Production of fungal and bacterial cellulase--free β-1,4-xylanase in submerged cultivation

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1. Introduction

With the exception of cellulose, β-1,4-xylan, is the most abundant hemicellulose fraction in both agricultural and forest plants, in which it constitutes about 15-35% of the total dry weight (1). Due to structural heterogeneity, xylan-degrading enzyme system consists of several hydrolytic enzymes (2). The best known of these enzymes are endo-β-1,4-xylanases (β-1,4-xylan xylanohydrolase EC 3.2.1.8), which attack the main chain of xylan and β-xylosidase (β-1,4-xyloside-xylohydrolase EC 3.2.1.37), that hydrolyses xylooligosaccharides to D-xylose. In addition to these two enzymes, several auxillary enzyme activities are also produced and are necessary for efficient debranching of substituted xylans (3-6). Conventionally, xylanases in conjunction with cellulolytic enzymes have been mainly utilised for bioconversion of lignocellulosic materials, especially residues and wastes from agricultural and forestry, into highly valued products of commercial applications (7). Nevertheless, this method has been shown to be less attractive for the production of fermentable sugars due to the fact that xylan can be easily hydrolysed by acidic process. Recently, research in this field seems to develop towards production of alkaline-active and hyper or thermostable cellulase-free β-1,4-xylanases by the use of wild, mutant or genetically engineered strains harbouring a xylanase gene of a given thermophilic microorganism. Examples of cellulase-free xylanase producing organisms include some species of *Clostridium acetobutylicum* (8), *Trichoderma longibrachiatum* (9), *Trichoderma reesei* (10), *Aspergillus awamori* (11), *Aspergillus tamarii* (5), *Thermomyces lanuginosus* (12), *Penicillium canescens* (13), *A. pullulans* (14) and genetically

modified fungal and bacterial strains such as *Streptomyces lividans* (15), *Bacillus subtilis* (16) and *E. coli* (4).

Such xylanase preparations are presently used in kraft pulp for bleaching processes as an alternative to chlorine-based bleaching methods, which generate chloroorganics (12,17). The liberated chloroorganic substances are hazardous to human and expensive processing methods are required to eliminate such effluents from the environment (7,18). In the food industry, cellulase-free β-1,4-xylanase is used to convert xylan to xylose and xylooligosaccharides (17,19). Moreover, this enzyme has also found application in improvement of bread-making quality, clarification of beer or juice and maceration of vegetables (13,20).

The aim of this article is to review current trends and biotechnological factors or parameters that affect the production of cellulase-free β-1,4-xylanase by fungal and bacterial strains during submerged cultivation.

2. Xylanase synthesis

The complexity of xylans requires the action of multiple xylanases and cellulases with overlapping yet different specificities to effect extensive hydrolysis (21), but the regulatory mechanism to overproduce a particular enzyme *e.g.* cellulase-free β-1,4-xylanase in large amounts in relation to other enzymes has not been fully elucidated, at least on the molecular level. Some of this multiplicity has been demonstrated to be genetically determined. The induction mechanism of xylanase (constitutive or induced) as well as regulation (under separate or common regulatory control system with cellulases) seems to differ between organisms. Some reports have shown that xylanases are inducible and specific induction can occur independently of cellulase synthesis (7, 21-23) in certain organisms. However, post-translational modifications, such as glycosylation, proteolysis or aggregation with other polysaccharides have been shown in certain cases to account for some of this multiplicity. Predominant enzymes, apart from cellulolytic enzymes within these systems, are endo-β-1,4-xylanase (EC 3.2.1.8) and β-xylosidase (EC 3.2.1.37). Both enzymes are inducible and subject to catabolite repression by glucose and glycerol (3). However, there are different opinions on the identity of the true inducers of these enzymes. According to the generally accepted view on the regulation of their synthesis, low-constitutive levels of hydrolases act on polymeric substrates to produce small soluble signal fragments, which enter the cell membrane and thereby induce the synthesis of these enzymes, thus allowing effective utilisation of the polysaccharides (5, 6). Xylose, xylobiose and higher homologous xylooligosaccharides have been implicated as the most probable potent low-molecular mass inducers. The enhanced effect of xylobiose on xylanase formation has been ascribed to prolonged periods of induction due to its slow utilisation (24). Furthermore, a series of non-metabolizable alkyl and aryl-β-D-xylosides, especially β-methyl-D-xyloside (β-MX), have also been defined as inducers in many

organisms, but it varies and appears not to function in all microorganisms. For example, *Aspergillus tamarii* produced biomass in a culture medium with β-methyl-D-xyloside (β-MX) as the only carbon source. Although the rate of absorption was slow in this case, an inducing effect was observed (5,6). This effect contrasted with the previous observation with other microorganisms, where β-MX induced the production of xylanolytic enzyme and seemed to function as non-metabolizable inducer (22,25). On the other hand, β-MX did not induce xylanase formation in *Thermomyces lanuginosus* (12). Nevertheless, it has also been shown that cleavage of xylobiose to xylose may result in catabolite repression of xylanase synthesis. Glucose and glycerol are well defined catabolite repressors in almost all examined microorganisms that produce xylanolytic enzymes *e.g. Trichoderma longibrachiatium* (9), *Aspergillus ochraceus* (23) and *Streptomyces* sp. (3), although constitutive xylanase activity has been observed. Besides glucose, the role of other, easily metabolizable substances, such as xylose, arabinose and lactose, as inducing substances differs in various strains. Furthermore, arabinose, galactose, maltose and lactose also show various effects in different organisms. Xylose acted as an inducer for *A. pullulans* (14,26), *Aspergillus terreus* and *Aspergillus niger* (22) and other microorganisms, whereas the same substrate acted as repressor during the cultivation of *Trichoderma longibrachiatum* (9) and *Cryptococcus albidus* (27). Also, other sources of simple sugars such as D-ribose and D-lyxose have recently been shown to induce xylanase formation in *Thermomyces lanuginosus* (12) with various effects, but β-MX had no inducing effect in the latter strain. In either case, the inducing effect of these simple sugars was explained to be caused by a non-specific binding to regulatory molecules.

