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Expression cloning of fungal enzyme genes; a novel approach for efficient isolation of enzyme genes of industrial relevance

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Abstract

Expression cloning is a relatively new method for fast and efficient cloning of enzyme genes from fungi that are known to make complex enzyme mixtures. In contrast to traditional cloning methods that are usually dependent on knowledge of at least a partial amino acid sequence in order to synthezise appropriate DNA probes or primers, the expression cloning method solely relies on access to reliable and sensitive enzyme assays. A representative expression cDNA library is made in *Saccharomyces cerevisiae* from the donor strain and relevant cDNA clones are detected directly based on the encoded enzyme activity. Thus, time-consuming enzyme purification and characterization steps are avoided. The method has been applied on the characterization of extracellular enzyme genes from the filamentous fungus *Aspergillus aculeatus* and has resulted in the isolation of 20 different enzyme genes such as endo-glucanases, xylanases, pectinases, proteases, hemicellulases and rhamnogalacturonan-degrading enzymes. All enzymes have been expressed in *Aspergillus oryzae*, purified and characterized. In the present review a description of the expression cloning technique will be given as well as examples of how the technique has been used in the exploration and characterization of a commercial enzyme product that is known to consist of a complex mixture of more than 25 different enzyme activities.

Keywords: Expression cloning; Fungal gene; Glucanase; Xylanase; Pectinase; Heterologous expression

Contents

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1. Introduction

Filamentous fungi have been used for more than 50 years in the production of industrial enzymes. However, most fungi are known to produce several enzymes simultaneously and the classic product is therefore most often a mixture of different enzymes that 'contaminate' the one enzyme of interest (Fig. 1).

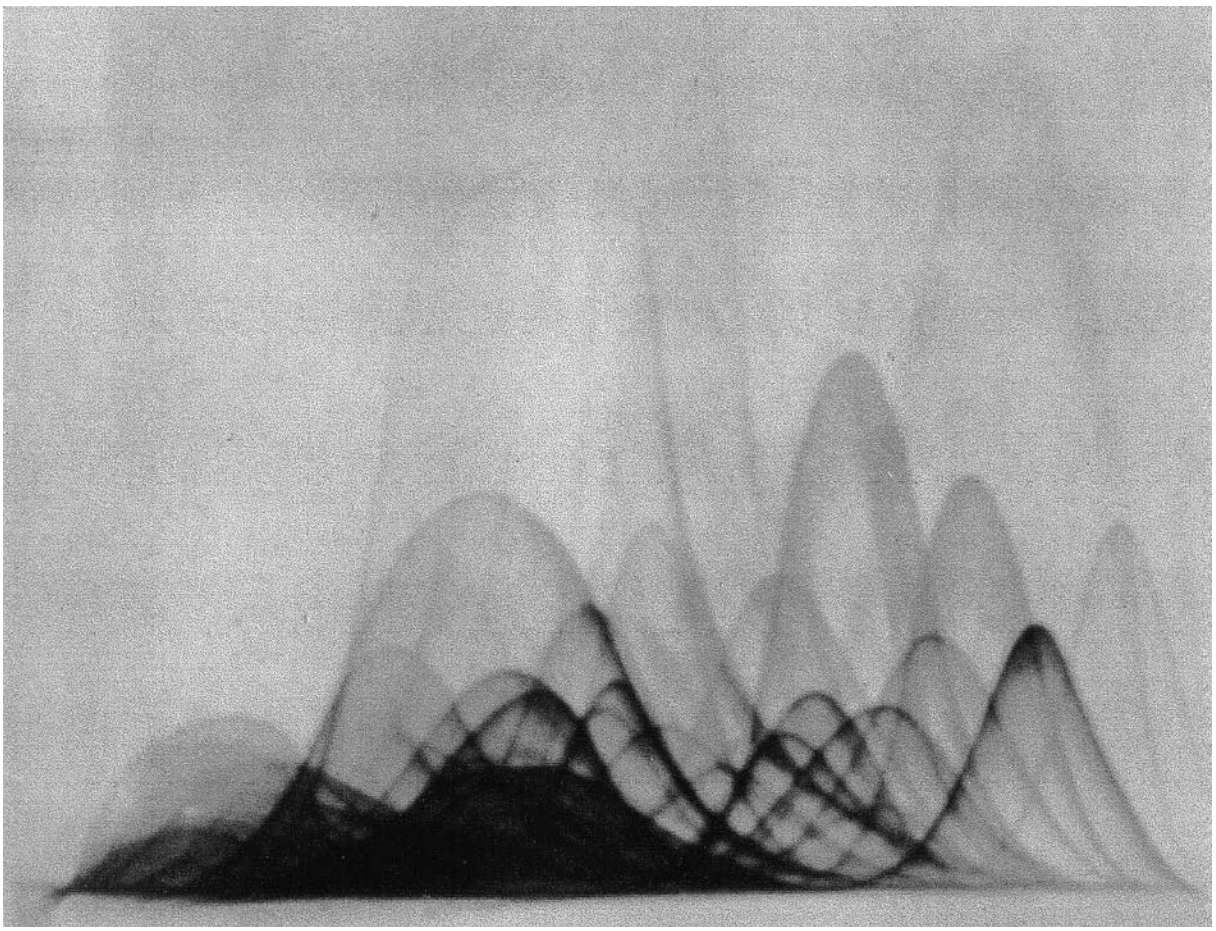

Fig. 1. Crossed immunoelectrophoresis of a classic enzyme product from *A. aculeatus*. First dimension electrophoresis was in a 1% agarose gel, 20 mM Tris/10 mM malein pH 7.0. Second dimension was carried out in the same buffer containing rabbit polycloned antibodies raised against *A. aculeatus* extracellular enzymes.

