



**HAL**  
open science

## CHAPTER 3. PRODUCTION OF ENZYMES: FERMENTATION AND GENETIC ENGINEERING

Henry Eric Spinnler

► **To cite this version:**

Henry Eric Spinnler. CHAPTER 3. PRODUCTION OF ENZYMES: FERMENTATION AND GENETIC ENGINEERING. Selim Kermasha; Michael N.A. Eskin. Enzymes: Novel Biotechnological Approaches for the Food Industry, 1, Elsevier, 2021, 978-0-12-800217-9. 10.1016/C2013-0-13507-6 . hal-04487606

**HAL Id: hal-04487606**

**<https://agroparistech.hal.science/hal-04487606>**

Submitted on 4 Mar 2024

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

# BIOTECHNOLOGICAL APPROACHES FOR THE USE OF ENZYMES IN FOOD INDUSTRY

Selim Kermasha & Michael N.A. Eskin

(Editors)

---

Henry-Eric Spinnler

*AgroParis Tech, Paris, France*

## CHAPTER 3. PRODUCTION OF ENZYMES: FERMENTATION AND GENETIC ENGINEERING

### Abstract

Producing a pure enzyme requires it to be overproduced. To obtain this overproduction, several drawbacks must be overcome. The first drawback is the amplification of a strain's capacity to use the biological machinery, to produce the targeted enzyme as the major protein of the metabolism. For ease of recovery, the secretion of the enzyme is researched. Consequently, organisms having specific post-translational systems of protein secretion are preferred. These systems are particularly well developed in fungi and as such, are preferred for industrial scale production. Moreover, fungi are able to grow at low pH and easily cultivated in liquid medium and/or by solid state fermentation. The recovery, the purification and the formulation of the protein preparation to maintain the enzyme activity, for as long as possible, are also described in this chapter.

### 3.1. Introduction

Because enzymes are largely used as processing aids in food production, in washing powders and in chemical manufacturing, their production by fermentation has been seen as an opportunity to produce enzyme concentrates on a large scale. The progress in genetic engineering and DNA editing has been used to build strains with a modified genome particularly efficient for these production processes. One of the goals has been to maximise the specific enzymatic activity of certain strains. This has been done by incorporating a gene or increasing the number of copies of the gene coding the enzyme protein sequence, into the genome. Another step has involved the overexpression of these genes in the transcription phase to obtain increased enzymic activity. However, as these operations have been intracellular, a third goal has been to improve the efficiency of the enzymatic recovery. This has been done by selecting organisms that have the capacity to secrete the protein outside the cell, into a culture broth. Figure 3.1 shows the key steps in a process using specific organisms. Bacteria such as *Bacillus sp.* (Cai et al., 2019) and fungi like *Trichoderma reesei*, *Aspergillus oryzae* or *Yarrowia lipolytica* (Archer and Peberdy, 1997) are species commonly used. By increasing the enzyme titre produced, it has reduced the risk of

unexpected secondary activities in the culture broth and has facilitated an efficient process of enzyme extraction/ purification, which is a fourth step. The fifth step which is not the easiest has been to harvest and stabilise the enzyme produced.

Due to their ability to secrete the protein outside the cell, manufacturing companies have preferred using organisms mentioned above. In addition, companies have controlled and optimized the culture media used for growing these organisms and, it has been done for safety reasons (no toxins produced), optimising harvest conditions and for general ease of use for industrial scale production. This chapter will attempt to cover the different aspects of this process so as to facilitate the commercialisation of these enzymes.

### Figure 3.1

## 3.2. Enzyme Screening

Several steps are necessary to achieve efficient enzyme production but the first is to develop an efficient screening method to easily select strains of micro-organism producing the researched enzyme.

Traditional systems using screening on agar plates were shown to be efficient. This has now been surpassed when automatic microplate readers with a capacity to read over ninety-six wells became available. With both systems the main challenge was to obtain a semi-quantitative indication of the enzyme activity, obtained via a signal such as a colour reaction for example. If necessary, a quantitative measurement of the activity could be obtained subsequently, by more accurate and reliable systems. This could include labelled substrates such as fluorogenic and chromogenic substrates that generate a reporter molecule by  $\beta$ -elimination, fluorescence resonance energy transfer (FRET) substrates and isotopic labels for screening of enantioselectivity.