Evidence has been gathered that positional isomers of xylobiose (Xylp-β-(1,4) Xylp) such as 1,2-β-xylobiose and 1,3-β-xylobiose added into the culture medium are more potent inducers than the cleavage products formed or derived from purified xylan during cultivation (28). Such isomers of xylobiose can be formed due to transglycosylation by xylan-degrading enzyme systems (27) and have been known as inducers *in vivo*. As mentioned above, xylan-degrading organisms secrete complex mixtures of hydrolytic enzymes but the ratio of these enzymes varies and depends on available polymeric substrates, conditions of cultivation and on existing regulatory mechanisms in a particular organism. Several organisms produce xylanase not only in the presence of xylan, but also when α-cellulose is present (1,29). This has been demonstrated for *Trichoderma reesei* (29) and *Sclerotium rolfsii* (1) during cultivation in an α-cellulose-containing medium. In some studies, it has been shown that for some organisms, the synthesis of xylanase and cellulase is regulated separately. But this mechanism of regulation is not common or predominant in other investigated organisms. Conversely, induction of xylanase, mannanase and cellulase has been demonstrated in fungus *Polyporous adustus* to be controlled by a single common regulatory gene (18). Therefore, it seems that the mechanism of regulation and ratio of xylanase produced in either case to other enzymes might depend on the source of

substrates or inducers produced synergistically during xylanase production as well as existing regulatory mechanisms. In *Aspergillus terreus*, the selective induction of xylanase synthesis was achieved using xylan, xylobiose or D-xylose as substrates, whereas in the presence of α-cellulose or cellobiose both cellulase and xylanase were produced (22).

3. Conditions affecting the selective synthesis of cellulase-free β-1,4-xylanase

3.1. Effect of inducing substrates

The choice of an appropriate inducing substrate is a prerequisite for effective and selective production of xylanases despite overlapping multiplicity of enzyme activities (21,30) that exist in many organisms. The substrate not only serves as carbon and energy sources, but also provides the necessary inducing compounds, formed synergistically during the synthesis of xylanase and this could lead to alteration of its rate of synthesis and ratio to the other enzymes under different conditions of cultivation. Most of these inducing substances such as β-methyl-D-xyloside (β–MX) and lyxose are known to extend the period of cultivation, and therefore result in a prolonged production phase and overall increase in the productivity of xylanase (12,31). Cultivation of different organisms on purified xylan has been shown to result not only in increased yields of xylanase but often caused selective induction of xylanase with no traces or only traces of concomitantly formed cellulase activities. Nevertheless, these purified compounds are expensive and not desirable for large-scale production. It has been reported that several organisms are capable of producing high levels of xylanase with low cellulase activity when grown on α-cellulose (24), viscose fiber or corn cobs, wheatbran and wheathusk (5,6,13,17). Furthermore, an alternative to the use of purified xylan is the addition of non-metabolizable synthetic xylobiose analogue β-methyl-D-xyloside (β-MX) to the culture medium. Addition of this compound at different concentrations (5–10 g/l) to various natural and purified xylan sources such as oat spelt xylan, wheat bran or husk gave a reasonable yield of xylanase activity by different *Aspergilli* (5,6,31). Royer and Nakas (24) achieved a higher ratio of xylanase synthesis to cellulase on oat spelt xylan-containing medium using *T. longibrachiatum* without β-MX supplementation. Cellulase activity in this oat-spelt xylan containing medium was extremely low. On the other hand, the authors obtained a much higher activity of xylanase on α-cellulose-containing medium 2303.83 nkat/ml with an increased yield of (7.17 nkat/ml) cellulase.

However, for more direct synthesis of a specific xylanase preparation such as cellulase-free β-1,4-xylanase, the cheapness of appropriate inducers and substrates is an important factor due to economic viability. Therefore, for commercial synthesis of a specific xylanase preparation, the relative cost of