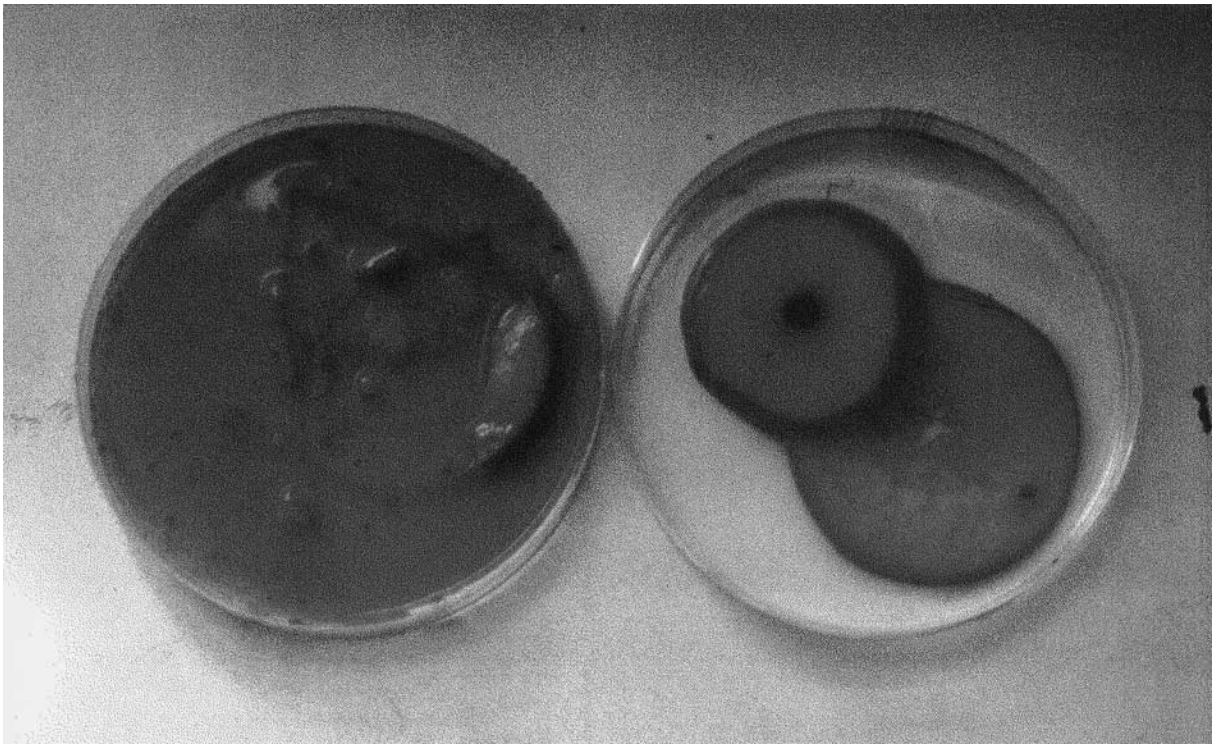

Fig. 2. Liquefaction of apple using a classic pectinase enzyme mixture. Slices of apples were incubated over night in 10% enzyme solution from *A. aculeatus* (left) and buffer (right).

Although the enzyme mixture can be used in certain applications and can give a significant effect, the complex nature of the enzyme mixture has several disadvantages. First, it is impossible to obtain any detailed knowledge of the action of the enzyme mixture: an effect is seen, but which enzyme is responsible or necessary for what cannot be elucidated. Second, a reasonable production economy can be difficult to obtain, as it may be impossible to optimize the production of a specific enzyme without knowing the target gene. Third, without understanding the effect and action of the enzyme/enzymes it is difficult to develop new and improved enzymes.

As a consequence, more and more enzymes are produced by recombinant technology. The recombinant enzymes can be produced in high yields thus providing new tools for functional studies through careful selection of the expression system in substantially higher purity. However, although the number of recombinant enzyme products on the market is increasing the introduction of cloned products is hampered by the relatively time-consuming standard cloning process, based on enzyme purification, amino acid sequence determination and subsequent probing of libraries with DNA probes. Recently a new method for fast and efficient isolation of enzyme genes from filamentous fungi was described [1]. The method combines the ability of *Saccharomyces cerevisiae* to express heterologous genes with the utilization of sensitive and reliable enzyme assays. A cDNA library is constructed in a *S. cerevisiae*/*E. coli* shuttle vector in *E. coli* from the fungi of interest. Plasmid DNA is isolated from sub-pools of the libraries and transformed into *S. cerevisiae*. Next the yeast transformants are replicated onto sets of agar plates containing appropriate enzyme substrates that allow detection of enzyme activity. After a subsequent characterization of the clones by DNA sequence analysis a representative cDNA for each enzyme is sub-cloned in an *Aspergillus* vector and expressed in high levels in *A. oryzae*.

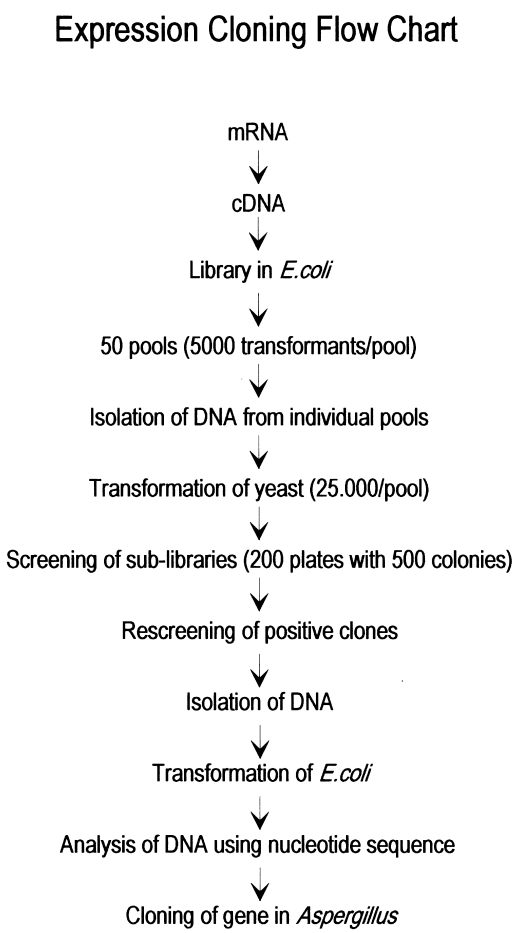

Fig. 3. Flow diagram of expression cloning.