Depending on the enzyme researched, several strategies to detect activity have been used (Goddard and Reymond, 2004). These include:

- using a substrate releasing a chromophore or a fluorophore when the activity is present, as is the case for sugar hydrolase (with paranitrophenyl glycosides), proteases (with azocasein), peptidases (using para nitro-anilide derivated peptides or amino-acids releasing a coloured nitro-aniline when a peptidase was present).
- using a specific reactivity of the reaction product (e.g.  $\text{CuCl}_2$  because  $\text{Cu}^{++}$  reacts with fatty acids, resulting in a bright blue colour, therefore enabling the detection of lipolitic activities)
- using pH indicators when pH change is expected and
- using fluorochromes such as Sybr Green or coumarin derivatives (Goddard and Reymond, 2004)

### 3.3. Microbial Strain Selection

When an efficient screening method is usable it becomes easier to screen culture collections. At first a diversity of wild type strains has to be screened to obtain high yielding strains. However, the ability to modify genomes opened up possibilities in the second part of the 20<sup>th</sup> century. Several companies used this technology to create strains dedicated to the production of specific enzymes. In addition, fungi were preferred in the process due to their ability to grow in low pH environments. Fungi were used to limit the growth of 'short generation time' bacterial contaminants that usually grow at neutral pH.

### 3.4. Strain Improvement

To develop the first generation of hypersecreting strains three main steps are necessary. One needs to i) prepare the receiving strain that is to be used industrially; ii) construct a vector in *E. coli* (the donor strain) and iii) transfer the researched gene(s) from the donor strain into the chromosomes of the receiving strain.

The strategy usually used is to insert the enzyme coding gene into cassettes built in the genome of the production strain. In *Trichoderma reesei* or in *Aspergillus niger*, genetic cassettes have been built by excision of known enzymes from the genome of the wild type strain (e.g. *Trichoderma* cellulases or *Aspergillus* glucanases) (van Dijck et al., 2003).

#### 3.4.1. Preparation of the Strain of Production (Cassette construction)

On the sites of known genes of the wild type fungal strain, a zone, sensitive to a different restriction enzyme was inserted, while the gene coding for the known enzymes was removed. Up to seven cassettes were built on the fungal genome which means, on each cassette a sequence sensitive to a different restriction enzyme was positioned. This zone was flanked by small nucleotidic sequences that helped the insertion of the gene of interest at the right place, including a nucleotidic zone sensitive to another restriction enzyme other than the one initially present in the cassette. The insertion was done from a vector and provided a high number of copies of the targeted gene in the production strain (up to 120 copies).

Figure 3.2

#### 3.4.2. Preparation of the Vector

The vector used to transfer the gene coding the enzyme is usually built in *E. coli*. If the original gene is coming from a species very different from the species used for the overproduction (e.g. initial gene coming from a prokaryote and produced by a yeast), a change of the sequence in order to avoid problems with codon bias that could happen between very different species should be applied using tables before to insert the gene of interest in *E. coli*. The plasmid usually bears a marker gene and is in targeted zones that can be opened with specific restriction enzymes, so that foreign genes could be inserted easily.

Markers need to be installed when constructing a vector so that the transformed clones could be easily identified.

For a while, genes generating resistance to antibiotics were used as markers but this approach should be avoided for enzymes used for food applications. If this approach is unavoidable, these genes should be deleted in the final strain.

It should be stressed that this marker gene can enable the use of a specific substrate. For example, the addition of the *amdS* gene in the plasmid coding the acetamidase, gives the strain the capability to uptake acetamide as carbon source. In this case, the capability of the transformed strains to use acetamide indicates the efficiency of the plasmid transfer (Selten et al, 1995). Figure 3.3 shows the construction of such a plasmid.