a required substrate versus the cost of enzyme purification should be considered for its efficient production (24). Subsequently, these different lignocellulosic materials have been assessed in relation to pure substrates like xylan or cellulose in different studies (1,11,13,24). Inexpensive substrates, mainly insoluble lignocellulosic materials such as barley husk, corn cobs, hay, wheat bran or straw have been investigated for this purpose (11,13,24,32). In some studies using different strains, these substrates gave a better yield of xylanase in comparison to the use of purified xylan. This also shows that there is a realistic future alternative to the use of these inexpensive C sources for large-scale production processes, but some biotechnological problems may arise at this level of production. Generally, these insoluble substrates were used at concentrations of 30–75 g/l for effective production of cellulase--free xylanase. The optimal concentration that resulted in high yield of xylanase was for example 31.2g/l of corn cobs for *Thermomyces lanuginosus* (32) with xylanase activity of 20004 nkat/ml, 32.4 g/l of wheat straw for *Thermoascus auriantiacus* (33) and 72.4 g/l of Avicel for *S. commune* (34). The cheapness of C substrates is an important factor but certain biotechnological problems such as high medium viscosity, insufficient agitation and limited mass transfer are drawbacks at large-scale production due to high concentration of incomplete breakdown of insoluble substrates. An alternative to solving the afore mentioned technical problems is the use of soluble substrates for large-scale production. In this regard, xylose is the most preferred soluble source of carbon, although the use of lactose separately or in combination with insoluble substrates has been reported (10,14). In addition to the source and nature of inducing insoluble substrates, several methods of pretreatment also influence the direction of enzyme synthesis with increased yield of xylanase. Gomes et al. (33) investigated the effects of physical treatment of wheat straw on the production of xylanase by *T. aurantiacus*. The wheatstraw pretreatment (190°C, 10 min.) and grinding of steamed substrates to a particle size of about 0.25 mm were found to increase the accessibility of wheatstraw with effective production of high xylanase activity, whereas untreated or raw wheat straw gave a low yield of enzyme. The authors suggested that modification of the wheat straw structure, resulting in changes of physical structure of lignin, increased surface area and pore sizes, partial decrystalization of cellulose, removal of acetyl groups of hemicelluloses or depolymerization of these compounds enhanced the productivity of xylanase. On the other hand, a negative effect of substrate pre-treatment on the level of xylanase synthesis was observed for *Thermomyces lanuginosus* grown on corn cobs as a substrate (17). The authors found that particle size of 2-7 mm was optimal for the raw substrate and obtained an activity of 23983.13 nkat/ml of xylanase, whereas the same substrate used as fine powder caused more than a threefold decrease in xylanase activity. These examples confirm the observed variability in xylanase production depending on the source, origin and method of substrate pre-treatments using a definite microorganism.

4. Effect of medium composition

Generally, media composition used in reported studies (1,5,8-10,12) for the production of xylanase are complex and contain apart from the inducing substrates, some organic and inorganic nitrogen sources as well as mineral salts. The complex nature of different media has been shown to affect the synthesis of xylanase in relation to the other enzymes. Therefore, alteration of the medium component concentrations could result in changes of the regulatory control system and therefore overproduction of xylanase in relation to other enzymes. Such an effect has been investigated with the use of different statistically designed experimental response surface methodologies (19,24), arranged either according to an orthogonal central composite design, described by Box and Hunter (35) or a folded Plakett and Burman design (36). These response surface methodologies were employed to find optimal conditions and identify optimum concentrations of substrates in order to attain maximum productivity. One of such studies relating to the effect of added inorganic nitrogen compounds on the synthesis of xylanase to other enzymes has been reported by Haltrich et al. (1) using a mutant strain of *Sclerotium rolfsii* during cultivation on α-cellullose-containing media. Ammonium nitrate, urea, ammonium sulphate, sodium nitrate and $(NH_4)_2HPO_4$ in sequence, were found to be the most preferred nitrogen sources for xylanase formation. Furthermore, they found no significant effect of added mineral salts such as KH_2PO_4, KCl and $MgSO_4 \cdot 7H_2O$ on xylanase formation in this organism. A xylanase activity of about 6567.98 nkat/ml with mannanase activity of 2583.85 nkat/ml and traces of other accompanying enzymes were reported on ammonium nitrate based medium after optimization processes. Pham et al. (37) used a strain of *Bacillus polymyxa* for the synthesis of cellulase-free endo-β-1,4-xylanase and obtained an activity of about 24 nkat/ml on lignocellulosic wastes and xylan media. The authors further applied the statistical experimental design procedure based on central composite design to search for optimal concentrations of examined variable factors on xylan containing medium. They obtained about 135% increase in xylanase activity after optimisation processes and the established optimal levels of examined factors were 3.16 g/l, 1.94 g/l, 0.8 g/l for xylan, casein hydrolysate and NH_4CL, respectively (38). On the other hand, it has been reported for *A. pullulans* that the kind of nitrogen sources (organic or inorganic) not only alters the formation of xylanase but also changes the morphology and physiology of the strain (26). Also, an addition of organic nitrogen sources into the culture medium has been shown to positively affect the rate of xylanase synthesis in comparison to some inorganic nitrogen sources (1,13). Ammonium nitrate lowered the pH value during cultivation, which markedly enhanced the xylanase formation by *S. rolfsii* (1). Recently, Flores et al. (3), reported a concentration-dependent stimulatory effect of 1% pyruvic acid in a medium on the ratio of xylanase to xylosidase formation in *Streptomyces* sp. CH-M.-1035. They obtained a 20-fold increase in xylosidase formation in relation to xylanase. Furthermore, an addition of 1% pyruvic acid facilitated the formation of β-xylosidase and had a negative effect on xylanase

