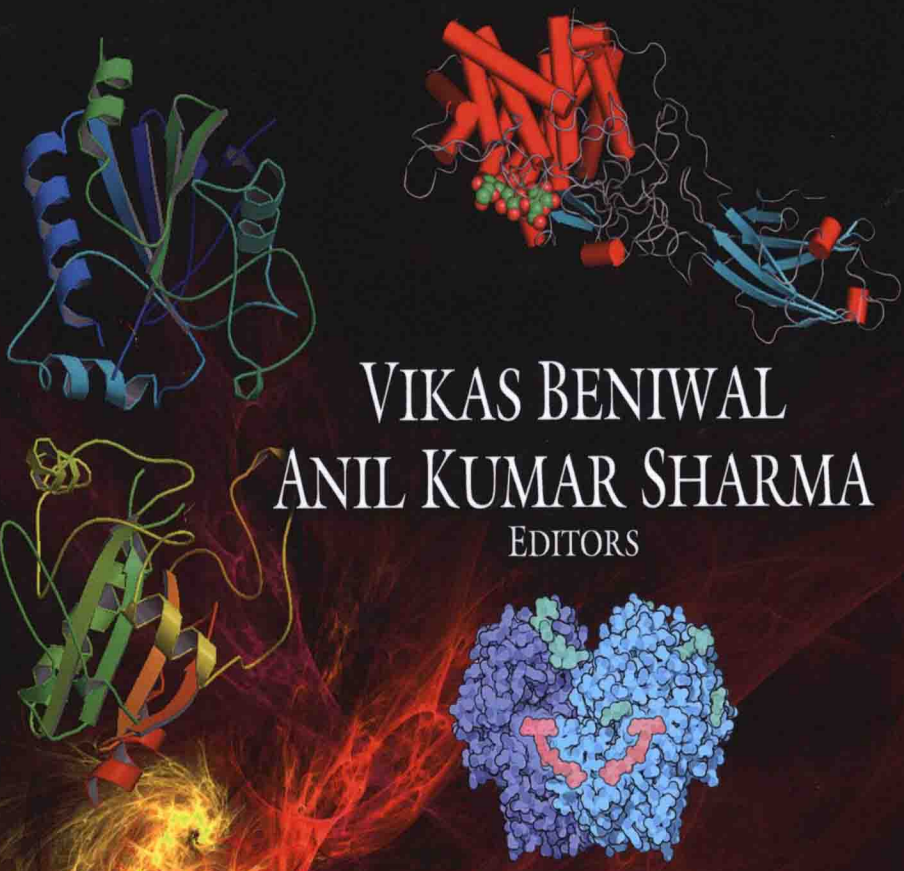
Industrial Enzymes

Trends, Scope and Relevance



VIKAS BENIWAL
ANIL KUMAR SHARMA
EDITORS

Biotechnology in Agriculture, Industry and Medicine

NOVA

BIOTECHNOLOGY IN AGRICULTURE, INDUSTRY AND MEDICINE

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PREFACE

Since the time immemorial enzymes have been known to catalyze variety of chemical reactions. Optimization and protein engineering are the key parameters to be ascertained to get an enzyme with desired activity under industrial conditions. The use of enzymes has been implicated in getting higher product quality and lower manufacturing cost, less waste and reduced energy consumption. However the enzymes found in soil and water may display the desired enzyme activity but may not be suited for industrial use because of numerous chemical processes undergoing in industry for which we require novel biocatalysts. It is well accepted now that industrial biocatalysis should be viewed as the 'third wave' of biotechnology following the pharmaceutical and agricultural waves. However the productivity, stability and availability are few of the major concerns to be addressed before a biocatalyst can be considered as an industrial enzyme. Efforts are underway to employ new enzyme technologies endeavoring to enhance cost efficiencies and productivity. There is a growing interest among consumers in substituting petrochemical products with other organic compounds such as enzymes. There is an increased demand of such beneficial enzymes from textile manufacturers, animal feed producers, detergent manufacturers, pharmaceutical companies, bioethanol producers and cosmetics vendors. We require sophisticated technological advances to produce these biocatalytic enzymes in bulk quantities at reasonable cost. The book begins with a general classification of industrial enzymes; their production and downstream processing part which is then followed by catalytic activities and other commercial applications of these enzymes. We also emphasized upon the usage of bioinformatics and genetic engineering as tools for these key industrial enzymes. We end the book with an excellent chapter on the global market scenario of these industrial enzymes just to apprise our readers of the latest trends and scope of such enzymes in the global market. The continued success of the books published under the banner of NOVA publisher is the result of a joint effort of a dedicated editorial and publishing team and we will continue to evolve progressively for the benefit of our contributors and readers. While thanking all the contributors, we reiterate our commitment for ethical and quality work published through this book on key industrial enzymes. This is a sincere gratitude to all the authors of this book for their valuable scientific contributions and appreciate the valuable efforts of the reviewers for their precious comments and suggestions for improving the quality and scientific perspectives of the chapters. We anticipate that this book would be able to provide a comprehensive, accessible, up-to-date information about industrial enzymes and their broad spectrum roles in modern therapies and other commercial applications for the global use of such enzymes.

Moreover this book offers an instant access to a wealth of key enzyme data for industrial enzymologists, biochemists, biochemical engineers, and students from diverse streams of biotechnology and industrial engineering.