Figure 3.3

### **3.4.3. Construction of the Overproducing Strain**

This construction should meet several objectives to ultimately obtain a stable overproducing strain. This means i) successfully inserting the 'gene coding the enzyme' on at least one of the chromosomes ii) being able to have a hyperproduction of the enzyme, rendering this protein the most significant one produced quantitatively, in the culture supernatant iii) avoiding disrupting the general metabolism of the producing strain, iv) obtaining a protein that will be processed by the post-translational modification system, v) finally showing that all foreign DNA, with the exception of the enzyme coding genes have been removed during the strain construction process.

Figure 3.4

The inserted sequences are amplified by PCR and sequenced in order to check that no sequence of *E. coli* remains in the production strain.

This approach was built for the construction of enzymes used as food processing aids and was done so following very strict safety assessment guidelines. The idea was to have strains produce enzymes which no longer needed to be tested on animals to demonstrate the safety of the products. Irrespective and, at least in Europe, the present legislation still obliges companies to test the enzyme preparations in animals to evaluate for different types of toxicity (e.g. subchronical toxicity for 9 weeks). These tests must be completed prior to permission being granted for use in food preparations.

### **3.4.4. The Revolution of CrispR- Cas9 Editing for the Construction of Fungal Strains that Hyperproduce Enzyme**

The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated (Cas) systems, are systems containing *cas* genes and CRISPR array(s) being naturally derived from the adaptive immune system of prokaryotes. The *cas* genes are short sequences that

originated from foreign genetic material, called spacers, interspaced with identical palindromic repeats (Jinek et al., 2012). The *cas* genes code proteins responsible for acquisition of new foreign sequences into the CRISPR array(s) as well as for disruption of exogenous DNA through the activity of Cas proteins bearing endonuclease activity, such as the Cas9, Cas12 or Cas 13 proteins (Mota et al., 2020). Over the past decade, the ability to reprogram Cas proteins by creating crRNAs (guide RNAs) designed to bind and/or cleave specific DNA or RNA sequences has made them useful in a variety of applications, including genome editing and modulation, specific control of gene expression and nucleic acid detection (Mota et al., 2020).

### Figure 3.5

In the CRISPR/Cas9 system, the Cas9 protein forms a complex with two RNA molecules: CRISPR RNA (crRNA), encoded by the random spacers found in the CRISPR array(s), and transactivating CRISPR RNA (tracrRNA). It has been shown, that these two RNAs form a dual-tracrRNA:crRNA, which can also be designed as a single guide RNA (sgRNA) for genome editing purposes (Jinek et al., 2012). The Cas9-sgRNA complex is guided to a target DNA via homology of the crRNA to the protospacer region of the target sequence (Fig. 3.5).

The Cas9 nuclease subsequently binds the target DNA through interaction with the protospacer adjacent motif (PAM) immediately downstream of the protospacer sequence. If the crRNA part of the sgRNA sequence successfully pairs with the target DNA, the Cas9 nuclease will perform a double strand break (DSB) three nucleotides upstream of the PAM motif, which can be repaired. The simple design and construction of a single guide RNA for precise genome editing has rapidly expanded the toolbox of molecular methods, such as Zinc Finger Nucleases (ZFN) and Transcription Activator-Like Effector Nucleases (TALENs) (Kim and Kim, 2014).

CRISPR/Cas9 is a cost-efficient and simple platform to perform genetic manipulations with a single enzymatic activity guided by a predesigned sgRNA molecule. Therefore, it has become a common genome editing method in a variety of organisms and has been reported to work in numerous filamentous fungi. In filamentous fungi, this system has been used to target genes involved in enzyme production (Q. Liu et al., 2017) (Katayama et al., 2018) (Liu et al., 2015) (R. Liu et al., 2017). The system can also be used to efficiently change functional properties of the proteins encoded by the target gene by on site genomic mutation (e.g. in *Aspergillus niger*) (Kun et al., 2020).

