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DISCUSSION

Marine environments, in general, are unique by virtue of their salinity, wide range of mineral content and well knitted ecosystem when compared to terrestrial environments which is constantly disturbed by human activities. The marine fungi have not been experimentally tried for their potential in many of the human endeavours in which they would have a major role to play as a biocatalyst, through their enzymes or whole cell systems.

*Aspergillus sydowii* BTMFS 55 isolated from sea water was found as a potential strain, that produced extracellular β-glucosidase among other fungal isolates obtained from marine environments. *Aspergillus sydowii* BTMFS 55 isolated from sea water produced extracellular β-glucosidase in Czapek-Dox medium and the process optimization for the maximal production of the enzyme was done under submerged and solid state fermentations.

5.1 Molecular identification of the fungal strain

Traditional identification methods of fungi such as morphological and cultural characteristics may be tedious, time consuming and require a great deal of skill. The PCR amplification of rDNA sequences using taxon-specific primers which were derived from sequence data and were checked for cross reaction with related fungi is thought to be the most powerful molecular tool for fungal diagnosis which has been developed so far (Sugita and Nishikawa, 2003). A portion of the 28S rRNA gene
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(~700 bp) was amplified from the genomic DNA using a primer pair for 28S rDNA (NL1F and NL4R) and the partial nucleotide sequence obtained after sequencing was of 494 bp. The identity of the sequence was determined by BLAST software (Altschul et al., 1990) and the resultant sequence showed 93% identity with the already available sequences of *Aspergillus sydowii* in the GenBank. A phylogenetic tree was constructed using nucleotide evolutionary model based on synonymous and non-synonymous nucleotide substitutions. *A. sydowii* shared a close affinity with most of the terrestrial species of *Aspergillus* suggesting that *A. sydowii* could have migrated to marine sediments through surface drain from terrestrial environments and could have adapted to marine environments in due course of time.

*Aspergillus sydowii* BTMFS 55 isolated from sea water produced extracellular β-glucosidase in Czapek-Dox medium and the process optimization for the maximal production of the enzyme was done under submerged and solid state fermentations.

5.2 Submerged Fermentation (SmF)

There are so many reports regarding the production of extracellular β-glucosidase enzyme from different fungal species and among the group *Aspergillus* sp. is considered as the best since it produce more β-glucosidase than the others (Asquieri and Park, 1992; Gupte and Madamwar, 1997). Among the genus, *A. niger* (Galas and Romanowska, 1997; Yan and Lin, 1997), *A. aculeatus* (Takada et al., 1999), *A. japonicus* (Sanyal et al., 1988), *A. nidulans* (Bagga et al., 1990), *A. oryzae* (Riou et al., 1998), *A. kawachii* (Iwashita et al., 1999), and *A. terreus* (Tavolibov et al., 1988) are the mostly reported ones. But there are no reports available about the
production, purification or characterization of \( \beta \)-glucosidase from *Aspergillus sydowii* either from terrestrial or marine sources.

*A. sydowii* BTMFS 55 produced extracellular \( \beta \)-glucosidase in CD medium utilizing all the substrates evaluated and the enzyme production was maximum with glucose (9.86 U/ml) as the sole source of carbon, thus seemed as a constitutive enzyme. Generally, most of the microbial cellulases are inducible enzymes and they are secreted when the microorganisms grow in cellulose (Kubicek et al., 1993; Mach and Zeilinger, 2003). According to Kang et al. (2004) the production of the \( \beta \)-glucosidase enzyme is affected by the nature of the substrate used in fermentation, hence the choice of an appropriate inducing substrate is highly important. But, in the case of *Candida peltata*, the extracellular \( \beta \)-glucosidase enzyme found as constitutive and produced regardless of the presence or absence of cellulosic substrate (Saha & Bothast, 1996). The \( \beta \)-glucosidase from *Pyrococcus furiosus* was isolated from a maltose based medium (Bauer et al., 1996) and *Aureobasidium pullulans* produced \( \beta \)-glucosidase activity constitutively when grown in liquid medium containing lactose as the carbon source (Saha et al., 1994). It has been reported that the presence of glucose in the fermentation medium was the most effective for promoting activity of the glucanase enzyme (Sharma et al. 1996). There are so many reports regarding the growth and production of \( \beta \)-glucosidase in presence of glucose as carbon source (Riou et al., 1998; Saha et al., 1996; Parry et al., 2001).