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Chapter 1

MECHANISM OF ACTION OF KEY INDUSTRIAL ENZYMES

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ABSTRACT

Enzymes, either in isolated form or as a part of the whole cell based system, are used to catalyze the synthesis of specific molecules of industrial interest. A large domain of pharmaceutical and food industries depends on enzyme mediated synthesis of industrial important molecules. Enzymatic synthesis has several advantages over other synthesis routes. The enzyme mediated reactions are eco-friendly, cost effective and can be controlled in a specific manner. Moreover, the reaction route and turnover of enzymatic reaction can be modulated by knowing its mechanism of action followed by designing enzymes with site specific functional mutations. Therefore, the first step towards improvement of enzymatic efficiency is to become familiar with the mechanism of action of particular enzymes. Large arrays of different enzymes are being employed in various industries. This chapter is focused on the mechanism of action of some industrially important enzymes.

Keywords: Enzymes, industrial importance, mechanism, mode of action, catalysis

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INTRODUCTION

Enzymes are the functional proteins which catalyze various chemical reactions. Enzymes increase the rate of a reaction by lowering the activation energy, selectively binding to the substrate (S) and converting it into products (P). At first it binds with the substrate at the active site and initiates the catalytic process. The active site is the specific domain of an enzyme which interacts directly with the substrate. In a typical enzymatic reaction, the substrate passes through the transition state before being transformed to the product (Cooper and Hausman, 2004; Mizobata and Kawata, 2007). The catalytic mechanism of an enzyme involves the binding of an enzyme to the substrate and successive formation of an enzyme-substrate (ES) complex (Figure 1). Binding of substrate alters the electron distribution in different chemical bonds of the substrate and ultimately results in the formation of products. Two simplest models for enzyme-substrate interaction are the lock and key model and induced fit model. According to the lock and key model, the substrate precisely fits at the active site of an enzyme while in induced fit model, the binding of a substrate distorts the confirmations of both enzyme and the substrate (Cooper and Hausman, 2004). Specific amino acids in active site may involve directly in the catalytic process. The mechanism of action of a particular enzyme depends on various factors including substrate, product, active site composition, acidic or basic amino acids in proximity of an active site and conditions during the enzyme substrate interaction.

Enzymes are the backbone of life; cells cannot survive without these functional proteins. Some fundamental and important characteristics of enzymes make them strong candidate molecules for industrial processes. These characteristics are (i) higher specificity and efficiency, (ii) enzymes can be recovered after the product formation and (iii) enzymes can be produced from rapidly growing microorganisms. A large number of enzymes have already been commercialized for different industrial processes. Already commercialized enzymes are particularly of interest for increasing their catalytic efficiency.