Once the overproducing strain is obtained a validation of the strain stability is necessary. Strains with a high number of copies of a gene are often not very stable. It seems that there is an economy of the cell that leads to the removal or inactivation of non-compulsory genes. The high number of copies is not necessary and some are often deleted after a limited number of generations. Irrespective, it is important to set up a proper strain stock of the efficient strain, in order to keep the best activity obtained

## **3.5. Growth Requirements of Microorganisms**

### ***3.5.1. Media Development***

The knowledge of the strain growth requirements and regulation systems are prerequisites to controlling the over-expression of enzyme production. Sugar and nitrogen substrates are quantitatively used during the process. By-products of the food industry such as molasses or corn steep liquors can be efficient substrates and more affordable than purer substrates such as pure sugars or protein hydrolysates (peptones or tryptones). Yeast extracts and their content in vitamins (especially B vitamins) are also commonly used. However, the complexity of the medium can be a drawback for the future purification of the enzymatic activity at the end of the fermentation process.

Another substrate that should be provided in significant amounts is oxygen. The oxygen transfer from gas to micro-organisms can be a limiting step especially when filamentous fungi are used. This will be discussed later.

### ***3.5.2. Nutritional Requirements***

#### ***3.5.2.1. Effect of Carbon Sources***

Apart from their influence on growth rates the availability of carbon sources has a significant metabolic regulation effect. It starts by the expression of the substrate uptake, but may also influence the pathway expression. Some of the enzyme activities are associated with growth and, in this case, easy to use sugars such as sucrose, fructose or maltose can be efficient for enzyme production. However, the availability of glucose, even if very efficient on cell growth is also one of the most repressive substrates for a large number of genes and as a consequence limits the production of many enzymes. So, the use of other sugars such as lactose or maltose or the use of glycerol can be a better choice to develop a full expression of the strain potentiality.

Arora et al, (2018) have shown the diversity of by-products that could be used for the production of enzymes by solid phase fermentation (see 3.5). Substrates as diverse as apple or tomato pomace, different types of bran, linseed oil cake, palm straw, citrus pulp or peel, etc. can be used and offer the advantage of being cheap substrates as well as being substrates easily used by fungi.

#### ***3.5.2.2. Effect of Nitrogen Sources***

Nitrogen is often added in the mineral form with salts such as ammonium sulfate or ammonium nitrate. Organic nitrogen can also be used with simple substrates such as urea but peptone or tryptone of different proteins, even if they are significantly more expensive are also very efficient. Other efficient complex sources of nitrogen are used for industrial processes such as sugar slop or spent or corn steep liquor which are also rich in nitrogen. However, a complete analysis of the flow sheet costs should be done to take the right

economical decision because the more complex the medium the more difficult and costly could be the purification process.

Yeast extract is providing nitrogen, but it is also providing a large diversity of vitamins especially B vitamins.

#### *3.5.2.3. Effect of salts and oligoelements*

Phosphate and sulphate sources are usually added to the culture medium as phosphorus and sulphur are significant constituent atoms of living cells.

Metal ions and halogens can be efficient bio-chemical mediators and necessary oligoelements for efficient enzyme production (e.g. sodium, potassium, calcium, manganese, magnesium, iron, copper, molybdenum, cobalt, iodine, chloride,...) however some contaminant metals must be absent in enzyme preparations for food applications. It is the case for lead, cadmium, arsenic, mercury and the concentration of these metals must be controlled regularly for these applications.

### **3.6. Process Design and Optimization**

Automated liquid fermentation equipment available on the market nowadays, enables organisations to control temperature, pressure, pH and weight during industrial scale production.

Sensors are also available for biomass measurement but are mainly adapted for monitoring bacterial or yeast growth rather than filamentous fungi. Such sensors are not adapted to measuring the biomass of fungi because the fungi usually form pellets when the liquid medium that it is in is agitated, a step that is necessary for providing oxygen to the microorganisms. Special geometry of reactors called "air lift" are favouring oxygen transfer without increasing the shearing stress associated to high oxygen transfer with propellers.