Although there was growth and enzyme production in the presence of additional carbon sources, the enzyme activity was comparatively lesser than the activity recorded with glucose (9.86 U/ml) as the only carbon source. Hence, it is inferred that there is no need for any additional carbon sources for enzyme production. Results obtained in the present study were in agreement with the earlier reports that glucose acts as a good growth substrate for production of \( \beta \)-glucosidase by yeasts.
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(Saha & Bothast 1996). Candida molischiana 35 produced an exocellular β-glucosidase with a wide substrate spectrum (Vasserot et al. 1995). The high level of enzyme production on glucose and the glucose tolerance observed in the present study are one of the most important characteristics of the A. sydowii and could have an application in the enzymatic hydrolysis of cellulose to glucose.

Among the various nitrogen sources, both organic and inorganic, tested as additional nitrogen sources, ammonium sulphate supported maximal enzyme activity (12.8 U/ml). Both peptone (11.2 U/ml) and malt extract (11 U/ml) were also equally good in enhancing enzyme production. May be the marine fungus preferred ammonium sulphate as source of additional nitrogen as well as sulphate for enhanced enzyme production compared to peptone and malt extract which are complex organic source of nitrogen. Further studies on this aspect may reveal the physiology of this marine fungus with particular reference to the role of ammonium sulphate in enzyme synthesis.

The requirement of NaCl for the production of β-glucosidase was tested by incorporating different concentrations of NaCl (0-5%) in the CD medium along with the already optimized parameters. Even though there was good growth in the presence of NaCl at all the concentrations tested, β-glucosidase production was maximal only at 0.5% of NaCl (13.9 U/ml), which was higher than that observed with 1% NaCl (10.5 U/ml) which was normally used in the CD medium. From the results it is inferred that this fungus though exist in sea water, where the NaCl concentration is around 3%, it does not secrete maximal enzyme at 3% NaCl as the organism may not require production of copious amount of this enzyme in its natural environment. Whereas, under controlled conditions at lesser levels of NaCl the enzyme synthesis machinery might have been induced for enhanced levels of enzyme production. May be it is possible that at higher concentration of NaCl above 1%, the glucose induction
of β-glucosidase production could have been repressed and hence level of enzyme production decreased along with increase in concentration of NaCl, in spite of the presence of optimal level of glucose in the medium. Since this is a new marine fungus this aspect needs to be investigated further particularly with respect to the relationship between glucose induction of enzyme and NaCl concentration at molecular level.

The surfactants play a role in the secretion of cell membrane bound exoenzyme in microbes by increasing the cell permeability of the micro organisms (Reese et.al., 1969). The effect of Tween 80 as surfactant was tested and it was found that low concentration of Tween 80 (0.1%) could enhance the release of the enzyme (24.3 U/ml) in the medium. The present results were in agreement with an earlier observation where the production of cellobiase from *A. niger* A20 was enhanced by the addition of a low concentration (0.2%) of Tween 80 as optimal concentration (Abdel-Fattah et al., 1997).