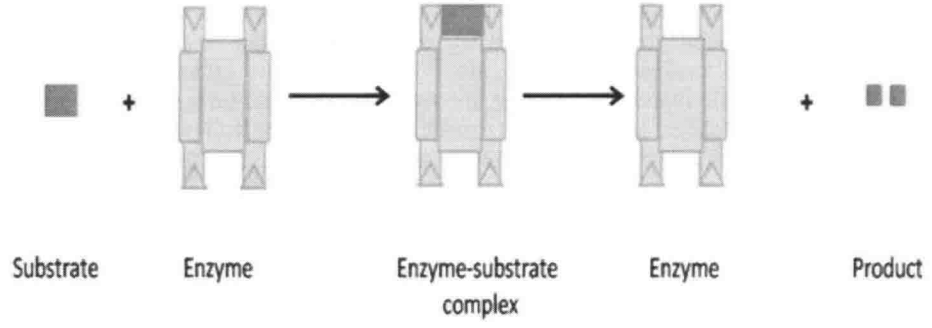

Figure 1. General mechanism of enzyme action.

Mechanism of Industrially Important Enzymes

Various enzymes have been used regularly for different industrial applications. The majority of enzymes have already been studied in detail before their commercialization.

Elucidation and understanding the mechanism of action is considered a critical aspect in enzyme research. Pioneer studies and decisive contributions are going on for better understanding of catalytic mechanisms of various enzymes. Random mutagenesis (directed evolution), site-directed mutagenesis, pH based evaluations, crystal structure and other different methodologies have been applied to resolve the catalytic mechanisms of different industrial enzymes. This chapter includes the mechanism of action of some industrially important enzymes.

Aspartase

Aspartase (L-aspartate ammonia lyase, EC 4.3.1.1) catalyzes the reversible reaction to produce aspartic acid or fumaric acid (Papierz et al., 2007; Singh and Yadav, 2012). L-aspartic acid or fumaric acid is widely used in different food and pharmaceutical industries (Singh and Yadav, 2013).

The aspartase mediated deamination reaction includes the initiation by removal of a proton from the L-aspartate at C3 position (Figure 2). Subsequently, the intermediate structural state is stabilized by carbanion formation (Mizobata and Kawata, 2007). The next step is a rate limiting step which includes detachment of ammonium group (NH_3) followed by protonation of the detached NH_3. This NH_3 is protonated by a general acid group and form NH_4^+ (Mizobata and Kawata, 2007). The NH_4^+ is the actual product released along with fumarate and divalent cation. The products are released in a random manner. Recently, structural basis for the catalytic mechanism of aspartate ammonia lyase has been elucidated by Fibriansah et al. (2011). It has been shown that aspartase (*asp*B) forced the substrate to adopt a high-energy enediolate-like conformation, which is stabilized by the hydrogen bonding among Thr101, Ser140, Thr141 and Ser139 residues. The Ser318 has been shown to act as a catalytic base, which removes the Cβ proton of substrate in the first step of the reaction mechanism. The small C-terminal domain of aspartase (*asp*B) has been suggested to be involved in regulation of catalytic activity.