#### **Figure 3.6**

It can be interesting to produce enzymes by solid state fermentation (SSF). A recent review (Arora et al., 2018) showed the diversity of enzymes and efficiency when using such a method for producing enzymes. Different types of solid state bioreactors are used : tray bioreactors, packed bed bioreactor, air pressure pulsation bioreactors and mixed SSF bioreactors. A diverse range of enzymes has been produced with such reactors. The best results were obtained for enzymes such as amylase, pullulanase and xylanase.

In this case the enzymes are more concentrated in the solution that can be obtained by pressing the solid substrate (such as sugar beet pulps). However such equipment is less common than the liquid fermentation ones. The control of sterility, relative humidity and temperature can be an issue when scaled up for industrial production. However this technique is used by several companies.

### **3.7. Downstream Processes**

#### **3.7.1. Concentration and Purification of Enzymes**

At the end of fermentation, the purification of enzymes is facilitated by the titre and can be hindered by the complexity of the medium. Most often enzymes in the culture supernatant are concentrated by tangential ultrafiltration and then precipitated using salts such as ammonium sulfate. It is followed by a desalination process, using membrane techniques.

As for the use in food, the regulations stipulate that there are maximum permissible limits for heavy metals and there must be an absence of pathogenic micro-organisms (such as *Salmonella*, *Staphylococcus aureus*, sulfite-reducing *Clostridia*,...).

#### **3.7.2. Product Formulation and Standardization**

The activity of the concentrated form is measured in order to standardise the number of enzyme units per pack. Some enzymes are sensitive to oxidation and they should be protected by antioxidants such as sulphites or ascorbic acid. They are often mixed with carriers agents such as dry starch or dextrans and granulated in order to facilitate their use.

### **3.8. Conclusion**

The screening methods are still prerequisites for selecting overproducing strains but the recent progress in genome knowledge and genome expression have made genetic engineering and automated protein engineering easier. It has contributed to the improvement of specific qualities and quantities of enzymes produced including producing them at reasonable prices. The revolutionary CRISPR/Cas gene technology also helps these operations, however in some cases the insertion sites are not as specific as expected.

One of the critical steps is to keep the built strain efficiency for a long duration. The number of copies of the interesting gene can be deleted when the number of generations become high. As far as we know the systems regulating the excision of genes with low interest for the producing cells are not completely elucidated and seem to be difficult to inactivate.

The production of enzymes in liquid medium are now quite controlled. However, production of enzymes by filamentous fungi, requires large amounts of oxygen. Oxygen transfer presents technical problems such as shear stress damaging the mycelia and energy cost that can be critical at large scale. New techniques of solid state fermentation of agro industry by-products are now at an industrial scale. Even if they are more difficult to control than liquid fermentation, obtaining high enzyme concentrations is possible. Moreover, solid state fermentations save water and has shown to reduce sterilisation, downstream processes and water treatment costs.

Downstream processes can be very specific depending on the type of enzyme and its sensitivity to environmental factors. Levels of purity is sometime a significant factor for

some applications and secondary enzymatic activities present in the preparation can be a problem.

The simultaneous development of these techniques has helped to develop a large number of applications first for cleaning powders, then for food and feed applications, and more recently for the lignocellulose breakdown and the production of specific chemicals.