2. A classic enzyme product

One of the first industries to benefit from the use of enzymes was the juice industry where the enzyme technology was first introduced in the 1930's [2]. An extensive use of enzymes, especially pectinases has since followed. A classic pectinase enzyme, or rather enzyme mixture as it consists of many different enzymes (Fig. 1), used in the juice processing, is derived from the filamentous fungus *Aspergillus*. The pectinases reduce the viscosity of the juice and cause precipitation of the pectic cloud particles thus ensuring good clarification and filtration rate of the juice. In addition the enzyme mixture improves the degradation of the plant cell walls and middle lamella of the fruits (Fig. 2) and can improve the juice yield from, e.g., apples by up to 40%. Although pectinases are the predominant enzymes, some of the other enzymes may act synergistically, and it may therefore be an advantage to be able to boost the classic enzyme mixtures with one or two key enzymes. Due to the complexity of the enzyme mixture substantial enzyme purification efforts have to be undertaken in order to identify and characterize which enzymes are of importance. However, the pricing of industrial enzymes sets some limitations on how much effort can be spent on development of a new product. It is therefore of importance that faster and more efficient methods can be applied in the characterization and production of new enzymes. The expression cloning method is an example of such a new method.

3. The principles of expression cloning

The method is based on isolation of enzyme genes from fungi by combining the ability of *S. cerevisiae* to express heterologous genes with the utilization of sensitive and reliable enzyme assays. The overall principles of expression cloning are outlined in Figs. 3 and 4. The donor strain is propagated in a complex media which can induce as many as possible of the enzyme activities of interest. On the precise day of maximal enzyme production the mycelium is harvested and used for mRNA isolation. A cDNA library is then constructed in a *S. cerevisiae*/*E. coli* shuttle vector, i.e., a vector which can replicate in both *E. coli* and *S. cerevisiae*. The cDNA is preferentially cloned in the vector in a directional manner between a strong yeast promoter and terminator and will thus be able to direct the expression of the fungal genes in the yeast cells. Due to the relatively low transformation frequency in yeast, the cDNA library is first made in *E. coli*. Plasmid DNA is subsequently isolated from sub-pools of the *E. coli* library and transformed into *S. cerevisiae*. The yeast transformants are replicated onto new sets of agar plates and screened on appropriate enzyme substrates that allow for detection of the interesting enzyme activity. The positive yeast colonies are subsequently restreaked by streaking on enzyme indicator plates. At this stage small scale cultures of the yeast transformants can be propagated for initial characteriza-

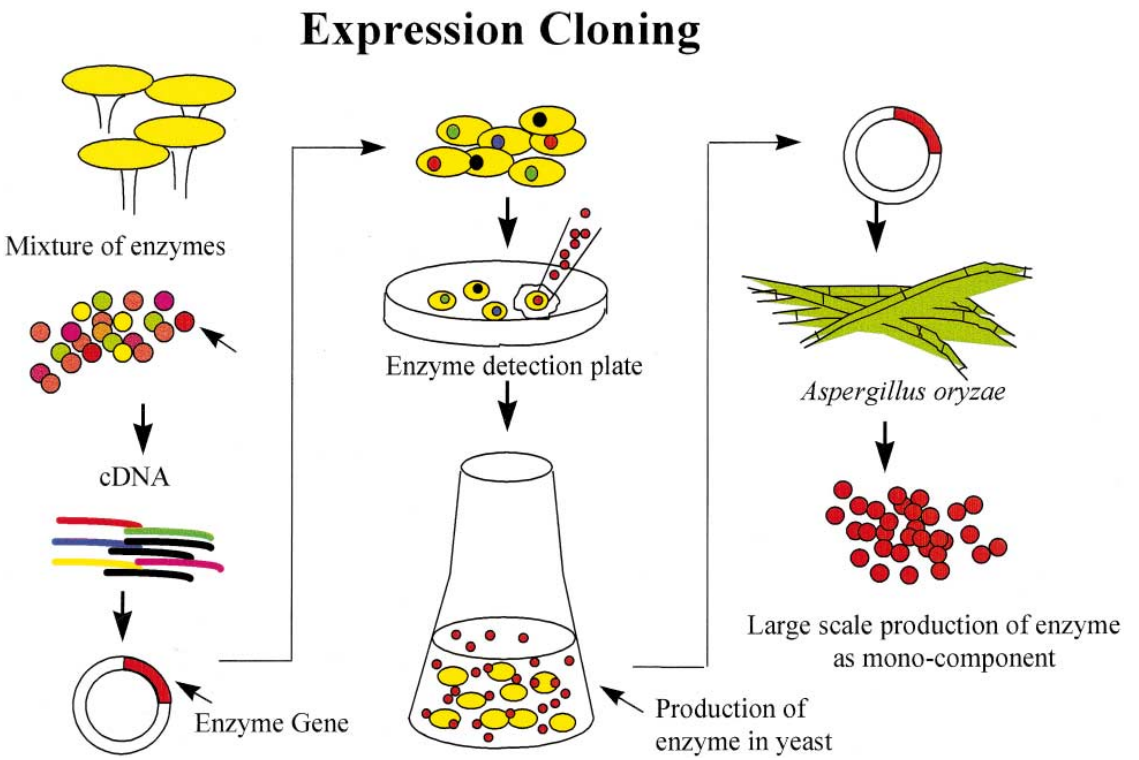

Fig. 4. Schematic representation of expression cloning.

tion of the enzyme. However, *S. cerevisiae* has been found not to be the optimal host as the production of enzymes by this organism is rather low (see 4.3). As a consequence it may be necessary to isolate the plasmid DNA from the yeast and to insert the cDNA fragment in an appropriate expression vector for subsequent expression in another host such as, e.g., *A. oryzae* [3]. In parallel the 5′ end of the cDNA sequence is determined in order to sort the clones not only according to enzyme activity but also by DNA similarity in different enzyme species.

The expression cloning technique has several advantages compared to the standard cDNA cloning. First of all it is totally independent of any knowledge of the enzyme to be cloned and the time-consuming purification and characterization steps required prior to cloning in the traditional technique can thus be omitted. Second, the system allows simultaneous screening for many different enzymes simply by making several replica of the yeast containing agar plates and applying different screening assays.

4. The three key factors of importance for efficient expression cloning

The three main factors to consider in order to establish an efficient expression cloning system are the synthesis of cDNA, the enzyme assays and the expression of the enzymes in yeast.

