network (ANN).

Medium composition

Several carbon and nitrogen containing substrates may be used as carbon and nitrogen sources respectively for TA lignocellulolytic enzymes fermentation (Table 3). These carbon containing substrates include glucose, CMC, soluble starch, wheat bran, rice bran, rice husk, maize bran and sugarcane bagasse. On the other hand, nitrogen sources may derived from organic (i.e. yeast extract) or inorganic (i.e. KNO_3 and NH_4NO_3) nitrogen containing substrates. For instance, high yield of TA cellulases by *B. licheniformis* AMF-07 was in fermentation using 0.1% glucose and 0.05% yeast extract (C/N ratio of 5:1) as carbon and nitrogen source respectively in 0.5% (w/v) carboxymethyl- cellulose (CMC) medium [25-27]. Although cellulase producing strain such as *B.subtilis* 4-1 can ferment several carbon and nitrogen sources such as glucose, cellulose, xylose, yeast extract, peptone and tryptone but the highest cellulase activity by *B.subtilis* 4-1 has been reported in fermentation using 1.0% of soluble starch and 0.1% yeast extract with C/N ratio of 10:1 in CMC medium [32]. High yield of cellulases (exoglucanases and β-glucosidases) by *Trichoderma reesei* can be obtained from in fermentation using wheat straw as carbon source [41]. Lignocellulolytic enzyme by Trichoderma Reesei could also be produced using cane molasses as an alternative and economical approach [58]. Under optimized condition, high yield of TA cellulase (100% of CMCase activity, 22% of avicelase activity and 15% of cellobiosase activity) by *B. licheniformis* AMF-07 was obtained in fermentation containing wheat bran and rice bran [25]. It also has been reported that rice bran was the most suitable carbon source for cell growth and TA cellulase production by other *Bacillus sp.,* while alkali-treated sugarcane bagasse was the most preferable carbon source for CMCase production by *Geobacillus sp*. HTA426 (103.67 U/mL) and *B. vallismortis* RG-07 (4105 U/mL) [7,36]. In

Table 3: The inoculums size, C/N ratios, temperature, pH, production duration time and activity of thermo–alkali-stable lignocellulolytic enzymes.

Strain /Origin	IS (%)	Medium composition	Temp(°C) / agit(rpm)	pH	Other minerals	Duration (h)	Activity (U/mL)	References
Bacillus sp. SMIA-2	-	Sugarcane bagasse (5 g/L), corn steep liquor (5 g/L)	50, 150	7.2	KCl, $MgSO_4$, K_2HPO_4, $CaCl_2$, ZnO, $FeCl_3$, $MnCl_2$, $CuCl_2$, $CoCl_2$, $NiCl_3$, H_3BO_3	120-168	0.83	[21]
B. licheniformis AMF-07	5	CMC (0.5%)	60, 160	6.0	-	72	-	[17]
B. subtilis 4-1	2	CMC (0.5%), soluble starch (1%), YE (0.1%)	60, 120	9	$(NH_4)_2SO_4$, NaCl, K_2HPO_4, KH_2PO_4, $MgSO_4 \cdot 7H_2O$, $CaCl_2$	24	170	[26]
Aneurinibacillus thermoaerophilus WBS2	2	Wheat (1%), rice straw (1%), NH_4SO_4 (0.2%), $NaNO_3$ (0.2%), YE (0.2%), beef extract (0.2%)	65, 150	9.0	-	40-60	0.46 IU/mL	[25]
Bacillus sp. KSM-S237	-	CMC (0.1%), YE (0.1%), meat extract (1%), Polypepton S (2%), sodium glutamate (0.5%)	30	-	K_2HPO_4, $CaCl_2$, $MgSO_4$, $FeSO_4$, $MnSO_4$, Na_2CO_3	40	-	[18]
B. vallismortis RG-07	-	Sugarcane baggase (2%)	65, 120	7	-	12-96	4105	[6]
Geobacillus sp. HTA426	-	Sugarcane baggase (1%)	60, 170	7	-	144	103.47	[24]
B. tequilensis ARMATI	1	Birchwood xylan (1.5%), YE (1%)	40	7	KH_2PO_4, $MgSO_4$, Na_2CO_3, NaCl	24	86 IU/mL	[29]
Amycolatopsis cihanbeyliensis Mut43	-	Wheat straw (1.5%), YE (0.6%)	32, 150	7.0	NaCl	72	5.21	[22]
Aspergillus terreus AUMC 10138	40	Corn Stover (1%)	45, SSF	9-11	-	168	1783	[39]
Anoxybacillus sp. Ip-C	-	Xylan (1%),NH4Cl (10 g/L)	60	7	KH_2PO_4, K_2HPO_4, $MgSO_4$, $CaCl_2$, $FeSO_4$, $CoCl_2$, $MnSO_4$, $ZnSO_4$	120	0.85 IU/mL	[7]
Bacillus sp. Ag12	5	Birch-wood xylan (1%)	35, 150	8.5	K_2HPO_4, $MgSO_4$, NaCl	24	3.7 IU/mL	[19]
B. tequilensis SN4	0.3	YE (0.6%), tryptone 0.2%)	30, 150	8.0	$MnSO_4$, $FeSO_4$ & ethanol	96	18,356 kats/ml	[13,14]
Micrococcus sp	2	Birch wood xylan (1%)	45, 200	10	-	84	2487	[34]