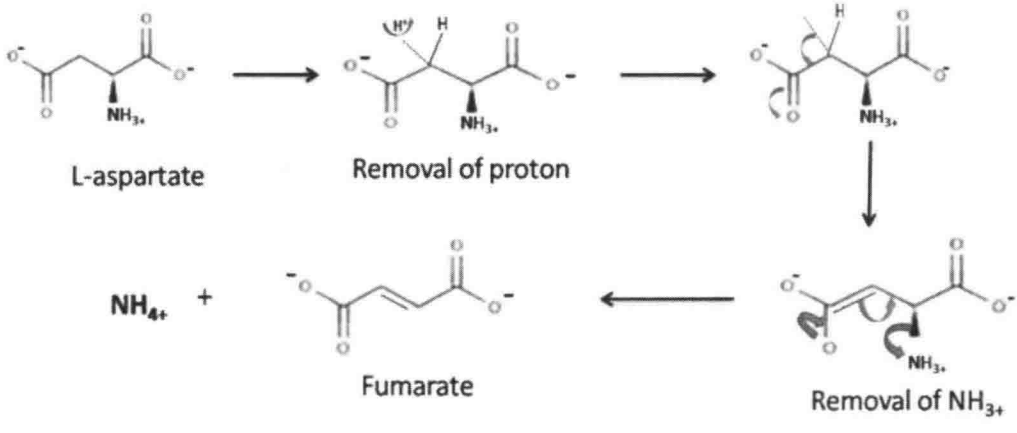

Figure 2. Mechainsm of aspartase mediated reaction.

Asparaginase

L-asparaginase (EC 3.5.1.1) are known as potent therapeutic agents against cancer including acute lymphoblastic leukemia and lymphosarcoma. Several reviews are available regarding the use of L-asparaginase in cancer therapy (Muller and Boos, 1998; Avramis and Panosyan, 2005; Sanches et al., 2007). The mechanism of asparaginase action has been reviewed by Sanches et al. (2007). The catalytic mechanism of asparaginase has been summarized in Figure 3. Asparaginase catalyzes the transformation of L-asparagine into L-aspartate and ammonia. The asparaginase mediated reaction proceeds through a covalently bound intermediate (Miller et al., 1993; Palm et al., 1996; Ortlund et al., 2000; Aghaiypour et al., 2001; Sanches et al., 2007). Firstly, a nucleophilic group of the enzyme attacks the Cγ of the substrate (L-asparagine) leading to a tetrahedral intermediate which subsequently breaks down to form an acyl-enzyme intermediate with the enzyme covalently bound to the substrate (Sanches et al., 2007). This is followed by the elimination of ammonia (Figure 3). This intermediate is then attacked by a second nucleophile (generally water) resulting in the hydrolysis of the acyl-enzyme intermediate yielding the acidic product and free enzyme (Sanches et al., 2007).

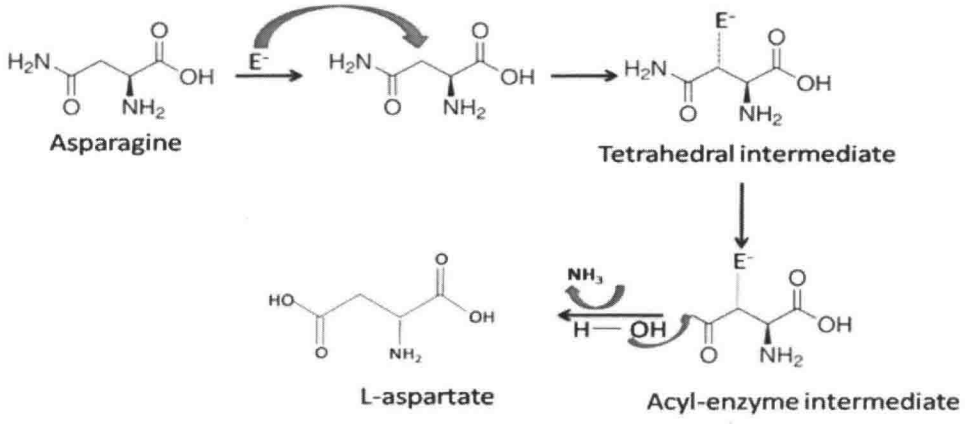

Figure 3. Mechainsm of asparaginase mediated reaction.