4.1. cDNA synthesis

Expression cloning is dependent on the presence of a signal peptide encoded by the heterologous cDNA gene. It is therefore a prerequisite for the successful outcome of the expression cloning that the cDNA is of full-length or at least covers the gene from the AUG initiation codon to the stop codon. Today many suppliers of fine chemicals are able to deliver a cDNA synthesis and cloning kit that may be applied. However, the commercial kits usually only contain reagent for a few cDNA synthesis/cloning experiments and there is therefore not sufficient ma-

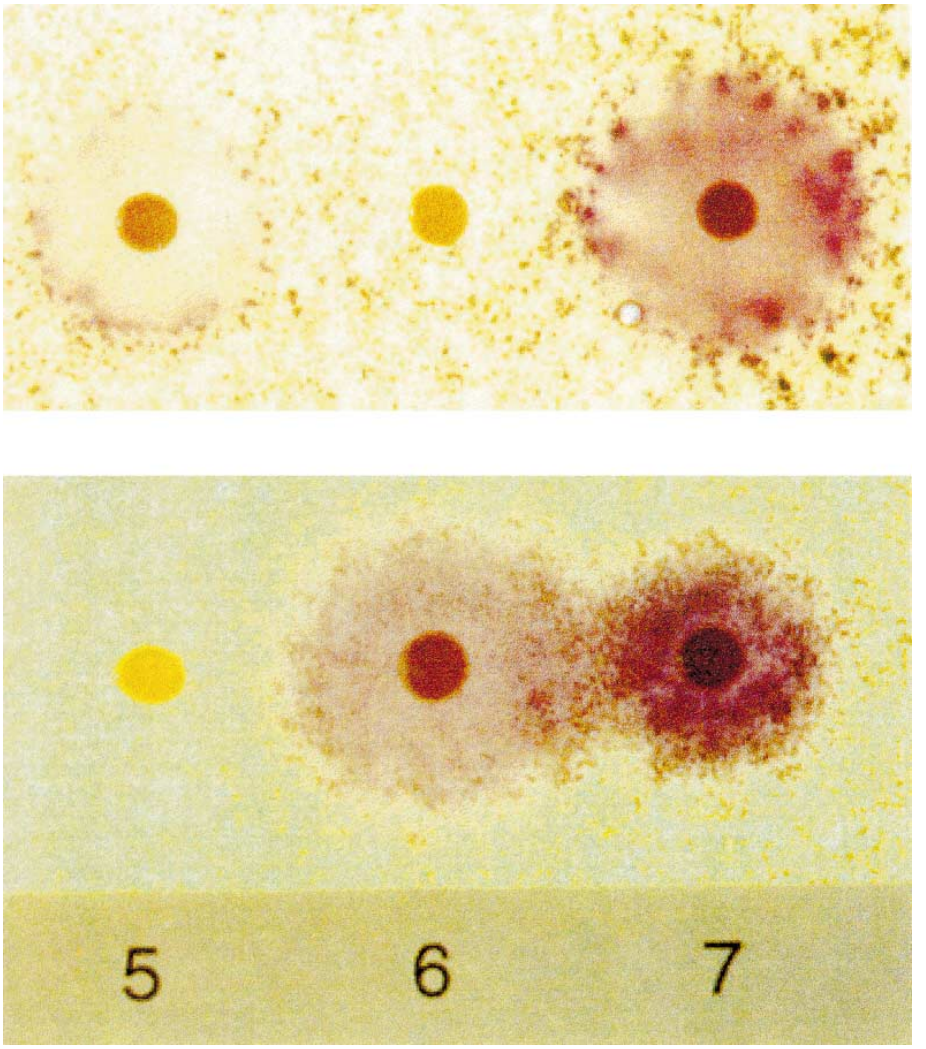

Fig. 5. Illustration of assays based on AZCL substrates. Three yeast colonies known to contain different cellulase genes were grown for three days on agar plates containing 0.1% AZCL HE-cellulose (upper) and 0.1% AZCL xyloglucan (lower). A clear difference in substrate specificity can be observed.

terial for proper optimization. Furthermore, variation can be expected from kit to kit and from batch to batch. It is therefore recommended to establish an in-house kit if the expression cloning method is to be established as a routine cloning method. Extensive testing of the different enzymes involved in the synthesis of the cDNA from different suppliers (reverse transcriptase, DNA polymerase, RNase H, T4 DNA polynucleotide kinase, T4 DNA ligase, mung bean nuclease, etc.) can be recommended. When the best enzymes are identified a reasonable amount of that particular batch should be purchased. Aliquots can be stored in the freezer and used for months without variation in the quality in the in-house kit. In order to test the enzymes involved in the cDNA synthesis it can be an advantage to make use of one of the commercial preparations of a long mRNA, e.g., the 7.5 kb mRNA from Gibco BRL.

Naturally the successful synthesis of cDNA requires the mRNA to be of high quality. Several methods have been described [4], but when reasonable amounts of mycelia are available, the classic

Table 1
Examples of plate screening assays

Substrate	Assay principle	Enzyme activity
AZCL arabinan	color release/halo formation	endo α-1,5-arabinanase
AZCL galactan	color release/halo formation	endo β-1,4-galactanase
AZCL mannan	color release/halo formation	endo β-1,4-mannanase
AZCL xylan	color release/halo formation	endo β-1,4-xylanase
AZCL HE-cellulose	color release/halo formation	endo β-1,4-glucanase
AZCL xyloglucan	color release/halo formation	endo β-1,4-xyloglucanase
Dyed-CL rhamnogalacturonan	color release/halo formation	endo rhamnogalacturonase
Casein	gelation of degraded casein/halo formation	protease
Lipid (olive oil)	degradation of lipid/halo formation	lipase
Pectin	precipitation of pectin with e.g. Ca^{2+}	endo α-1,4-polygalacturonase
MUF arabinofuranosid	fluorescent*	α-1,3 arabinofuranosidase
MUF galactosid	fluorescent*	α-galactosidase
MUF-GlcNAc	fluorescent*	exochitinase

MUF, 4-methylumbelliferyl; GlcNAc, *N*-acetylglucosamine; AZCL, azurin cross-linked; CL, cross-linked.
*The substrate fluoresces when cleaved.

guanidinium thiocyanate method followed by ultracentrifugation through a CsCl cushion is preferred. The cDNA synthesis can be based on a modification of the Gubler Hoffman method [5] using mung bean nuclease as a substitute for S1 nuclease. With a proper optimized cDNA system the average library size should be $>10^7$ clones/μg cDNA in a plasmid based system. If the original mRNA is isolated at the proper time for maximum production of the extracellular enzyme activities, the enzyme cDNAs will be reasonably represented compared to cDNAs representing household genes. It is therefore only necessary to plate a fraction of the *E. coli* cDNA library in order to isolate even low abundant enzyme cDNA's.