Note: IS, inoculums size (%,v/v); Temp, optimal temperature for TA enzyme production; agit, optimal or best agitation speed; SSF, solid state fermentation; YE, yeast extract; CMC, carboxymethyl cellulose; NH_4SO_4 , ammonium sulfate; $NaNO_3$, sodium nitrate; KCl, potassium chloride; $MgSO_4$, magnesium sulfate; K_2HPO_4, dipotassium hydrogen phosphate; $CaCl_2$, calcium chloride; ZnO, zinc oxide; $FeCl_3$, ferric chloride; $MnCl_2$, manganese chloride; $CuCl_2$, cooper chloride; $CoCl_2$, cobalt chloride; $NiCl_3$, nickel chloride; H_3BO_3, boric acid; NaCl, sodium chloride; $MgSO_4$, magnesium sulfate; Na_2CO_3, sodium carbonate; % for medium compoistion, w/v.

other studies, rather than using organic nitrogen sources such as yeast extract and beef extract, uses of inorganic nitrogen source such as KNO_3, $(NH_4)_2SO_4$ and NH_4NO_3 has been reported for cellulases production by several strains such as by *A. thermoaerophilus* WBS2, *Thermomonospora fusca* and *Cellulomonas flavigena* [39,41]. The TA cellulase activity from *Gracilibacillus sp*. SK1 was also high when using 27.1 g/L corn stover as substrate (with fermentation time up to 48 h) as compared to 20.4 g/L rice straw (with fermentation time up to 64 h) [39]. In the production of xylanase by *Anoxybacillus sp*. Ip-C, the use of 1% xylan, 10 g/L ammonium chloride (NH_4Cl) with an addition of some minerals in fermentation medium can obtained about 0.85 IU/mL endoxylanase activity [9]. A very high laccase yield by *Bacillus sp* was obtained using birch-wood xylan, but relatively high enzyme production was also obtained on wheat straw and corncob when cultivated at pH 8.5 [20].

Other minerals

Additional minerals were required for TA lignocellulolytic enzymes stability and help in TA lignocellulolytic enzymes production. For instance, minerals in the production of xylanase by Anoxybacillus sp. Ip-C are such as KH_2PO_4, K_2HPO_4, $MgSO_4$, $CaCl_2$, $FeSO_4$ $MnSO_4$, $ZnSO_4$ and $CoCl_2$ which may range from 2 to 0.02 g/L [9]. Some minerals and metal ions was added in the fermentation medium to enhance the stability of TA enzymes (i.e. Ca^{2+}) and facilitate in cell membrane stability while ion like potassium ions are largely required for ATP synthesis and transportation system, export TA enzymes out from the cells.