After optimization of all the parameters a time course experiment was performed and it was noted that the considerable levels of β-glucosidase could be recorded after 4 days of incubation (9.3 U/ml) and the enzyme activity gradually increased to a maximum after 7 days (14.3 U/ml). The maximal biomass (dry weight) was recorded on the 3rd day of incubation (11.21 mg/ml), which decreased later and remained in a steady state from the 6th day onwards (5-9 mg/ml). Whereas, maximal enzyme production at considerable level was recorded from the 6th day onwards, this remained more or less steady with slight variations during the rest of the incubation period. These observations testify that the β-glucosidase production by *A. sydowii* is not growth associated and enzyme synthesis took place only during the stationary phase. A similar observation was made with *Aspergillus oryzae* for β-glucosidase production where enzyme production increased from 6th day to 14 days of incubation and remained steady (Gunata & Vallier, 1999).
5.3 Solid State Fermentation (SSF)

In the production of microbial exoenzymes, solid state fermentation (SSF) has several economic advantages over conventional submerged fermentation such as use of agro industrial wastes as simpler substrates, minimal requirement of water, production of metabolites in a more concentrated form and making the downstream processing less time consuming and less expensive (Sreeja et al., 2006). Among the various groups of microorganisms used in SSF, filamentous fungi are the most widely exploited owing to their ability to grow and produce a wide range of extracellular enzymes on complex solid substrates (Moo-young, 1983). Among processes used for enzyme production, SSF is an attractive one because it presents many advantages, especially for fungal cultivations (Weiland, 1988). In SSF, the productivity per reactor volume is much higher compared with that of submerged culture (Grajek, 1987). Also, the operation cost lower, because simpler plant, machinery and energy are required (Roche and Durand 1996).

In recent years, SSF has received more attention by the investigators, since several studies for enzymes, flavours (Ferron et al., 1996), colourants (Johns & Stuart, 1991) and other substances of interest to the food industry have shown that SSF can support higher yields (Tsuchiya et al., 1994) or better product characteristics than submerged fermentation. In addition, costs are much lower due to the efficient utilization and value-addition of wastes (Robinson & Nigam, 2003).

The marine fungi *A. sydowii* BTMFS 55 produced β-glucosidase as extracellular enzyme even without the addition of any nutrients to the wheat bran (WB). Wheat bran is considered as a complete medium for producing various industrially important enzymes (Smits et al 1996). It was observed that the fungus started to produce the enzyme after 48 h of incubation (353 U/gIDS) and there was a
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double fold increase in the yield after 72 h with an enzyme activity of 696 U/gIDS. There are reports which supported the low activity during the first 24 h since the time needed for enzyme production was found to be longer when original substrate contained no compounds stimulating enzyme production. The action of both endoglucanase and exoglucanase during the early stages of growth resulted in cellobiose production that can induce β-glucosidase biosynthesis at later stages (Godden et al., 1989).

Though there were good growth and production of enzyme in wheat bran media with all the moisture levels tested, the maximal enzyme activity was observed with 60% moisture content (681 U/gIDS). In solid state fermentation, the demands of moisture level differ according to the nature of enzyme to be produced, substrate, microorganisms as well as the particle size of the substrate (Muniswaran and Charyulu, 1994; Nandakumar et al, 1994; Krishna and Chandrasekaran, 1996; Fadel, 1999). Besides, the moisture content and relative humidity of the medium are the key factors, which determine the outcome of the process and moisture content affects both aeration and nutrients solubility and suitability to be utilized by microorganisms (Nigam, 1990). The moisture content, incubation temperature and oxygen supply are considered as the most critical parameters for the growth and enzyme production in SSF (Barrios-Gonzalez et al., 1993).

Incorporation of different carbon sources as additional nutrients showed an increased enzyme yield compared to SSF with Wheat Bran alone. The addition of CMC showed maximal enzyme activity (1651 U/gIDS) followed by glucose (1492 U/gIDS) and cellobiose (1472 U/gIDS). CMC, glucose and cellobiose could have played the role of an inducer for β-glucosidase. Apparently it seems this fungus is induced for it β-glucosidase production by its own metabolites rather external carbon sources for enhanced enzyme production.
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The addition of various nitrogen substrates showed both better growth and production of \( \beta \)-glucosidase enzyme compared to the control (WB + CMC). Among the substrates evaluated, addition of peptone showed maximal \( \beta \)-glucosidase activity (1392 U/gIDS). Fang and Zhong (2002) reported that organic nitrogen sources were efficient in *Ganoderma lucidum* fermentations for mycelial growth and polysaccharide production.