Lipase

Lipases (EC 3.1.1.3) are important class of industrial enzymes. These are being produced by most of the organisms across the microbial, plant and animal kingdom (Gilbert, 1993; Jaeger et al., 1994). Lipases are used not only for enzymatic hydrolysis, but also for esterification and transesterification reactions in pharmaceutical industries (Brockman et al., 1988; Chênevert et al., 2009; Zimmermann et al., 2009; Sabbani and Hedenström, 2009). Lipase catalyzes either (i) hydrolysis or (ii) synthesis reactions (Gandhi, 1997). The synthesis reactions are further classified as (a) esterification, (b) interesterification (c) alcoholysis and (d) acidolysis. Lipases are the serine hydrolases which act at the lipid-water interface (Gupta et al., 2003). They act on carboxyl ester bonds present in acyl-glycerol and release organic acids and glycerol (Jaeger et al., 1994). The catalytic triad of lipase is composed of serine-histidine and aspartate or glutamate. The aspartate residue is connected through hydrogen bond (Schmid and Verger, 1998). Serine acts as a nucleophile and aspartate as an acid residue. The serine residue is activated by a hydrogen bond in relay with histidine and

aspartate/glutamate and tetrahedral intermediate is formed. This intermediate form is stabilized by an oxyanion hole (Schmid and Verger, 1998).

Polygalacturonases

Polygalacturonases constitute a major group of enzymes which are used for degradation or depolymerization of pectin by hydrolytic cleavage of glycosidic bonds (Palanivelu, 2006). Palanivelu (2006) has proposed mechanism of action of polygalacturonases. The author proposed that NTD178 and RIK256 motifs interact with the substrate on both sides of the susceptible glycosidic bond through hydrogen bonds (Figure 4). The interaction through hydrogen bonds produces necessary strain and distortion on the susceptible glycosidic bond. The catalytic amino acid residues His223 and Asp201 are positioned on the susceptible glycosidic bond. The glycosidic bond is cleaved with the release of first product. Simultaneously, covalent bond formation takes place between the substrate and nucleophile Asp201 at catalytic active site. Another active site residue (Asp202) positions a water molecule for nucleophilic attack. The nucleophilic attack by the water molecule results in release of second product and also restores the enzyme active site (Figure 4).

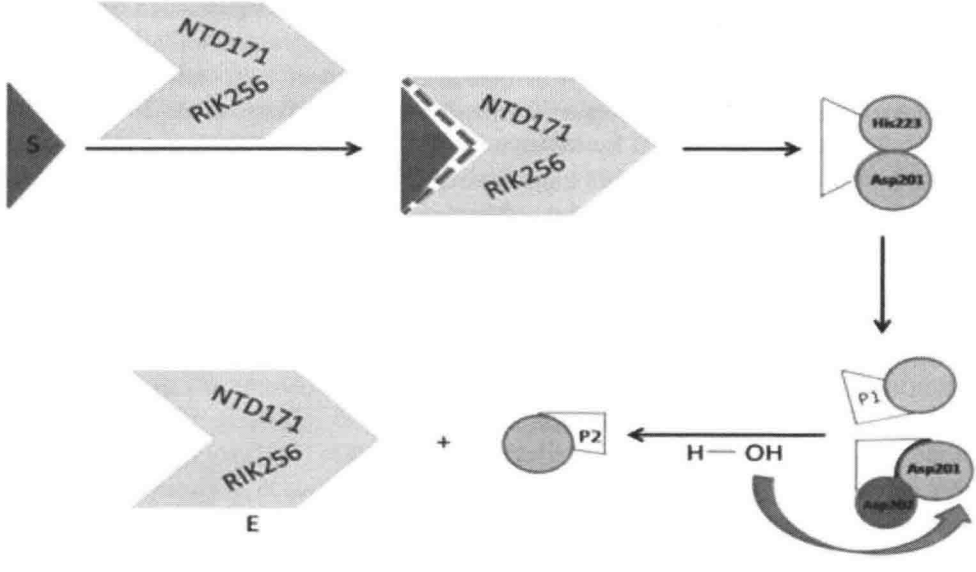

Figure 4. Mechanism of polygalacturonase mediated reaction.