synthesis, and thus altered both the ratio and yield of these enzymes. Udeh et al. (26), have recently demonstrated a similar stimulatory effect of EDTA on cellulase-free β-1,4-xylanase formation at 0.06% on a medium containing 3% xylose with about 16.5-22 and 1.5-22-folds increase in both volumetric and specific activities. The stimulatory effect of EDTA was attributed to its ability to chelate metal ions, alteration of cell permeability and activation of a specific intracellular protein, leading to a metabolic shift and enhanced secretion of cellulase-free β-1,4-xylanase. Balakrishnan et al. (39) examined separately the effects of different concentrations of DL-norvaline, glycine and casamino acids on the synthesis of alkaline protease in relation to xylanase, both in glucose and wheat bran media using *Bacillus* sp. (NCL-87-6-10). The authors reported about 1.5-fold higher activity of xylanase (from 733.48 – 1183.57 nkat/ml) in the presence of 0.5% of norvaline with an accompanying decrease of protease activity (from 3817.43 – 533.44 nkat/ml). Similar tendency was observed for 2-2.5% of casamino acid. On the other hand, the addition of glycine to the medium containing 3% of wheatbran increased the xylanase activity up to the concentration of 0.25%, and later diminished with further increase in its concentration. A 5-fold increase in xylanase activity was observed in glucose media, which contained 0.25% of glycine. In addition to the components mentioned above, different surfactants such as Tween 80 and fatty acids in the form of oleic acid have also been shown to increase the permeability of cell membranes of certain xylanase producing organisms (fungi and bacteria) and their enhanced secretion. For example, the addition of Tween 80 at concentrations of 0.5-3 g/L into culture media during cultivation of *A. pullulans* (40) and *Anthrographis* sp. (41) enhanced xylanase production by approximately 12-60%. The positive effect of Tween 80, oleic acid and olive oil, alone or in combination were further demonstrated in a clone of *Streptomyces lividans* IAF 18. An addition of 0.2% of Tween 80 or 0.2% of an emulsion containing Tween 80 and olive oil (1:1) to the culture medium, increased the synthesis of xylanase from 10002.0 to 21671.0 nkat/ml (15).

5. Effect of cultivation conditions

5.1. Effect of pH value on cultivation

An effect of pH value on the β-1,4-xylanase synthesis is very significant and differs from strain to strain. Generally, pH value of 6–7 has been reported to be optimal for the production of β-1,4-xylanase by strains such as *Trichoderma reesei* (10) and *Thermomyces lanuginosus* (12). The former author observed that such pH value influenced the production of both β-1,4-xylanase and cellulase and favoured xylanase synthesis. At this pH, the latter author obtained only cellulase-free xylanase on 3% corncobs. The pH of 6.5–7.7 generated higher yield of xylanase and cellulase using *T. longibrachiatum* (9). It was suggested that this higher yield of the enzymes ob-

tained at an elevated pH value of 6.5–7.7 may be the result of relatively slow release and utilisation of soluble end-products of cellulose or xylan hydrolysis. The authors (10,12) further explained that such conditions diminished the growth rate of examined microorganisms due to unfavourable pH or poor utilisation of substrates such as arabinose and therefore resulted in an increased yield of an extracellular xylanase activity. Generally, these studies revealed that lowering of the pH value of culture medium from 6.5 to 5 or 3, using the strains mentioned above, facilitated the production of oxalic acid. This oxalic acid produced inactivated or rather inhibited the enzymes and thus lowered the activity in comparison to culture run at higher pH value. On the other hand, fungi such as *Aspergillus niger*, *Aspergillus tamarii*, *Aspergillus awamori* and *Aureobasidium pullulans* (5,6,14,35,42) are known to synthesize considerable levels of xylanase at lower pH values (3-4.5) on different substrates. In this regard, the production of xylanase by various strains differs and depends on their genetic traits, physiology, pH of the culture medium and more specifically on the type of secreted protein as well as the existing regulatory mechanism. Most strains that prefer an acidic pH value range for growth, produce low levels of xylanase at elevated pH values. The reason for omitting higher pH values during cultivation, as reported, was in part due to the higher synthesis of proteolytic enzymes, which inhibited the activity of secreted xylanolytic enzymes as well as the aforementioned inhibitory effect of oxalic acid produced during cultivation (18). The pH value of culture medium in most cases has been shown to markedly increase the synthesis of a particular enzyme, *e.g.* xylanase, in relation to the other enzymes and moreover to affect the general level and ratio or pattern of protein secreted.

5.2. Effect of growth temperature

The temperature of cultivation not only affects the growth rate of microorganisms but also influences the level of xylanase produced in relation to cellulase. An example of this temperature effect in augmenting or varying the ratio of xylanase to cellulase was observed for *Trichoderma reesei* Rut C-30, which grew equally well both at 17,28°C and 37°C on lactose (43,44). As reported, an increased synthesis of xylanase was obtained at higher temperatures, whereas the production of cellulase was reduced. At lower temperatures, a reversible effect was observed. Suh et al. (10) studied also the effect of temperature on protein secretion and enzyme activities (xylanase and endoglucanase) by *T. reesei* QM6a and a mutant *T. reesei* RL P-37 at 25, 30 and 37°C, both on lactose or xylan-containing media. Both strains showed reduced secretion of overall protein at higher temperatures on lactose. The secretion of xylanase was reduced in QM6a, but increased in RL P-37 at 37°C. An increased specific activity of xylanase, three-fold for QM6a (361.17 nkat/mg protein) and 70-fold for RL P-37 (17420.15 nkat/mg protein) was observed at 37°C in comparison to the results obtained at 25°C.