Temperature

Fermentation temperature regulates the level of mRNA transcription, translation and protein (enzymes) stability. The optimum temperature for TA lignocellulolytic enzymes production may sometimes corresponds to optimal growth temperature of strain producer. High production of several TA enzymes was observed at moderate to slightly elevated temperature ranged from 28°C to 37°C. For examples, strains producers such as *Amycolatopsis cihanbeyliensis* Mut43, *Bacillus sp*. Ag12, *Bacillus sp*. KSM-S237 and *B. tequilensis* SN4. Other TA enzymes are highly produced at high temperature and this includes TA cellulase by *B. subtilis* 4-1 and *B. licheniformis* AMF-07 was significantly high produced at 60°C [25,32]. The production TA cellulase by *A. thermoaerophilus* WBS2 was optimal at 65°C [39]. Most of the microbes are thermopiles or hyperthermophiles and capable to withstand and grow at a very high temperature. High TA enzymes at these temperatures usually related to high kinetic rate and mass transfer rate at high temperature. Other possible reason for production at elevated temperature may be temperature influences their secretion; possibly by changing the physical properties of the cell membrane. Some mRNAs of bacteria species are containing a temperature-sensitive region in the 5' untranslated region (UTR) which prevents the mRNA from binding to a ribosome and being

Table 4: The production of TA lignocellulolytic enzymes in bioreactors.

Bioreactor	strain /origin	IS (%)	Medium composition	Temp (°C)	pH	Agit (rpm)	DOT (%)	Other minerals	Duration (h)	Activity (U/mL)	References
5 L	Recombinant *Pichia pastoris* habouring xylanase XynHB 188As gene	0.5 -5	2 L BSM supplemented containing 8 mL/L PTM1	28°C	6	~200	10 - 20	Glycerol, PTM1, methanol	96	48,241	[15]
150 L	*Rhodothermus marinus* ITI-378	5	YE (5 g/L), trinitrilotriacetic acid (134 mg/L), CMC (0.3 g/L)	65°C	7	~200-500	-	NaCl, $MgCl_2.6H_2O$, Na_2HPO_4, KH_2PO_4	16	97.7	[30,31]

Note: IS, inoculums size; Temp, optimal temperature for TA enzyme production; agit, optimal or best agitation speed; SSF, solid state fermentation; YE, yeast extract; CMC, carboxymethyl cellulose; NH4SO4 , ammonium sulfate; BSM, Basal Salt Medium; NaCl, sodium chloride; MgSO4, magnesium sulfate; K2HPO4, dipotassium hydrogen phosphate; PTM1, Pichia Trace Minerals.

translated at normal temperature. At an elevated temperature, however, the loop opens and ribosome is available to bind with mRNA and translated into proteins.

Agitation rate

Fermentations of TA lignocellulolytic enzymes at laboratory scale are usually conducted in shake flask at respective agitation rate to ensure efficient oxygen transfer in the culture medium. In shake flask, agitation rates between 100 to 250 rpm have been reported for the production of TA enzymes. The production TA glucanases by *A. thermoaerophilus* WBS2 can be optimized at agitation of 150 rpm [3,39]. TA cellulose from *B. subtilis* 4-1 and *B. licheniformis* AMF-07 was produced at agitation rate of 120 rpm and 160 rpm respectively in optimum medium supplemented with CMC [25,32]. Relatively high agitation rate has been reported using Micrococcus sp which was at 200 rpm [60]. In pilot scale fermentation of TA enzymes, the agitation was conducted using impeller (i.e. rushton turbine, concave disc, pitched blade turbine) with certain number of blades in stirred tank reactor to provide a simple radial flow pattern that moves biomaterial from the center of the vessel outward. Agitation rate has an effect on mixing quality and substrates availability to microbial cell and also it influences the amount of dissolve oxygen level in the fermentation medium.