4.2. Enzyme assays

A typical *E. coli* library pool consists of approximately 5000 clones (Fig. 3). In order to have a reasonable probability for screening of most of the clones the corresponding library in yeast must be approximately 5× higher. Thus the number of agar plates to be screened will be in the order of 50–100 each containing 300–500 clones. This relatively large number sets some prerequisites to the assay systems. In addition to being sensitive and reliable, they must also be easy to handle.

General methods for detection of enzyme activity on plates have been described in literature. For example carbohydrases acting on β-1,4 glucosidic bonds can be assayed in the so-called Congo red assay [1]. Enzyme samples are applied on agar plates containing the relevant substrate. The agar plates are incubated at an appropriate temperature, stained with Congo red and subsequently washed in 2 M NaCl. Enzyme activity is visualized as colorless or pale red clearing zones on a red background. Although this assay is sensitive it has some disadvantages. It can be difficult to identify which of the yeast colonies are the positives as the halos tend to get diffuse if the colonies are too dense. Furthermore the enzyme activity cannot be followed and once

Table 2
Selected yeast strains and expression systems

Host	Promoter	Selection	Origin of replication
Saccharomyces cerevisiae	*gal1*	*ura 3*	*2μ*
Schizosaccharomyces pombe	*adh*	*ura 3*	*ars*
Kluyveromyces lactis	*lac 4*	*ura 3*	pKD 1
Hansenula polymorpha	*mox*	*leu 2*	*2μ*
Yarrowia lipolytica	*xpr 2*	*leu 2*	*ars 18*

Table 3
Test genes

Gene Encoded enzyme	Origin of gene
pga I α-1,4-Polygalacturonase	*Aspergillus aculeatus*
gal I β-1,4-Galactanase	*Aspergillus aculeatus*
cel I β-1,4-Endoglucanase	*Aspergillus aculeatus*
cel II β-1,4-Endoglucanase	*Humicola insolens*
xyl I β-1,4-Xylanase	*Humicola insolens*
lip I Lipase	*Thermomyces lanuginosus*

the plate has been 'developed' no further incubation is possible. A good alternative is to use azurine dyed and cross-linked (AZCL) substrates. Several substrates are commercially available [6] or can be made relatively easily [7]. Due to the cross-linked nature of the substrate it can be dispersed in the agar plates as grains. (see Fig. 5). If a yeast colony secretes an enzyme with activity towards the substrate the enzyme will degrade and convert the insoluble substrate to a soluble form revealing the activity by formation of coloured haloes around the colony due to the release of soluble, dyed substrate fragments. Compared to the Congo red assay this type of assay is easier to handle, as the substrate can be added to the agar plate and enzyme activity can be detected without further treatment. The possibility of incubating and following the colour formation for days can be used to adjust the sensitivity of the assay, and to allow identification of the positive colonies at the optimal time for detection. When the positive clones have been identified it is possible to apply a second assay in the form of an agarose overlayer containing a new cross-linked substrate. Unfortunately not all substrates can be azurine dyed and cross-linked into a suitable substrate and other assay principles based on precipitation of undegraded substrates, halo formation or release of fluorescense or colour may then be applied [8–10]. Examples of some of the most convenient plate assays are listed in Table 1.

Several of the assay principles can also be used in liquid assays making such assay set-ups possible where clones after propagation in small cultures can be screened in pools. Such systems can be automated for high throughput screening systems.

4.3. The yeast expression system

The most widely used expression system for expression cloning has been based on *S. cerevisiae* as host and an episomal *E. coli*/*S. cerevisiae* shuttle vector. However, in contrast to the significant activity that can be observed on the screening plates, only a very limited amount of enzyme has been found to be produced when the yeast clones are propagated in liquid medium [1]. This problem requires the enzyme genes to be transferred to another host system with a higher production capability before sufficient en-

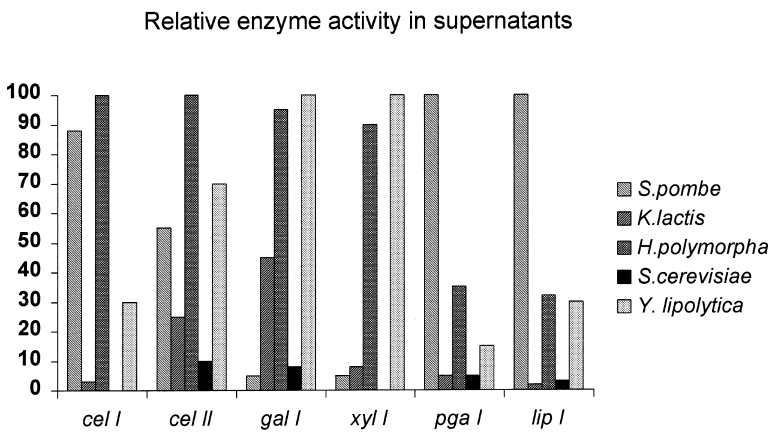

Fig. 6. Expression studies of the test genes in different yeast samples. Relative enzyme activity per volume was measured in culture supernatants taken at the time of maximal activity. For *cel I*, *cel II gal I*, and *xyl I* the expression activity was measured in liquid assays. The degradation of AZCL substrates was measured spectrophotometrically at OD_{620} and related to corresponding standard samples. For *pga I* and *lip I* the expression activity was measured on substrate containing agarose plates where the area of the clearing/precipitation zones were related to titrations of standards of the respective enzymes.