In contrast to the lactose grown-cultures, *T. reesei* QM6a showed no significant changes in the pattern of protein secreted on xylan-grown media, but overall protein secretion increased three-fold in RL P-37. Both strains exhibited an increased specific activities of xylanase and endoglucanase (7356.47 nkat/mg protein; 230.05 nkat/mg protein) in RL P-37 and (5584.45 nkat/mg protein; 134.03 nkat/mg protein) in QM6a at 37°C. These results confirm the above mentioned positive effect of higher temperatures of cultivation on the regulation of the ratio of xylanase formation to cellulase (i.e. increase in xylanase and decrease in cellulase). The authors (10,43,44) in agreement concluded that by increasing the cultivation temperature, it is feasible to improve the yield of xylanase in relation to other enzymes or obtain a more strictly directed synthesis of cellulase free-xylanase. These observations also imply that the regulation of secretion and/or synthesis of xylanase, cellulase and endoglucanase may not be under a common regulatory control at least in *T. reesei* RL-37. Stölinberger et al. (45) extended these studies and examined an effect of temperature using a temperature shifting method to regulate the synthesis and the ratio of the enzymes secreted. They showed that during an initial phase of cultivation of *T. reesei* Rut C-30 at 37°C, followed by a shift of temperature to 28°C, at the beginning of enzyme formation both the level of xylanase produced and its ratio to cellulase increased. On the other hand, the thermophillic fungi generally grow at elevated temperatures up to 60°C, but it is unclear whether the lowering or increasing of the temperature of cultivation leads to an increased synthesis of enzymes. Furthermore, its effect on the ratio of xylanase to cellulase formation has not yet been reported.

Additionally, other cultivation conditions apart from previously mentioned effects of higher temperature of cultivation are also important for effective production of xylanase. Panda et al. (46) reported a mixed culture technique which involved cultivation of *T. reesei* D-1-6 and *Aspergillus venti* Pt 2804 on α-cellulose containing medium. This method was based on an initial cultivation of *A. venti* for 15 h., followed by inoculation or addition of *T. reesei* cells into the same culture medium. This method of cultivation facilitated cellulase-free xylanase production in comparison to separate cultures of *T. reesei* or *A. venti*. It was concluded that *A. venti* produced a glucose polymer which enhanced the activity of xylanase in *T. reesei*. As shown, this glucose polymer had no effect on cellulase or β-glucosidase synthesis. These authors further demonstrated that the culture filtrate of *A. venti* obtained after 72 h. of cultivation, filtered and heated for 60 min. at 30°C or 100°C stimulated the formation of cellulase-free β-1,4-xylanase by *T. reesei* when added to the culture medium. Hydrolysis and analysis of this "heat stable" compound revealed that it consists of glucose or glucose polymer (46).

5.3. Effect of mixing

Optimization of culture conditions generally involves changes of a single variable factor, while maintaining fixed levels of all other variables. The effect

of mixing and aeration is not an exception. The effect of shear forces and mechanical forces on filamentous fungi is known to cause disruption of mycellium or earlier sporulation of fungi resulting in the leakage of intracellular materials and lowering of the enzyme production (18). It also affects the morphology of fungi *i.e.* mycellium or pellet form, which in turn might affect the metabolism and the rate of xylanase synthesis. Recently, it has been reported that the type of impellers and its speed imparted shear stress and thus markedly affected xylanase formation in *Thermomyces lanuginosus* (33) and *P. canescens* (18). Gaspar et al. (47) applied a hub-mounted pitched blades impeller (PBTS), instead of a disk-mounted blade impeller (DTA) for the synthesis of hemicellulase and xylanase by *P. canescens* 10-10c. The hub-mounted pitched blade impeller reduced the lag time of hemicellulase production and increased xylanase productivity 1.3-fold. In a 15 m^3 bioreactor, an impeller speed of 30 rpm and increased aeration rate from 0.1 vvm to 0.3 vvm allowed xylanase formation of 11669.0 – 13002.6 nkat/ml, when the pO_2 level was maintained at 5-8% (32). Gaspar et al. (13), found that the increase of the impeller speed up to 1200 rpm decreased by almost half not only the biomass formation but also the production of xylanase. This confirms the sensitivity of fungi to hydrodynamic stress and the dependence of xylanase production on the rate of agitation. Nevertheless, an impeller speed of 450 rpm was found to be optimal for *P. canescens* 10-10c and about 17503.5 nkat/ml of xylanase activity was produced in a 6 dm^3 fermentor (13).

5.4. Strain modifications

Most studies on the regulation of synthesis of xylanolytic enzymes have focused on an induction of their activity under various cultivation conditions, rather than on gene technology (48). However, genetic modifications of fungal and bacterial organisms for effective or strictly controlled synthesis of cellulase-free β-1,4-xylanase activity have also been reported, and are generally based on traditional mutation or genetic engineering techniques. In the case of *Aspergillus awamori* (32), *Penicillium canescens* (13) and *A. pullulans* (14) mutation has been used to tailor xylanase formation with high yields and in consequence to regulate the relative overproduction of this enzyme in comparison to cellulases and proteases or to achieve a cellulase-free β-1,4-xylanase activity. For this purpose, Smith and Wood (32) obtained a mutant of *A. awamori* AANTG43 that produced cellulase-free xylanase (19320 nkat/ml) and β-xylosidase (58.35 nkat/ml) with low protease activity. Gasper et al. (13) used a mutant strain of *Penicillium canenscens* 10-10c, which produced a cellulase-free xylanase on corn cobs, wheat bran or husk with maximum activity of 1750.35 nkat/ml and a low protease activity. Suh et al. (10), used also a hyper-secretory mutant of *T. reesei* RL-P37 to obtain higher xylanase (916.85 nkat/ml) activity in comparison to the wild type of *T. reesei* QM6A that secreted 323.4 nkat/ml. In the field of gene technology, *Bacillus subtilis* harbouring a xylanase gene from *Clostridium thermocellum* produced

xylanase with an activity of about 1233.58 nkat/ml, when grown on a synthetic medium containing 4% of maltose as a carbon source. A xylanase gene from *Bacillus* sp. cloned on plasmid pCX311 and expressed in *E. coli* was used for overproduction of xylanase. A significantly higher xylanase activity of about 7.33 nkat/ml was produced by *E. coli* clone in the culture medium in comparison to zero activity obtained with wild *E. coli* strain (16). Despite this genetic modifications, other accessory enzymes such as β-galactosidase and β-lactamase (16) were produced in this case. Bertrand et al. (15) used a clone of *Streptomyces lividans* IAF18 obtained by homologous cloning of the xylanase gene from *S. lividans* 66 using the multicopy plasmid pIJ702 for the production of cellulase-free xylanase. A yield of about 26672.0 nkat/ml on 2% xylan after 120 h. of cultivation at 34°C was achieved, whereas the wild type yielded 446.8 nkat/ml.