The \( \beta \)-glucosidase production showed a linear increase along with increase in inoculum concentration. The inoculum concentration of 10% was taken as optimal (2411 U/gIDS). The same was also reported with *A. niger* grown under SSF (Fadel, 2000). Inoculum size controls and shortens the initial lag phase, and while a smaller inoculum size led to extended lag phase, larger inoculum size contributed to increase in the moisture content to a significant extent (Sharma et al., 1996).

The time course experiment presented an interesting picture on the potential of this *A. sydowii* for \( \beta \)-glucosidase production. In SSF, the solid substrate Wheat bran not only supplies the nutrients to the culture but also serves as an anchorage for the microbial cells. The addition of 0.5% CMC as the additional carbon source and 0.5% peptone as the additional nitrogen source along with 0.5% NaCl, at pH of 8.0 and at room temperature, supported enhanced \( \beta \)-glucosidase production. The optimal size of the wheat bran particle size was found to be 425\( \mu \) with an optimum of 60% moisture content and 10% inoculum for the enzyme production. It has been reported that the particle size, moisture content and the substrate used are the most critical factors in solid state fermentations (Liu & Tzeng, 1999; Roussos et al., 1993; Sarrette et al., 1992; Smail et al., 1995; Zadrazil & Punia, 1995). During the course of incubation for 14 days, under optimized conditions, maximal enzyme production occurred only on the 4\(^{th} \) day (1401 U/gIDS) at 30°C at a pH of 8.0. As per Godden et al (1989) and
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Fadel (2000), the low activity for β-glucosidase during first 24 h may be due to the fact that original substrate contained no compounds stimulating enzyme production. The enzyme production declined after 4 days. It was noted during the course of the present study that A. sydowii took 7 days for maximal enzyme production under SmF conditions compared to the 4 days under SSF. It is reported literature that the incubation time needed for enzyme production was shorter on SSF compared to SmF (Illanes et al 1992, Jiafa et al 1993).

5.4 Media optimisation by statistical analysis under Solid State Fermentation

Medium optimization by the one-factor-at-a-time method involves changing one independent variable (i.e., nutrients, temperature, pH, etc.) while fixing others at certain levels. This method is not only time-consuming, but also often leads to an incomplete understanding of the behaviour of the system, resulting in confusion and a lack of predictive ability (Xu and Yun, 2003). Hence statistical approach was attempted towards optimization of bioprocess variables for β-glucosidase production.

Optimization through factorial design and response surface methodology is a common practice in biotechnology for the optimization of media components and culture conditions (Chen, 1996). In order to obtain optimum yield of an enzyme, development of a suitable medium and cultural conditions is obligatory. Statistical optimization not only allows quick screening of a large experimental domain, but also reflects the role of each of the components. Basically this optimization process involves three major steps: performing the statistically designed experiments, estimating the coefficients in a mathematical model, and predicting the response and checking the adequacy of the model. Using the mathematical model, the levels of variables giving maximum response can then be determined.
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There are a number of reports in which the Plackett-Burman design has been used to screen the factors in a fermentation medium to be optimized in subsequent experiments (Krishnan et al., 1998; Reddy et al., 1999; Son et al., 1998; Srinivas et al., 1994; Yu et al., 1997).

In the present study, the Plackett-Burman experimental design was used to optimize the solid state culture conditions for the production of β-glucosidase by A. sydowii. The experiment was done according to the Plackett-Burman experimental design and the statistical analysis was carried out using fisher’s test for ANOVA. Among the eleven parameters tested, moisture content, concentration of peptone and inoculum only showed positive significance for the β-glucosidase enzyme production. The test model was statistically significant with a confidence levels above 95%, and by the model, 91.62% of the variability in the response could be explained. A validation run was carried out under the conditions predicted by the model. The experimental values (1355.6, 1202.5, and 1338.3) were found to be close to the predicted values (1257.1, 1262.8, and 1255.7 respectively), and hence, the model was successfully validated. The results obtained from the statistical optimizations studies confirm the observations made during the conventional optimization studies conducted under SmF and SSF, particularly in the case of peptone more evidencingly.