α-Amylases

α-amylase (EC 3.2.1.1) is an important industrial enzyme isolated from a wide variety of microorganisms (Pandey et al., 2000; El-Fallal et al., 2012). It is an endoamylase having ability to cleave the internal (endo) α,1-4 glycosidic bonds present in the amylose or amylopectin molecules. The end products are oligosaccharides of varying length with α-configuration and α-limit dextrins (El-Fallal et al., 2012). Depending on the degree of substrate hydrolysis, α-amylases are generally divided into two categories (Fukumoto and Okada, 1963; Rameshkumar and Sivasudha, 2011; El-Fallal et al., 2012; Yadav et al., 2013). These two models for amylase action pattern includes the random action and the multiple attack action. Random action has also been referred as a single attack or multi-chain attack

action (Azhari and Lotan, 1991; El-Fallal et al., 2012). In case of "single attack", the substrate polymer molecule is hydrolysed completely in a consecutive manner before the dissociation of an enzyme-substrate complex. While, in case of "multiple attack", only one bond is hydrolyzed per effective enzyme-substrate encounter (El-Fallal et al., 2012). The "multiple attack" is generally an accepted concept to explain the differences in action pattern of amylases (Kramhøft et al., 2005; Svensson et al., 2002; El-Fallal et al., 2012). Majority of the endoamylases have shown low level of multiple attack action (Bijttebier et al., 2008; El-Fallal et al., 2012). The level of multiple attacks of numerous endoamylases has been reported to increase with the increase in temperature up to a degree and this depends on the amylase itself (Bijttebier et al., 2008; El-Fallal et al., 2012).

Papain

Papain (EC 3.4.22.2) is an endolytic plant cysteine protease enzyme isolated from papaya (*Carica papaya* L.) latex. Papain has been successfully used to overcome the allergies associated with leaky gut syndrome, insufficient stomach acid and intestinal symbiosis like gluten intolerance. It has also been reported to have considerable analgesic and anti-inflammatory activity against the symptoms of acute allergic sinusitis and toothache pain (Mansfield et al., 1985; Amri and Mamboya, 2012). Recently, its mechanism of action has been reviewed by Amri and Mamboya (2012). According to these authors, the cysteine-25 portion of the triad attacks the carbonyl carbon in backbone of the peptide chain and this attack releases the amino terminal portion (Menard et al., 1990; Tsuge et al., 1999; Amri and Mamboya, 2012). The mechanism by which it breaks peptide bonds involves deprotonation of Cys-25 by His-159. Asparagine-175 helps to orient the imidazole ring of His-159 to allow this deprotonation to take place. Cys-25 then performs a nucleophilic attack on the carbonyl carbon of a peptide backbone (Menard et al., 1990; Tsuge et al., 1999). In the active site of papain, Cys -25 and His -159 are thought to be catalytically active as a thiolate-imidazolium ion pair. Papain can be efficiently inhibited by peptidyl or non-peptidyl N-nitrosoanilines (Guo et al., 1996; 1998; Amri and Mamboya 2012) and this inactivation is due to the formation of a stable S-NO bond in the active site (S-nitroso- Cys25) of papain (Xian et al., 2000).

Catalase

Dounce (1983) proposed a detailed mechanism for catalytic action of catalase. According to the author, the mechanism includes the formation of Chance's catalase compound I in the first step and hydride ion transfer in the second step (Dounce, 1983). The first oxidative step includes direct reaction of the hematin iron with an ionized H_2O_2 molecule followed by oxidation of the iron to Fe IV. The second step is supposed to depend on the reductive action of a second H_2O_2 molecule on Chance's compound I through a catalyzed hydride ion transfer, resulting in the regeneration of uncomplexed catalase. Catalytic mechanism of *Burkholderia pseudomallei* catalase has been proposed by Vlasits (2009). According to author, the first substrate hydrogen peroxide (H_2O_2) enters the main channel into the heme site by binding between Arg108 and Asp141 (according to *Burkholderia pseudomallei* amino acid numbering). This is one of the two substrate paths proposed by Deemagarn et al. (2007). Compound I formation is started by movement of H_2O_2 molecule to interact with Arg108 and His112. Two electrons each from iron and porphyrin are transferred to the oxygen during compound I formation and in this way catalyze the heterolytic cleavage of H-O-O-H and form