## References

- Archer, D.B. and Peberdy, J.F. (1997). The Molecular Biology of Secreted Enzyme Production by Fungi. *Crit. Rev. Biotechnol.* **17**, 273-306.
- Arora, S., Rani, R. and Ghosh, S. (2018). Bioreactors in solid state fermentation technology: Design, applications and engineering aspects. *J. Biotechnol.* **269**, 16=34.
- Baker, S.E. (2018). Protein hyperproduction in fungi by design. *Appl. Microbiol. Biotechnol.* **102**, 8621-8628.
- Cai, D., Rao, Y., Zhan, Y., Wang, Q. and Chen, S. (2019). Engineering *Bacillus* for efficient production of heterologous protein: current progress, challenge and prospect. *J. Appl. Microbiol.* **126**, 1632-1642.
- Fiedler, M.R.M., Barthel, L., Kubisch, C., Nai, C. and Meyer, V. (2018). Construction of an improved *Aspergillus niger* platform for enhanced glucoamylase secretion. *Microb Cell Fact.* **17**, 95 (Doi: 10.1186/s12934-018-0941-8).
- Goddard, J.-P. and Reymond, J.-L. (2004). Recent advances in enzyme assays. *Trends Biotechnol.* **22**, 363-370.
- Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J.A. and Charpentier, E. (2012). A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Sci.* **337**, 816-821.
- Katayama, T., Nakamura, H., Zhang, Y., Pascal, A., Fujii, W., and Maruyama, J., (2018). Forced Recycling of an AMA1-Based Genome-Editing Plasmid Allows for Efficient Multiple Gene Deletion/Integration in the Industrial Filamentous Fungus *Aspergillus oryzae*. *Appl. Environ. Microbiol.* **85**, e01896-18 (Doi: 10.1128/AEM.01896-18).
- Kim, H. and Kim, J.-S. (2014). A guide to genome engineering with programmable nucleases. *Nat. Rev. Genet.* **15**, 321-334.
- Kun, R.S., Gomes, A.C.S., Hilden, K.S., Cerezo, S.S., Makela, M.R. and de Vries, R.P. (2019). Developments and opportunities in fungal strain engineering for the production of novel enzymes and enzyme cocktails for plant biomass degradation. *Biotechnol. Adv.* **37** (Dot: 10.1016/j.biotechadv.2019.02.017).
- Kun, R.S., Meng, J., Salazar-Cerezo, S., Makela, M.R. de Vries, R.P., and Garrigues, S. (2020). CRISPR/Cas9 facilitates rapid generation of constitutive forms of transcription factors in *Aspergillus niger* through specific on-site genomic mutations resulting in increased saccharification of plant biomass. *Enzyme Microb. Technol.* **136** (Doi: 10.1016/j.enzmictec.2020.109508).

- Liu, Q., Gao, R., Li, J., Lin, L., Zhao, J., Sun, W. and Tian, C. (2017). Development of a genome-editing CRISPR/Cas9 system in thermophilic fungal *Myceliophthora* species and its application to hyper-cellulase production strain engineering. *Biotechnol. Biofuels*. **10** (Doi: 10.1186/s13068-016-0693-9).
- Liu, R., Chen, L., Jiang, Y., Zhou, Z. and Zou, G. (2015). Efficient genome editing in filamentous fungus *Trichoderma reesei* using the CRISPR/Cas9 system. *Cell Discov*. **1** (Doi: 10.1038/celldisc.2015.7)
- Liu, R., Chen, L., Jiang, Y., Zou, G. and Zhou, Z. (2017). A novel transcription factor specifically regulates GH11 xylanase genes in *Trichoderma reesei*. *Biotechnol. Biofuels*. **10**. (Doi: 10.1186/s13068-017-0878-x).
- Mota, D.S., Marques, J.M., Guimarães, J.M., Mariúba and L.A.M. (2020). Research Article CRISPR/Cas Class 2 systems and their applications in biotechnological processes. *Genet. Mol. Res*. **19** (Doi: 10.4238/gmr18478).
- Selten, G.C.M., van Gorcom, R.F.M. and Swinkels, B.W. (1995). Selection marker gene free recombinant strains : a method for obtaining them and the use of these strains. *Eur. Patent Appl*. EP 00635574 A1.
- van Dijck, P.W.M., Selten, G.C.M. and Hempenius, R.A. (2003). On the safety of a new generation of DSM *Aspergillus niger* enzyme production strains. *Reg.Toxicol. Pharmacol*. **38**, 27-35.