5.5 Enzyme purification and characterization

The native PAGE analysis of the ion exchange purified β-glucosidase enzyme from A. sydowii showed a single band and the molecular weight and purity was determined by SDS-PAGE electrophoresis. The electrophoresis showed a single polypeptide band having a mass of ~95 kDa. The β-glucosidase activity of the corresponding band was confirmed as monomer by zymogram analysis. It should be
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noted that the molecular mass of most fungal β-glucosidases are often greater than 80 kDa (Woodward and Wiseman, 1982). Extracellular β-glucosidases which have molecular masses of 96 kDa (Witte and Wartenberg, 1989), 100 kDa (Galas and Romanowska, 1997) and 105 kDa (Yan and Lin, 1997) have been purified from three different strains of *A. niger*. The β-glucosidases with molecular masses of 106 kDa, 114 kDa and 85 kDa from *Neurospora crassa* (Yazdi et al., 2003), *Phanerochaete chrysosporium* (Lymar et al., 1995) and *Stachybotrys* sp. (Amouri and Gargouri, 2006), have also been reported, respectively.

The temperature and pH optima for the activity and stability of the extracellular β-glucosidase of *A. sydowii* BTMFS 55 were comparable with respective values of other fungal β-glucosidases. The maximal enzyme activity was recorded at 50°C and it was active over a range of 30-70°C. The optimal pH and temperatures of β-glucosidases from various microbial sources range between 3.0-7.0 and 40-105°C, respectively (Coughlan, 1985; Kengen et al., 1993; Saha et al., 1995; Woodward and Wiseman, 1982). The optimum temperature (50°C) of the purified enzyme is similar to that of *A. oryzae* (Riou et al., 1998), *A. nidulans* (Kwon et al., 1992), *Candida peltata* (Saha and Bothast, 1996), *Stachybotrys* sp. (Amouri and Gargouri, 2006), *Volvarialla volvacea* (Li et al., 2005) and *T. reesei* (Cascalheira and Queiroz, 1999). The thermal stability (30-70°C for 12 h at pH 8.0) of the purified enzyme is sharing some what similar pattern reported for this enzyme from other fungal strains. The β-glucosidase of *Thermoascus auranticus* showed a thermal stability of 40-70°C (Parry et al., 2001) whereas, *Aureobasidium* sp. showed a range of 30-80°C (Hayashi et al., 1999), with an optimum at 80°C.

The optimal pH (5.0) activity of the purified enzyme was seemed to be acidic, ranging from 4.0-7.0. It is similar to that of *A. oryzae* (Zhang et al., 2007), *A. niger*
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(Yan and Lin, 1997), Neurospora crassa (Yazdi et al., 2003), Candida peltata (Saha and Bothast, 1996), A. fumigatus (Ximenes et al., 1996), and Stachybotrys sp. (Amouri and Gargouri, 2006). The pH stability of the enzyme (4.0-6.0) supported with that from Aureobasidium pullulans (Saha et al., 1994) and Candida peltata (Saha and Bothast, 1996). It was observed that the β-glucosidase of Thermomyces lanuginosus (Lin et al., 1999) and Aureobasidium sp. (Hayashi et al., 1999) exhibited enzyme stability in a wide range of pH such as 5.0-12.0 and 2.2-9.8, respectively.