Table 4
Genes cloned by expression cloning from *A. aculeatus*

Enzymes from *A. aculeatus*	No. of clones	kDa	p*I*	pH opt.	Temp. opt.
Arabinanase I	44	38	4.3	7–8	50°C
Arabinofuranosidase I	3	60	8.5	4.5	50°C
Arabinofuranosidase II	1	75	5.7	3.5	50°C
Endoglucanase I	50	38	3.5	5.5	70°C
Endoglucanase II	67	35	3.4	3.0	50°C
Endoglucanase III	4	26	5.5	5.0	50°C
Endoglucanase IV	22	50	5.2	4.5	70°C
Galactanase I	42	42	2.8	4.0	60°C
α-Galactosidase I	16	70	n.d.	5.0	60°C
Mannanase I	57	45	4.5	5.0	60°C
Pectin lyase I	3	42	3.9	6.5	50°C
Pectin methyl esterase I	17	43	3.8	4.5	45°C
Polygalacturonase I	105	43	4.7	5.5	60°C
Polygalacturonase II	4	62	3.0	4.0	50°C
Polygalacturonase III	3	56	4.3	4.0	n.d.
Protease I	4	29	7.3	5.0	50°C
Protease II	2	36	5.1	5.0	45°C
Rhamnogalacturonase B	9	55	n.d.	6.0	40°C
Xylanase I	10	32	8.5	6.0	40°C
Xylanase II	6	56	4.6	4.5	75°C
Xylanase III	3	24	5.7	5.5	50°C

As demonstrated, the number of clones representing the different enzymes vary from 1 to 105. If parameters such as mRNA 1/2 life and capability of the mRNAs to be converted to cDNA and cloned are not taken into consideration, these numbers in part reflect the proportion of the individual enzymes in the enzyme mixture; n.d., not determined

zymes can be made for initial characterization and application studies. The filamentous fungus *A. oryzae* has been demonstrated to be very useful for expression of most fungal enzyme genes. Although in the end this host might be the preferred choice for production it is not an ideal host for expression cloning since only a limited number of *A. oryzae* transformants can be present on the plates because of the large size of the colonies, and due to the fact that the colonies are difficult to replicate. Furthermore the transformed DNA is integrated in the chromosome thus making it difficult to rescue, and finally the transformation frequency is orders of magnitude lower than in *S. cerevisiae*.

The requirements to the optimal host are that the transformation frequency is high, that most enzymes can be made in reasonable amounts, i.e., in the range of 10–50 mg/l, and that no post translational modification that may hamper the enzyme activity takes place. In order to identify other 'yeasts' that might be more suitable as hosts *Yarrowia lipolytica, Schizosaccharomyces pombe, Kluyveromyces lactis* and *Hansenula polymorpha* expression systems were developed and compared to *S. cerevisiae* (Table 2) (Sandal et al. 1995 unpublished results; Müller et al., in preparation). Five enzyme genes, *cel I, cel II, gal I, pga I* and *lip I*, were selected for the expression studies (Table 3). Each gene was for convenience of cloning and in order to maintain identical 5′ and 3′ sequences adapted with an *Sfi*I and an *Not*I site in the 5′ and 3′ ends respectively. The plasmids were introduced into the respective hosts and grown in media for optimal induction of the respective promoters. Several samples were taken during the exponential and stationary phases and used for measurement of different parameters such as enzyme yield, cell density, plasmid copy number, and plasmid stability. The optimal enzyme production for all strains was found to be in one of the samples from the stationary growth phase. The obtained maximum cell density varied for the different yeast strains when grown in shake flasks and the enzyme activity data therefore have to be corrected for variation in the cell density (Fig. 6). The preliminary results from

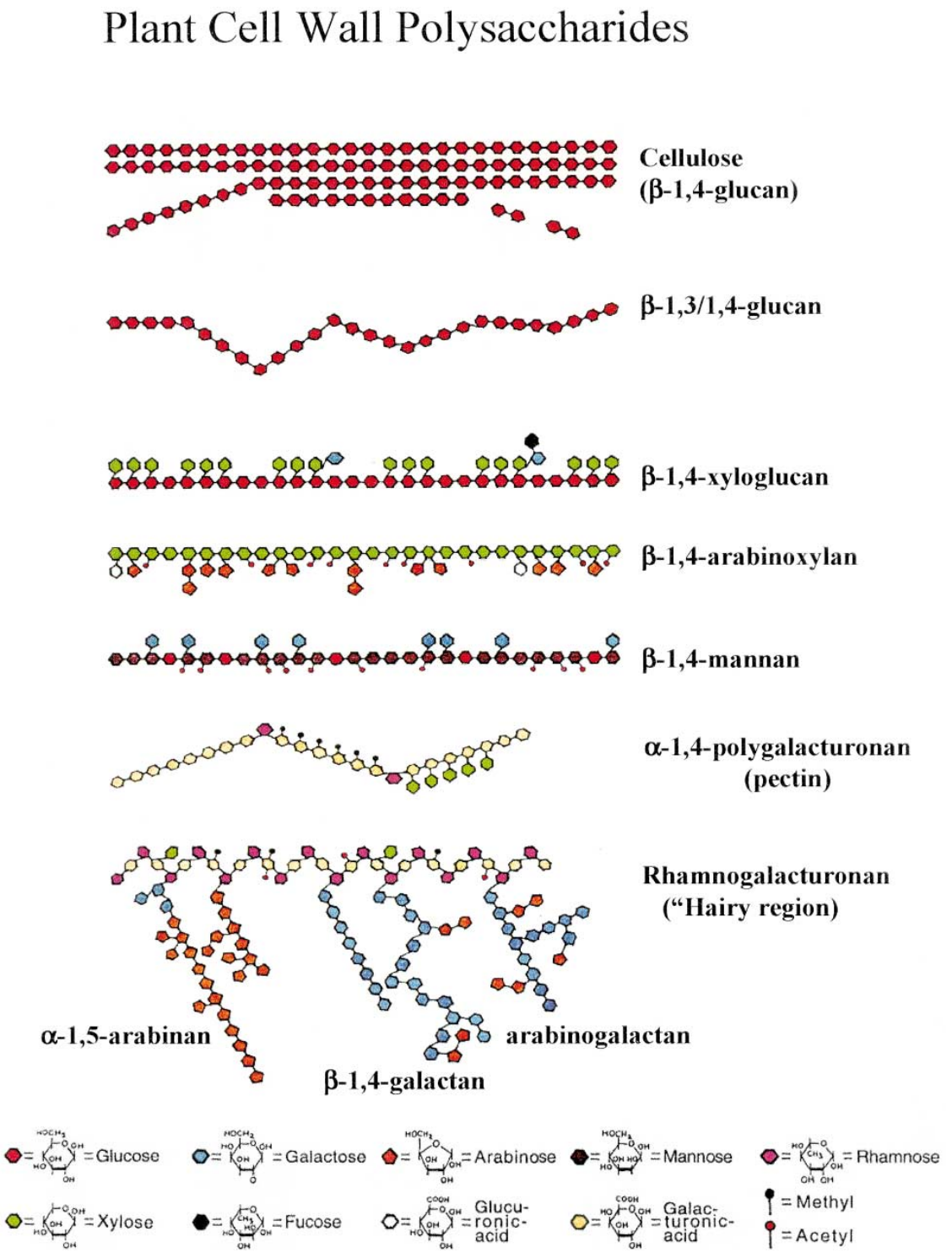

Fig. 7. Schematic drawing of the plant cell wall polysaccharides.

these studies indicate that it is possible to find host systems that are better producers of enzymes than *S. cerevisiae*.