6. Effect of pH, temperature and other factors on the stability and activity of xylanase

Temperature and pH activity as well as the stability of xylanases produced by various fungal and bacterial organisms have been studied (49-52). Results proved that instability of xylanase preparations was in part due to the presence of proteolytic enzymes, whereas the degree of thermostability depended on the enzyme origin *i.e.* structure based on conserved amino acid sequences or terminal aromatic residue interactions, which can significantly enhance the protein stability due to the formation of hydrophobic clusters or "sticky patches" on the surface of the enzyme. Different reagents added to xylanase preparations during incubation such as cysteine, N-bromo-succinimide, glycine and polyol in form of sorbitol acted as thermal stabilisers (49-52). For example, the thermostability of endo-β-1,4-xylanase from *Bacillus circulans* was enhanced by the addition of disulfide bonds *e.g.* cysteine (50). In the case of xylanase preparation from *Bacillus D3*, which in its structure lacks cysteine residues for the formation of disulfide bonds, its thermostability was ascribed to other factors such as electrostatic or hydrophobic interactions and the enzyme concentration in the substrate (51). The oxidation of indole group of tryptophan was also reported to increase the thermostability of certain proteins or enzymes. Xylanase from *AT Bacillus* was protected against thermal inactivation by N-bromo-succinimide, which is a tryptophan specific reagent with an increased stability. Sorbitol was also reported to increase the thermostability of an endo-β-1,4-xylanase from *Bacillus amyloliquefaciens*. This enzyme showed maximum activity of hydrolysis for 4 days at 50°C and retained 100% of its initial activity in the presence of sorbitol (250-400 mg/ml) depending on the pH values (5.25-8.0) of hydrolysis (52). On the other hand, the negative effect of proteolytic enzymes on the activity of cellulase-free xylanase has been confirmed by the results obtained in the presence of a serine protease inhibitor — phenyl methanesulphonyl fluoride

(PSMF) (1mM), which markedly enhanced the stability of endo-β-1,4-xylanase preparation from *Aspergillus niger* (42). Furthermore, an alkalophilic thermophilic *Bacillus* sp. produced xylanase, which was stable at 60°C and at pH 7 and 8 in the presence of neutral amino acids such as glycine (0.5 M) and β-alanine (0.5 M). The addition of glycine to the enzyme at these pH values showed a four to six-fold increase in the half life. The stabilizing effect of glycine on the xylanase preparation was ascribed to the effect of increased surface tension of water, which stabilised the protein structure or preferentially induced intermolecular hydrophobic interactions (49).

The characteristics of fungal and bacterial xylanases have been extensively studied and vary according to molecular weights, isoelectric points (pI), K_m, V_m, pH and temperature activities, as well as substrate specificities (51,52). They also exhibit a great homology at the molecular level on the basis of conserved amino acid sequences and hydrophobic clusters. They are classified into two main families of glycosyl hydrolases, and belong either to family F (previously known as 10) or G (previously known as 11). Both use ion pair catalytic mechanism and both retain anomeric configuration following hydrolysis (51-55). This indicates that they use a double-displacement mechanism in which the reactive intermediate is bound to the enzyme. This enables them to carry out trans-glycosylation reactions. The family F has high molecular weight of around 55-120 kDa, with acidic isoelectric points (pI 4.8-5.4). The family G has low molecular weight and basic (10.3–6.5) pI (51,52). Chen et al. (54) recently purified to homogeneity three xylanases from *E. coli* carrying a xylanase gene of a hyperthermophilic bacterium *Thermatoga maritima*, which had higher molecular weights of approximately 120, 95, and 85 kDa, respectively. All purified xylanases belonged to family F on the basis of amino acid sequence analysis.

Generally, the optimum pH of xylan hydrolysis is around 4-5 for most fungal xylanases and they are usually active at pH values from 2 to 7. The pH optima (pH 6-10) of bacterial xylanases are generally slightly higher than those observed for fungal xylanases (7,21,54). The cleavage products distribution after incubation of different endo-β-1,4-xylanase preparations vary according to different substrates used, time, pH and temperature, although exo-fashion mechanism of substrate hydrolysis exists (55,56). Analyses of the hydrolysis products obtained using most fungal and bacterial xylanases show that they are mostly endo-β-1,4-xylanase. There are differences in the composition of saccharides and oligosaccharides liberated during the course of incubation using these enzymes. For endo-β-1,4-xylanase, within a short period of incubation the proportion of xylooligosaccharides increased, whereas after a long duration (24 h.) of incubation, (it was observed that) only xylotetrose, xylotriose, xylobiose and relative amounts of xylose were obtained (53,57). For exo-β-1,4-xylanase, the proportion of liberated sugar composition was reverse with clear rise in xylose concentration within the first few minutes. The hydrolysis products of xylanase include xylose, xylobiose, xylotriose, xylotreaose, xylopentaose and higher xylooligosaccharides and xylose being the most pronounced reaction product. Lama et al. (57) examined