Fermentation pH

Medium pH is very important for efficient nutrients absorption and growth of strain producers, stimulation of enzyme production via signaling pathways and release of extra cellular enzymes based on certain mechanism of signal. Initial pH of the fermentation medium serves as starting pH for the microbes to grow. The initial pH favorable for the growth of strain producer may differ from a pH required to enhance the synthesis of TA lignocellulolytic enzymes. The optimum initial pH for maximal productions of TA enzymes by various microbes is varied may due to their nature and habitat where they were isolated. In shake flask, the effect of pH on the production of TA enzymes was solely conducted at initial pH of the fermentation culture and pH was not controlled throughout the fermentation time. The optimum pH for endoglucanases and exoglucanases production by thermophilic *A. thermoaerophilus* WBS2 was at pH of 9.0 while CMCellulase by *B. pumilus* S124A and *B. licheniformis* AMF-07 was highly produced at initial pH of 7.0 and 6.0 respectively [25,31,39]. The pH of fermentation medium was usually change overtime due to excretion of organic acids. The microbes will slowly adapt to the changes in fermentation medium as pH drop or rise which this explain the reason that medium was only set to an initial pH. Initial pH was usually enough for high production of TA enzymes and addition of acid or base to control pH medium at certain value may inhibit growth and production of TA enzymes. In few cases, some TA enzyme production can be optimized at controlled pH of the fermentation medium. In many situations, a very acid and alkaline will reduce the production sharply which due to instability of cell membrane potential, later lead to cell death.

Aeration rate and dissolved oxygen tension (DOT)

Aeration rate and DOT are important parameters especially in fermentation process involving aerobic microorganisms. In shake flask, the aeration rate cannot be controlled, thus it was greatly affected by agitation rate. The DOT level in shake flask fermentation was also difficult to be monitored due to lack of DOT probe. This is not the case in bioreactor (i.e. stirred tank bioreactor with submerged mode of operation) where the agitation, aeration and DOT can be set at certain values using rotameter or flow meter and DOT probe. In bioreactor system, the production of TA lignocellulolytic enzymes was enhanced at aeration rate of 1.5 to 3.0 L/min in 2 to 150 L bioreactor which is equivalent to air flow rate of 1.5 to 3.0 vvm (volume of air under standard conditions per volume of liquid per minute) [36]. High TA lignocellulolytic enzymes production was obtained in the fermentation where dissolved oxygen tension (DOT) was controlled at 20% saturation during production phase [49,50,61].

Bioreactor

On the other hand, fermentations of TA lignocellulolytic enzymes at pilot and industrial scales are usually conducted using stirred tank fermenters to ensure mixing of substrates and increase efficient oxygen mass transfer into the culture. In general, simplicity of the production procedure was needed at industrial scales; microbial enzymes may be produced in very different types of reactors such as packed beds, fluidized beds, and basket reactors but several studies demonstrated that high yield of TA enzymes may be produced via stirred tank reactor (STR). On the other hand, submerged fermentation was used as mode of operation although solid state fermentation (SSF) in tray bioreactor has also been described (Table 4). It has been demonstrated that recombinant Pichia pastoris with xylanase XynHB 188As gene can be synthesized up to 48,241 U/ml in 5 L STR using optimal medium and experimental controls. The production of TA enzyme in 150 L STR has also been demonstrated using strain *Rhodothermus marinus* ITI-378 with an obtained yield of 97.7 U/mL [49,50].

Conclusion

Lignocellulolytic enzymes which are stable at high temperature and resistant to alkaline pH are important and demanding in today's Kraft-pulp industry. Thus, the isolation of TA lignocellulolytic enzymes producing microbes is necessary and essentially useful for the production of TA lignocellulolytic enzymes. The optimized fermentation condition and strategy (i.e. microbes, C/N ratio, minerals, temperature, pH, agitation rate, aeration rate, DOT) are vitally important for the high production of commercial TA lignocellulolytic enzymes in industrial scale bioreactor. Several studies showed that different TA producing microbes for different TA lignocellulolytic enzymes required different optimization strategy. Fail to understand the microbial behavior and their 'needs' during fermentation process may lead to low production of TA enzymes. Some mutant and recombinant strains showed their advantages over wild type strains as well as enzyme modification in producing high amount and stable form of TA lignocellulolytic enzymes. This review also showed that chemical agents and process can be reduced and replaced by enzymatic process using TA lignocellulolytic enzymes in Kraft-pulp industry. Proper formulations (i.e. using suitable combination of surfactant, detergents, chelating agent and ions) by incorporating these TA lignocellulolytic enzymes in respective products are crucial for optimum reaction in industry.

Conflict of interest

Author claims that there is no conflict of interest in this article.

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