an oxoferryl heme with a porphyrin cation radical (Vlasits, 2009). The second substrate H_2O_2 enters the heme cavity on a proposed second path (as reported by Deemagarn et al., 2007) between the Asp141 and main chain atoms of Ile237, Tyr238 and Val239 (according to *Burkholderia pseudomallei* amino acid number). It finally interacts with Trp111 and His112 residues and reduces compound I to oxyferrous heme, which decomposes to ferric enzyme and superoxide (Vlasits, 2009). The superoxide being quantitatively oxidized at the adduct radical, closing the adduct shell and release the dioxygen (Jakopitsch et al., 2007; Suarez et al., 2009; Vlasits, 2009).

Inulinase

Inulinase catalyzes the hydrolysis of inulin into fructose. It targets on β-(2→1) linkages of the polymer and splits off terminal fructosyl units, releasing fructose and glucose. To convert inulin into fructose, the most rational and economic option is its enzymatic hydrolysis. Microbial inulinase are an important class of enzymes that catalyzes the hydrolysis of polysaccharides for production of fructose. Depending on the mode of action on inulin, inulinase can be divided into exo-inulinase and endo-inulinase (Nakamura et al., 1988; Fernandes and Jiang, 2013). Exo-inulinase (β-D-fructan fructohydrolase, EC 3.2.1.80) breaks off successive terminal fructose units from the non-reducing end of the inulin. Endo-inulinase (2, 1-β-D-fructan fructanohydrolase, EC 3.2.1.7) hydrolyze the internal β-(2→1)-fructofuranosidic linkages to yield different oligosaccharides. Yeasts are important sources of inulinases. Purified inulinase from *Saccharomyces fragilis* hydrolyzes inulin by an endwise action (Snyder and Phaff, 1962). The action commences at the D-fructose end of the polymer and yields fructose until the last linkage is broken, which yields a D-glucose molecule per molecule of inulin (Snyder and Phaff, 1962). It has been shown that the degradation of inulin occurs largely by the single-chain mechanism (Snyder and Phaff, 1962). Inulin has been shown as an ideal substrate to study the mechanism of action of the terminally acting inulinase (Snyder and Phaff, 1962). The substrate ends with a glucose molecule and inulinase hydrolyzes the substrate from the opposite end of the chain. Therefore, the rate of appearance of free glucose in relation to the total increase in reducing value can provide information regarding the degree of single- or multi-chain action (Snyder and Phaff, 1962). Studies on hexose to glucose ratios (H:G) of the inulinase mediated reaction established that under most conditions the hydrolysis occurred largely by the single-chain mechanism (Snyder and Phaff, 1962)., The multi-chain mechanism has been favored at pH below the optimal point (Snyder and Phaff, 1962).

α-Rhamnosidase

α-L-Rhamnosidase (EC 3.2.1.40) belongs to a group of hydrolases, which hydrolyse the terminal non reducing L-rhamnose from the natural and synthetic rhamnoside flavonoids. Rhamnosidase has been considered an important industrial enzyme due to its potential applications in food and pharmaceutical industry (Del Nobile et al., 2003; Yu et al., 2002). Naringinase is sometimes used as another name for α-L-rhamnosidase (Kupou et al., 1989). The glycoside hydrolases have been classified into EC 3.2.1 because of their potential of O- or S-glycosides hydrolysis. Hydrolysis of the glycosidic bond occurs by either with retention or inversion of the anomeric configuration. Both mechanisms employ a pair of carboxylic acids in active site. Inverting enzymes use one residue as a general acid and other as a general base catalyst and these are suitably placed (about 10.5 Å apart) to allow substrate and water

molecule to adjust between these residues. In retaining enzymes, one residue acts as a nucleophile and other as a general acid/base catalyst (Sinnott, 1990).