the product distribution of xylanase produced by a new thermophillic *Bacillus thermoantarcticus* that showed optimum temperature of activity at 70°C and at pH optimum of 5.6. The composition of reaction products varied with the increase of the temperature of the assay. At 70°C, the main product of reaction was xylose after 24 h. of incubation, whereas between 80 to 90°C, the main products of reaction were xylose, xylobiose and xylotriose with changes in the pattern of reaction products favouring the release of higher contents of xylobiose and xylotriose, respectively as the temperature of hydrolysis was increased up to 90°C (57). For substrate specificity, activity has been found for *A. pullulans* xylanase towards pullulans, laminarin, lichenen and carboxymethylcellulose but it was not detected for chitin, starch, cellulose and cellobiose (58).

Metal ions also affect the stability and yield of xylanase activity with different effects. Dűsterföft et al. (59) tested the effect of different metal ions at 1 mM concentration on the activity of purified xylanases Xyn1 and Xyn2 from *Humicola insolens*. The results obtained showed that Xyn1 activity was only affected by Hg^{2+} and Mn^{2+} ions, but retained 100% of its initial activity in the presence of other examined metal ions. The second enzyme Xyn2 was inhibited by several metal ions such as Zn^{2+}, Ni^{2+}, Cu^{2+}, Pb^{2+}, Mn^{2+} and Hg^{2+}, whereas 100% of its activity was observed for Ca^{2+}, Fe^{2+}, Al^{3+} and EDTA. In addition, the effects of crystalline cellulose (Avicel), carboxymethylcellulose and lignin were studied and the activities of either xylanase were unaffected by these lignocellulosic model compounds. Okazaki et al. (60) also studied the effect of metal ions and other compounds such as EDTA, iodoacetic acid and sodium *p*-chloro-mercuribenzoate on the activities of four isoenzymes of xylanase from *Bacillus* sp. at 5 mM concentrations. Among tested compounds, Hg^{2+} completely inhibited the activities of the four enzymes. Cu^{2+} also markedly affected their activities. Other metal ions tested such as Ca^{2+}, Ni^{2+}, Mg^{2+}, Ba^{2+}, Co^{2+} and Zn^{2+} had no inhibitory effect on any of the enzymes. On the other hand, Lama et al. (57) investigated the effect of certain compounds on the xylanase activity from *Bacilus thermoantarcticus* at 1 mM concentrations. In these studies, Cu^{2+}, Fe^{3+}, sodium docedyl sulphate (SDS) and N-bromo-succinimide had a partial inhibition. Mg^{2+} increased the activity by 11% above the maximal activity and it was not affected by Ca^{2+}, EDTA and *p*-hydroxymercuribenzoate.

Fungal endo-β-1,4-xylanases usually show optimal activity at about 50°C and are often totally inactivated at temperatures above 65°C. Nevertheless, there are exceptions and recently efforts are made to search for highly thermostable enzymes showing activities up to 95°C or above, and at a neutral pH value. A broad pH activity (pH 3.5 – 6) and temperature (30 – 70°C) has been recorded for different endo-β-1,4-xylanases for *A. pullulans* (58), *S. rolfsii* (1), *T. reesei* (53), *T. lanoginusus* (12) *and H. insolens* (59). On the other hand, alkali thermostable xylanases from certain bacterial strains such as *T. maritima* (61,63), *B. polymyxa* (37), *T. reesei* (57) and *Bacillus* sp. (60) show broad pH activity (5–11) and are active at the temperature 70-105°C, although there are exceptions with optimal pH 5-6.5 such as *E. coli* clone

carrying a gene from *T. maritima* (55,61). An example is provided by a xylanase from recombinant *E. coli* harbouring a xylanase gene of thermophilic *Thermoanaerobacterium* sp. strain JW./SL-YS 485 which showed optimal pH activity at 6.5 and temperature optimum activity of 80°C (4). Likewise, an alkali tolerant *Aspergillus fischeri* Fx n1 produced a cellulase-free β-1,4-xylanase that was active from pH 5-9.5 with temperature optimum at 60°C and gave a 57% reduction of activity at pH = 9.5 (62). Recently, a third endo-β-1,4-xylanase (Xyn 111) produced on sorbose by *T. reesei* PC 3-7 was purified to homogeneity. This enzyme was active between pH 4.5 and pH 8.5 at temperature optimum of 55°C and retained more than 80% of its maximal activity at pH 8.5 (53). Also, *Bacillus* sp. 3M. showed optimum activity at pH 6-7.5, active up to pH 9.0 and retained 100% of its activity at 55°C for 3 days, whereas at 80°C it had 47% of maximal activity (63).