5. Expression cloning from *Aspergillus aculeatus*

From *A. aculeatus* 20 enzymes have been cloned by expression cloning (see Table 4). All the enzymes have been expressed in *A. oryzae*, purified and characterized with respect to molecular mass, p*I*, pH, temperature optimum and stability, substrate specificity, K_m and V_{max} and specific activity. Below a more detailed description of the applied assays and the isolated genes will be given.

5.1. Plant cell wall polysaccharides as substrates

As mentioned above *A. aculeatus* produce a wide range of enzymes that are able to degrade plant cell wall material. The plant cell wall consists of a complex matrix of polysaccharides and it is necessary to have access to each of the individual polysaccharide in order to screen for as many different enzymes as possible. The polysaccharides from plant cell walls are traditionally divided into cellulose, hemicellulose and pectic substances. The cellulose is usually found as microfibrils consisting of parallel strings of glucan molecules which again consist of hundreds of glucose molecules linked by β-1,4 bonds (Fig. 7). The cellulose is not soluble in water and a part of the glucose molecules therefore have to be modified, e.g., by addition of methyl groups (as in Carboxy methyl cellulose) or a hydroxy ethyl group as in HE-cellulose (see below). In contrast to cellulose and due to the presence of some β-1,3 bonds the β-1,3/1,4 glucan is soluble in water and can as such be used directly as substrate. The major hemicelluloses are the xyloglucans and the arabinoxylans (Fig. 7). Xyloglucan consists of a β-1,4 glucan backbone on which xylose molecules are attached as the predominant side chains. In arabinoxylan the backbone is composed of β-1,4 linked xylose units on which single units of α-1,2 or α-1,3 linked arabinose are attached. The xylan backbone can be further modified by attaching acetyl groups (Fig. 7). Pectin or polygalacturonan consist of long stretches of α-1,4 linked galacturonic acid residues that are occasionally interrupted by rhamnose residue. The long pectin strings may also be interrupted by stretches of rhamnogalacturonan. This is a polymer of alternating rhamnose and galacturonic acid residues where arabinan and galactan strings may be linked as side chains on the rhamnose. Due to the presence of the long side chains the rhamnogalacturonan is often referred to as 'hairy regions'. The galacturonic acid residues in both polygalacturonan and rhamnogalacturonan may be further modified by methyl and acetyl groups.

5.2. Endo-glucanases

Screening of an expression library from *A. aculeatus* for endo-glucanases was performed on azurine-dyed and cross-linked (AZCL) hydroxy-ethylcellulose (AZCL HE-cellulose), AZCL β-glucan and AZCL xyloglucan and resulted in the isolation of four different classes of endo-glucanases (L. Andersen et al., unpublished results). The enzymes were shown to be very different in their specificity towards their substrates as well as in their basic physical and biochemical properties. Endoglucanse I (EG I) was most active towards HE-cellulose. Endoglucanase II (EG II) had high specificity towards xyloglucan whereas Endoglucanase III (EG III) and Endoglucanase IV (EG IV) were very active against β-glucan, but EG III was also active towards xyloglucan and HE-cellulose.

5.3. Endo-xylanases

Three endo-1,4-β-D-xylanases (EC 3.2.1.8) were identified from *A. aculeatus* by screening on AZCL Xylan [13]. Xyl II was characterized as being more acidic and more thermostable and having a slightly higher specific activity than Xyl I and Xyl III when measured on birch xylan. Analysis of the activity of these xylanases against soluble and insoluble xylan revealed that Xyl II had very low activity on insoluble wheat arabinoxylan compared to Xyl I and Xyl III. The different specificity of the xylanases was also demonstrated when wheat suspensions that contain both soluble and insoluble xylan were treated with the xylanases. In accordance with the fact that soluble xylan is known to contribute the most to the viscosity, Xyl II was capable of reducing the viscos-

ity to a significant higher extent than were Xyl I and Xyl III. Xylanases are today applied in different industrial processes such as, e.g., animal feed, wheat processing, paper and pulp production, and baking, and the access to xylanases with different specificity on soluble and insoluble xylan substrates increases the possibility of selecting the optimal enzyme for a given process.

5.4. Other hemicellulases

Cloning and characterization of the endo-1,4-β-D-galactanase (EC 3.2.1.89), and the endo-1,4-β-D-mannanase (EC 3.2.1.78) was recently reported [11,12]. The galactanase was screened using AZCL galactan and is apparently the first galactan degrading enzyme to be cloned. It is specific against unsubstituted galactan which is degraded to galactose and galactobiose. Galactanases are probably not a key enzyme for the maceration of vegetables [14], or in the liquefaction of apple cell walls [15,16] but may be required to achieve complete saccharification of solubilised cell wall polysaccharides in synergy with arabinofuranosidases and arabinanases. They may also be applied in the extraction of pectin from apple pomace.

Mannanase was screened using AZCL mannanan. More than 50 positive clones were identified indicating that this enzyme is relatively highly expressed in *A. aculeatus*. As the content of mamman is low in most fruits the enzyme will probably not find any application in the juice industry, but may be of interest in the processing of mannan containing gums as, e.g., guar gum and locust bean gum.