On the basis of substrate specificity, β-Glucosidases may be divided into three groups: (i) aryl-β-glucosidases, which have a strong affinity for aryl-β-glucosidases; (ii) cellobiases, which hydrolyze only oligosaccharides; and (iii) broad-specificity β-glucosidases, which exhibit activity on many substrate types and are the most commonly observed β-glucosidases. The purified β-glucosidase from A. sydowii hydrolysed both oligosaccharides such as cellobiose, maltose and lactose and glucosides such as pNPG. The enzyme showed high affinity towards the pNPG than the cellobiose and other disaccharides tested. Plant et al (1988) suggested that the preference of β-glucosidases for aryl glycosides is due to the high electrophilicity of the aglycone moiety, which enhances the stability of the ortho or para nitrophenoxide anion generated during the first step of catalysis. The β-glucosidase from thermophilic fungus, Talaromyces thermophilus showed an affinity of lactose and maltose than cellobiose with a relative activities of 75, 61 and 6%, respectively (Nakkharat and Haltrich, 2006). The β-glucosidase of A. sydowii also showed such a result that it hydrolyses maltose and lactose more efficiently than cellobiose. β-glucosidases with very broad specificity have been isolated from many fungi (Gueguen et al., 1995; Park et al., 2005; Pitson et al., 1997; Watanabe et al., 1992; Yan and Lin, 1997).
The reaction kinetics of the purified enzyme was determined from Lineweaver-Burk plots under optimal conditions (30 min, pH 8.0, 50°C). The enzyme had an apparent $K_m$ value of 0.67 mM and a $V_{\text{max}}$ value of 83.3 μmol min$^{-1}$ mg protein$^{-1}$ for the hydrolysis of pNPG. The $K_m$ value of *A. sydowii* β-glucosidase (0.67 mM) supported with that of *A. oryzae* and *A. phoenicis* which have a lower $K_m$ of 0.55 mM and 0.58 mM, respectively. *Thermomyces lanuginosus*, *A. fumigatus* (0.075 mM each) and *Phanerochaete chrysosporium* (0.096 mM) were also exhibited a much lower $K_m$.

Competitive inhibition by glucose is a common characteristic of fungal β-glucosidases (Gueguen et al., 1995; Saha and Bothast, 1996; Saha et al., 1995; Yan and Lin, 1997) and most microbial enzymes show inhibition constants of 0.6 to 8 mM for glucose (Ozaki and Yamada, 1992). The $K_i$ values of *Aspergillus* sp. have been reported to range from 3 to 14 mM (Yan and Lin, 1997). The β-glucosidase of *A. sydowii* BTMFS 55 showed an inhibition constant ($K_i$) of 17 mM. The availability of β-glucosidase insensitive to glucose have a significant impact on the enzymatic conversion of cellulosic biomass to glucose for the subsequent production of fuel ethanol. Glucose tolerant fungal β-glucosidases were reported from *A. oryzae* (136 mM), *A. niger* (543 mM), *Candida peltata* (140 mM), *Pyrococcus furiosus* (300 mM) and *A. tubingensis* (470 & 600 mM) by various authors (Decker et al., 2001; Kengcn et al., 1993; Riou et al., 1998; Saha and Bothast, 1996; Yan and Lin, 1997).

The β-glucosidases from *Sporotrichum thermophile*, *Monilia* sp., *Fusarium oxysporum*, *Neocallimastrix frontalis*, *Botrytis cinerea*, and *Streptomyces* sp. strain QM-B814 were competitively inhibited by glucose, with $K_is$ of 0.5, 0.67, 2.05, 5.5, 10.5, 65 mM, respectively (Bhat et al., 1993; Christakopoulos et al., 1994; Dekker, 1981; Gueguen et al., 1995; Li and Calza, 1991; Perez-Pons, 1994). Glucose inhibited
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the β-glucosidase-catalyzed reaction of *T. viride* cellulase in a mixed inhibition pattern with a competitive character (Montero and Romeu, 1992). The inhibition of β-glucosidase from *Pyrococcus furiosus* by glucose was almost negligible, with a $K_i$ of 300 mM (Kengcn et al., 1993). Aryl-β-glucosidase of *Trichoderma* spp. was totally inhibited by 1% glucose, and *Microbispora hispida* aryl-β-glucosidase was 35, 66, and 79% inhibited by 10, 20, and 30% glucose, respectively (Waldron et al., 1986). A cloned β-glucosidase from *Microbispora hispida* was activated two- to threefold in the presence of 2-5% glucose and tolerate up to 40% concentration (Wright et al., 1992). β-glucosidase from a *Streptomyces* sp. was activated twofold by 1.8% glucose (Ozaki and Yamada, 1992). In *Streptomyces* sp., the rate of $p$NPG hydrolysis has been enhanced by glucose at a concentration of 25 to 200 mM (Perez-Pons et al. 1995).