Cellulase

Microbial cellulase has gained importance in different industries including textile, pulp and paper, biofuel industry, laundry, food/feed industry, brewing, and also in agriculture field. The cellulolytic enzyme comprises of modular multidomain proteins. These proteins have catalytic domain (CD), cellulose binding domain (CBD) and an inter-domain linker which connects cellulose binding domain to catalytic domain (Figure 5). Depending on mode of action, cellulase is grouped in three categories (Kuhad et al., 1997; 2011; Himmel et al., 1999; Zhang et al., 2006; Deswal et al., 2011). These three groups include endo-(1,4)-β-D-glucanase (EC 3.2.1.4), exo-(1,4)-β-D-glucanase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21). The exoglucanase acts on the terminal ends of the cellulose chain and releases β-cellobiose as the product while endoglucanase randomly attacks the internal *O*-glycosidic bonds and results in glucan chains of different lengths (Bayer et al., 1994; Singh, 1999; Kuhad et al., 2011). The β-glycosidase acts specifically on the β-cellobiose disaccharides and produce glucose (Bayer et al., 1994; Singh, 1999; Kuhad et al., 2011). Earlier, Reese et al. (1950) proposed the mechanism of cellulase action. According to authors, at least two steps are involved, first is prehydrolytic step in which anhydroglucose chains are swollen/hydrated and in second step, hydrolytic cleavage of the susceptible polymers. The hydrolysis may occur either randomly or endwise (Reese et al., 1950). The first step would involve an enzyme designated C1 and the second hydrolytic enzymes termed as Cc (Figure 5). A third type of enzyme is β-glucosidase (cellobiase). The C1 component attacks highly ordered or crystalline cellulose (cotton fibers or Avicel) but has little effect on soluble derivatives such as carboxymethyl cellulose (CMC). Spano et al. (1975) suggested that C1 decrystallizes or hydrates cellulose chains while Cc consists of exo and endo β-1,4 glucanases that attack soluble derivatives or cellulose that has been treated with acid or alkali (Wood and Philips 1969.)

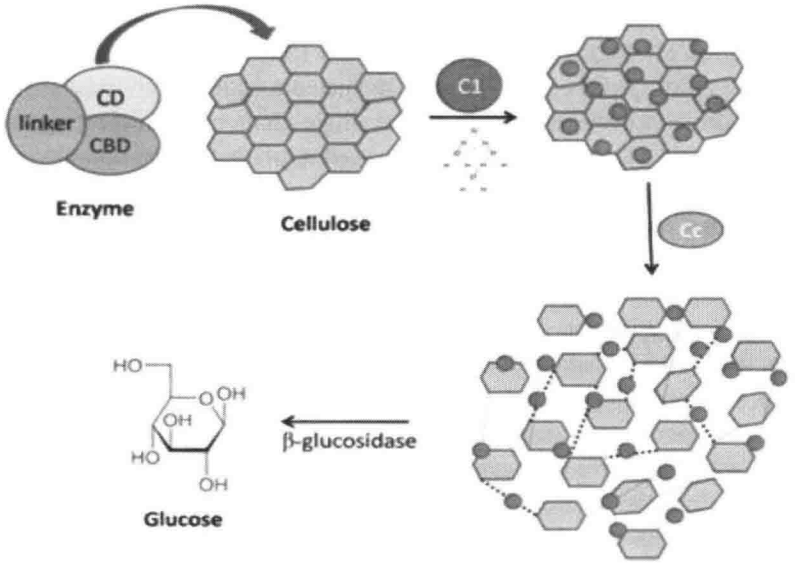

Figure 5. Mechanism of cellulase mediated reaction.