5.5. Pectinases

Pectic substances are a major component of the primary cell wall and middle lamella of fruits and vegetables and pectinases are of significant importance in the juice industry. The polysaccharides are composed of smooth regions containing mainly homogalacturonan, interrupted by so-called 'hairy regions' where multiple side-chains of neutral sugars such as galactan and arabinan are attached (Fig. 7). The backbone in the hairy regions is composed of alternating α-1,2 linked L-rhamnosyl and α-1,4 linked galacturonic acid residues [17]. Several different pectin degrading enzymes were cloned and characterized from *A. aculeatus*. Three endo-polygalacturonases (poly-1,4-α-galacturonide glucanohydrolases (EC 3.2.1.15), a pectin lyase (EC 4.2.10), a pectin methyl esterase (EC 3.1.1.11), two arabinofuranosidases (EC 3.2.1.55), one arabinanase (EC 3.2.1.99) and a rhamnogalacturonan degrading enzyme.

The polygalacturonase (PG) positive clones were identified using a precipitation assay. 1% apple pectin (35% degree of methylation) was used as substrate in an agarose overlay. Following incubation for 24 h, the activity was visualized by precipitation of the substrate with 1% mixed alkyl-trimethyl-ammonium bromide (MTAB). From the number of clones obtained for the three polygalacturonases it seems that the PG I is much more abundant than the two other PG's. Steady state studies of the *A. aculeatus pga* mRNA's during growth in pectin containing medium further confirm that the *pga* 1 mRNA is the most abundant (Kauppinen et al., unpublished results).

The pectin methyl esterase (PME) expressing yeast clones were identified using the same principle as for the polygalacturonases, except that here the substrate was 75% methyl-esterified apple pectin. The enzyme was specific for methyl groups on methyl-esterified galacturonic acid and was able to remove 85% of the methyl groups from highly methylated pectin. This enzyme is a key enzyme in the degradation of plant cell walls as the removal of the methyl groups is required prior to the action of the polygalacturonases [18].

In the screening for a rhamnogalacturonan degrading enzyme no AZCL substrate was available and a useful substrate was therefore made from the hairy regions from apples [7]. The side chains which on apple hairy regions mainly consist of arabinan were first removed by treatment with trifluoracetic acid. The resulting rhamnogalacturonan was dyed with a mixture of Cibacrom C blue and Cibacrom C yellow and subsequently cross-linked into insoluble grains using divinyl sulfone before it was used as substrate. The isolated clones were found to encode a novel type of a rhamnogalacturonan degrading enzyme which in contrast to the Rhamnogalacturonase A [19] cleave the Rha-α1-4GalUA linkage in the backbone. Recently, the enzyme was further

characterized and found not to be a hydrolase but a lyase (rhamnogalacturonan α-L-rhamnopyranosyl-(1,4)-α-D-galactopyranosyluronide lyase) [20].

5.6. Cloning of exo-acting enzymes

In contrast to endo-acting enzymes such as the endo glucanases, xylanases, galactanases and the endo-arabinanases which can be identified using AZCL substrates the exo-acting enzymes such as glycosidases, xylosidases, galactosidases and arabinofuranosidases cannot be detected on this type of substrate. Instead exo-enzymes can be detected by using the corresponding mono-sugars which are chemically linked to a fluorophor or color releasing compound. For example para-nitrophenol-labeled compounds may be used. However, the resulting yellow color can be very difficult to detect on agar plates and it may therefore be preferable to use sugars which are labeled with methylumbelliferyl. This type of substrate releases a fluorescence that can be detected under UV light and has successfully been used in the cloning of two arabinofuranosidases and an α-galactosidase from *A. aculeatus* [21].

6. Pectin methyl esterase; a new enzyme for industrial applications.

The smooth regions in pectin can be methyl-esterified to a varying degree and the degree of methylation has a significant impact on the physico-chemical and thus gel-forming properties of the pectin. Unmethylated pectin is able to form gels in the presence of Ca^{2+} ions and is being extensively used in the food industry as a gelling or conditioning agent. As pectin methyl esterases hydrolyze the ester linkage between methanol and galacturonic acid in esterified pectin, an improvement of the gelling properties of the pectin can be foreseen when treated with this type of enzyme. Until recently commercially available pectin methyl esterases were substantially contaminated with other pectin degrading enzymes such as polygalacturonases and pectin and pectate lyases and it has therefore not been possible to use the enzyme for in situ gellation. Cloning of the pectin methyl esterase from *A. aculeatus* and production of the enzyme as a mono component enzyme without significant side activities has allowed the development of the in situ gellation concept, e.g., in the jam industry. In the traditional method for jam production 0.3–1.0% pectin is added to the jam in order to obtain the wanted firmness. The same effect can be obtained by adding 8–10 units of pectin methyl esterase per kg fruit. After a few minutes of incubation the same firmness can be obtained as is obtained with the classic pectin addition. In contrast to pectin-added jam, jam produced using enzymes does not require labeling for additive addition as enzymes are regarded as a processing aid. Furthermore several of the quality parameters such as flavour, smoothness, firmness and mouth feel seem to be better in jams produced with pectin methyl esterase than in jams produced with pectin. A somewhat similar application of the enzyme has been demonstrated in the ketchup manufacturing, where significant improvements have been observed especially on viscosity and syneresis. The enzyme may also be of use in the juice industry for removal of soluble pectin polymers which are known to give unwanted haze formation in the juice. Addition of pectin methyl esterase will allow the pectic polymers to be precipitated by a simple calcium treatment or degraded into small oligos by the concerted action of a polygalacturonase.

7. Conclusion

The expression cloning method has contributed significantly to an increase in the speed with which new enzymes can be cloned and thus be produced in a pure form in large quantities. During the last few years expression cloning has been applied on several fungi and has proven very efficient in the cloning of enzyme genes [1,8,9,12,13] and more than 150 different fungal enzyme genes such as arabinanases, endoglucanases, galactanases, mannanases, polygalacturonases, pectin lyases, pectin methyl esterases, proteases, rhamnogalacturonases, lipases and xylanase have been cloned from different donor organisms within a short period of time. Together with the classic cloning methods this has opened new ways of getting a better understanding of the enzymatic processes, especially when complex mixtures of enzymes are being used, and of developing better en-

zymes for new and existing concepts. Today approximately 50% of the industrial enzymes on the market are made by recombinant organisms. However, in the near future this figure is expected to come close to 100%. The fast isolation of new genes, e.g., by expression cloning from natural occurring microorganisms will be an invaluable tool for the fast developing protein engineering and random mutagenesis field, as it will supply these programs with new interesting back-bones. Furthermore, the new enzyme back-bones may contribute significantly to a better understanding and determination of amino acid residues that may be of importance for the enzymatic characteristics.

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