It was observed in the present study that organic solvents had a stimulating effect on β-glucosidase activity especially alcohols at lower concentrations. Among the solvents tested, ethanol was the most effective one which could enhance the activity 41% at a concentration of 10% (v/v) followed by methanol (30%) and n-butanol (21%) except for propanol. This activation decreased with increasing alcohol concentration. The enzyme activity was decreased with an increase of propanol concentrations. Methanol could enhance the enzyme activity at lower concentrations of 10 and 20% while butanol and ethanol led to increased enzyme activity only at 10% level. The activation increased with the chain length of alcohols and the branched alcohols gave the more activation fold than the normal alcohols. The alcohol tolerant enzymes were reported to have some transglucosylation activities (Yan and Lin, 1997). The β-glucosidase could preferentially utilize alcohols rather than water as acceptors for the glycosyl moiety during catalysis of $p$NPG, resulting in elevated reaction rates, which suggests that ethanol increases the hydrolysis rate of $p$NPG by
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acting as an acceptor molecule for glucose (Saha et al., 1994). Therefore, it is inferred that the purified β-glucosidase from *A. sydowii* may have transglucosylation activity.

Activation of enzyme by ethanol was observed from *A. tubingensis* (Decker et al., 2001), *A. oryzae* (Riou et al., 1998), *Candida peltata* (Saha and Bothast, 1996) *Fusarium oxysporum* (Christakopoulos et al., 1994), *Dekkera intermedia* (Blondin et al., 1983), and *Aurebasidium pullulans* (Saha et al., 1994). This activation may be due to glucosyltransferase activity of the enzyme (Pemberton et al., 1980). In *F. oxysporum* the presence of ethanol increased the β-glucosidase activity 1.5 fold (Christakopoulos et al., 1994). The initial β-glucosidase activity of *C. peltata* was stimulated 11% by ethanol at a concentration of 0.75%.

Various metal ions and potential inhibitors modified the activity of the purified enzyme. The enzyme was indeed greatly inhibited by Mg²⁺ and Ag²⁻. This may indicate that thiol groups are involved in the active catalytic site. However, Mn²⁺ did significantly stimulate enzyme activity at 1 and 5mM and completely inhibited at 10mM concentration. Since Mn²⁺ is not involved in the stability of the enzyme, this specific cation could play a role in the enzyme function (e.g., by modulating its activity according to environmental conditions). In the case of metal ion effects, Cu²⁺ has been generally reported as a strong inhibitor for fungal β-glucosidases (Cao and Crawford, 1993; Gueguen et al., 1995; Li and Calza, 1991; Sasaki and Nagayama, 1995), but here, this metal ion activates the enzyme at a concentration of 1 mM. Cu²⁺ ions are generally involved in the enzyme reaction sequence. Yazdi et al. (2003) reported the activation of β-glucosidase of *Neurospora crassa* by Cu²⁺ ions at 1 mM level. The Ca²⁺ activated the enzyme at 1 mM concentration. The Ca²⁺ appears to play a role in maintaining the structure required for catalytic activity and enzymes requiring Ca²⁺ for activation are mainly extracellular one.
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The enzyme exhibited moderate inhibition with reducing reagents such as β-mercaptoethanol, chelating reagent (EDTA), and detergent (SDS). The inactivation of DEPC observed indicates that tryptophan and histidine residues are important in the catalytic action of the enzyme. The chelating agent EDTA did not inhibit β-glucosidase activity, indicating that divalent cations are not required for enzyme activation.