7. Application of cellulase-free β-1,4-xylanase

One of the most important applications of cellulase-free β-1,4-xylanase is its use in the pulp industry for bleaching. Pulp pretreatment with this enzyme after peroxide or chlorine bleaching has led to the development of new ecologic chlorine-free chemical bleaching technology (11). Conventional bleaching with chlorine or chlorine dioxide causes environmental damage due to the formation of chloroorganic substances in the effluents. These chloroorganics as mentioned previously are hazardous to humans and require expensive processing methods to be eliminated or detoxicated from the environment. In view of this, the use of this enzyme is an alternative to the chlorine-based bleaching methods and makes it more viable economically. Generally, the desirable features of cellulase-free β-1,4-xylanase for industrial application in kraft pulp industry to boost bleaching include low molecular weight (to assure easy access to the pulp matrix); thermal stability (to assure activity at up to 70°C); alkaline activity (pH 6-10); specificity for xylan (to avoid cellulose viscosity losses) and affinity for acidic side chains in order to maximize hexenuronic acid removal (7,53). Although these features have been termed "ideal parameters" for effective prebleaching of pulps with xylanases, recent studies have shown that neither differences in pI nor molecular weights of these enzymes could explain their bleachability on pulps. Gübitz et al. (56) studied the efficacy of different molecular weights of xylanases (26,35,41 and 53 kDa) from *T. laniginosus*, *P simplicissimum*, *S. commune* and *S. rolfsii*, respectively, which differed in pI, pH and temperature of activities for their bleachability. They found no correlation between molecular weights of investigated xylanases and their ability to bleach softwood kraft pulps. Instead, the mode of depolymerization defined as the increase of specific fluidity per released reducing sugars ($1m^{-1} \cdot Pa^{-1} \cdot S^{-1} \cdot g^{-1}$) was correlated to brightness effect and method of pulp pretreatment with peroxide. Chen et al. (55) also compared the effects of three xylanases obtained from *E. coli* encoded with a xylanase gene from *T. maritima*, which

had molecular weights of 120, 95 and 85 kDa for their bleachability with a commercial Pulzyme HC xylanase preparation. The enzymes showed comparative bleaching effects to Pulzyme HC xylanase preparation in releasing reducing sugars and aromatic substances from both hardwood and softwood suspensions. To ensure adequate bleaching effect in the kraft pulp industry, it is required that this enzyme should be absolutely free from other enzymes. The presence of cellulase in some of these xylanase enzyme preparations despite purification processes deteriorates or damages the structure of pulp fibre under process even at very low concentration (7,64,65). Cellulase-free β-1,4-xylanases have been reported to increase the extractability of lignin, release chromophores from pulp, improve viscosity and enhance brightness after bleaching (54,64,65). On the other hand, this enzyme has found applications in conjunction with cellulase in bioconversion of hemicellulose into renewable sources of fermentable sugars for microbial production of highly valued products such as liquid fuels and solvents (21,66). Besides, the cleavage products derived during enzymatic reactions with xylanase free from cellulase, such as xylobiose, xylotriose and xylooligosaccharides are useful as functional food additives or alternative sweeteners in the food industry (19,67). In addition, the cellulase-free β-1,4-xylanase has also found application in the improvement of bread quality *i.e.* the modification of cereal flours to enhance the volume and texture of bread through the hydrolysis of arabinoxylans (21,67). For effective utilisation of this enzyme in the baking industry, it is required that this xylanase enzyme preparation should not contain any protease or such an activity should be extremely weak, since proteases liquefy the dough (13). Furthermore, xylanase is used for maceration of vegetables in fruit and vegetable industry, together with endopolygalacturonase (at an appropriate ratio) for more effective release of protopectins that are linked to xylan (20,21). Xylanases are also useful for the clarfication of beer or juice and the pretreatment of grass and hay resulting in the improvement of nutritional value of animal feeds. Another potential application of the cellulase-free β-1,4-xylanase is its involvement in liberation of plant fibres from flax and hemp. At the moment, the retting process is used for this purpose, but the replacement of this slow and natural process caused by *in situ* microorganisms with treatment by the use of purified cellulase-free β-1,4-xylanase separately or in combination with other enzymes could lead to the development of a new rapid fibre liberation technology (48).

8. Conclusion

According to the results of reported data, the synthesis of cellulase-free β-1,4-xylanase by various microorganisms (fungi and bacteria) has been achieved using wild, mutant and genetically modified strains. Apart from the use of gene technology to tailor the xylanase formation, production of cellulase-free β-1,4-xylanase generally has been a function of an existing

regulatory system of control in a particular strain generated by the availability of inducers as well as an alteration of cultivation conditions. However, further studies are needed to elucidate the exact mechanism of regulation at the molecular level, in order to control strictly the direction of its synthesis. Furthermore, efforts should be directed towards searching for hyper or thermophillic alkalo-tolerant organisms that are able to produce highly thermostable cellulase-free β-1,4-xylanases. Such xylanase preparations should be active at alkaline conditions and relatively stable to fulfil the stringent requirements in kraft pulp bleaching processes. This could further reduce costs of their application in pulp and food industries. The synthesis of cellulase-free β-1,4-xylanase not containing protease or containing low protease activity should be studied due to its novel potential application in the baking industry.

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Production of fungal and bacterial cellulase-free β-1,4-xylanase in submerged cultivation

Summary

An interest in cellulase-free β-1,4-xylanase production has markedly increased in recent years due to its bio-friendly applications in the pulp and food industry. In the presented paper, the synthesis and function of its regulatory control system in different organisms were highlighted. The influence of various cultivation conditions such as substrate availability, temperature, pH, inducers and agitation on the formation of xylanase in relation to other enzymes was also discussed. Strain modifications by mutation and gene technology, followed by cultivation to obtain a cellulase-free β-1,4-xylanase were also presented. In addition, applications of xylanase for bleaching in the pulp industry, clarification of beer or juice, maceration of vegetables, improvement of bread quality, liberation of fibres from hemp and flax as well as improvement of animal feeds were mentioned.

Key words:
application, fungal and bacterial cellulase-free-β-1,4-xylanase, submerged cultivation, mechanism of regulatory control, synthesis.

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