5.6 Application studies

Bioethanol has significant environmental advantages over petroleum as a liquid fuel (Duff and Murray, 1996) if produced from cheap, renewable lignocellulosic feedstocks. Among the systems for ethanol production from cellulose, the simultaneous saccharification and fermentation (SSF) process has attracted many investigators (Hari Krishna et al., 1998). The SSF process offers benefits such as improved ethanol yields by reducing the product inhibition exerted by saccharification products and also eliminates the need for separate reactors for saccharification and fermentation, which results in cost reductions.

The production of ethanol from wheat bran and rice straw were performed after a pretreatment with 0.5% H$_2$SO$_4$ followed by autoclaving for 30 min. Lignocellulosic biomass cannot be saccharified by enzymes to high yields without a pretreatment mainly because the lignin in plant cell walls forms a barrier against enzyme attack (Sewalt et al., 1997). An ideal pretreatment reduces the lignin content and crystallinity of the cellulose and increases surface area (Takagi et al., 1977).

The production of ethanol from wheat bran and rice straw with help of baker’s yeast in the presence of the purified β-glucosidase from Aspergillus sydowii BTMFS
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55 showed a considerable yield of ethanol production in range of 24-48 h of incubation.

A considerable level of yield of ethanol from wheat bran was obtained after 48 h (11.2 g/L) at 37°C. Whereas, in the fermentation medium containing rice straw, the production was maximal at 24 h with an ethanol yield of 10.32 g/L. It was reported that the ethanol production rate was much higher at the initial stage of the fermentation such as 22 h (Saha et al., 2005). An incubation period of 24 h has been found to be optimum for production of ethanol by *S. cerevisiae* from acid and enzymatic hydrolysate of agricultural residues (Tewari et al., 1987).

The ethanol production from wheat bran with an enzyme yield of 17 g/L by simultaneous saccharification and fermentation with a recombinant *E. coli* strain, after 112 h was reported (Saha et al., 2005). Ethanol has been produced from a variety of substrates such as sunflower hull (Sharma et al., 2004), raw corn flour (Wang et al., 2007), wheat bran (Saha et al., 2005), lignocellulose from a weedy creeper, *Antigonum leptopus* and sugar cane leaves (Harikrishna et al., 2000, 1998), barley husk (Adrados et al., 2005) and water hyacinth (Nigam, 2007).

Temperature is a crucial factor for Simultaneous Saccharification and Fermentation because of the differences in saccharification optima (50°C) and that of the yeast (35°C). A temperature range of 39-40 °C was observed to be optimum for maximum ethanol yields. Slininger et al. (1987) reported that optimum fermentation rates with *Pachysolen tannophilus* were obtained at 32°C. They suggested that at high temperatures either the enzyme is not induced and/or once formed the enzyme degrades rapidly. According to Philippidis (1995), the optimal temperature for simultaneous saccharification and fermentation is around 38°C, which is a compromise between the optimal temperatures for hydrolysis (45-50°C) and
fermentation (30°C). The effect of initial pH value ranging from 3.5 to 5.5 on fermentation of enzymatic hydrolysate of sunflower hulls revealed maximum ethanol yield of 0.455 g/g at a pH of 5.0. A pH value lesser than 4.0 and higher than 5.0 resulted in sharp decrease in the ethanol yield (Sharma et al., 2004). The temperature, pH, substrate concentration, pretreatment of the lignocellulosic substrates etc are the significant factors that govern the ethanol production in large scale for the industrial use.

It is concluded that the production of ethanol from wheat bran and rice straw facilitated by the action of β-glucosidase from *A. sydowii* has potential for utilization of the process as a preliminary step for the ethanol production in large scale for industrial purposes. Further work is warranted for further optimization of various process parameters for increasing the yield of ethanol from these substrates.