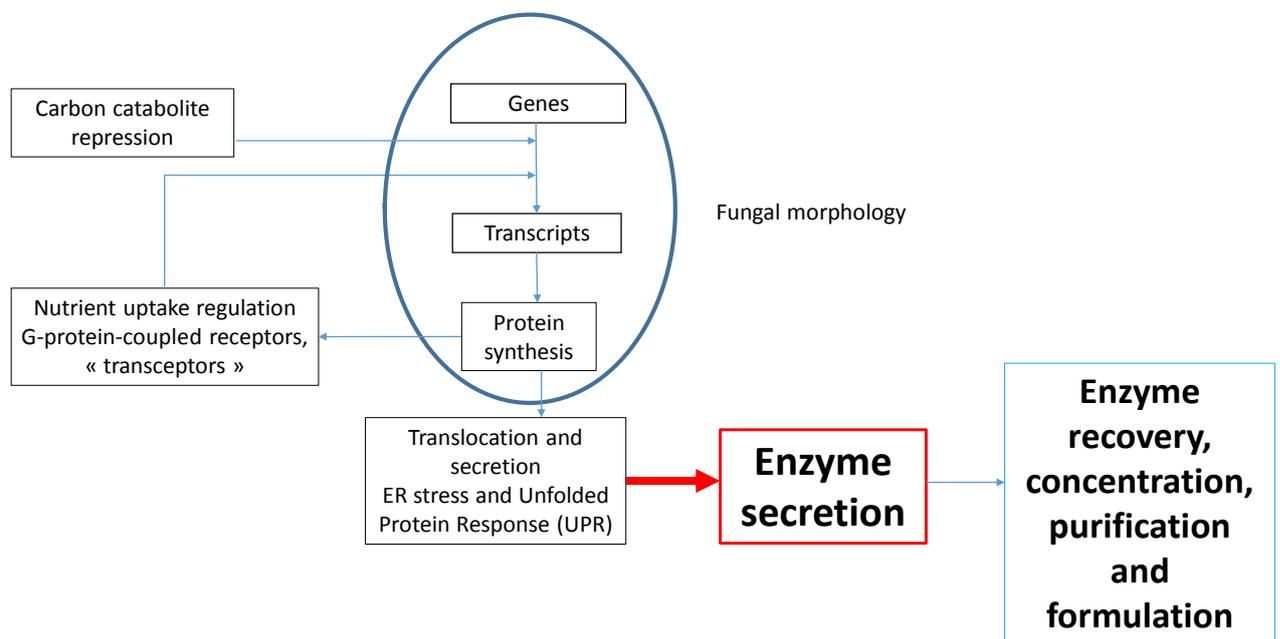


Figure 3.1. Key steps to overproduce a ready to use specific enzyme (Baker, 2018; Fiedler et al., 2018).

copies).

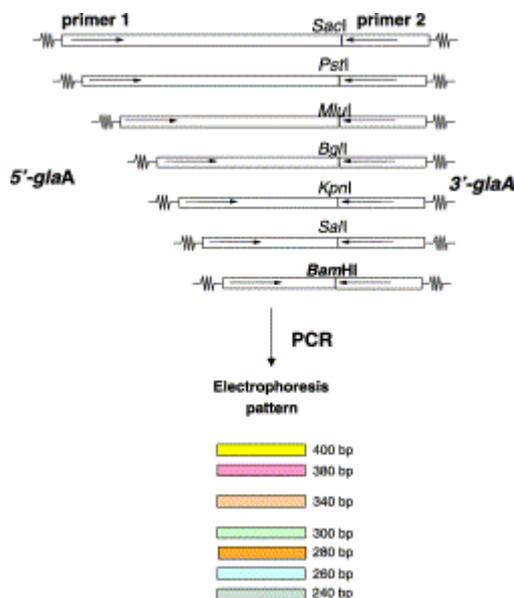


Fig. 3.2. Visualization of all seven marked *glaA* loci of *A. niger* strains, in which the *glaA* genes were removed. All gene sequences of the seven glucoamylase loci were removed by using proprietary technology. Subsequently they were partly filled using the same methodology with sequences from the 30 regions of the original locus. Due to the fact that these sequences varied in length and were decorated with a specific restriction site the seven marked loci can be detected individually by PCR using a set of specific primers and separation on a gel electrophoretic system (van Dijck et al, 2003).

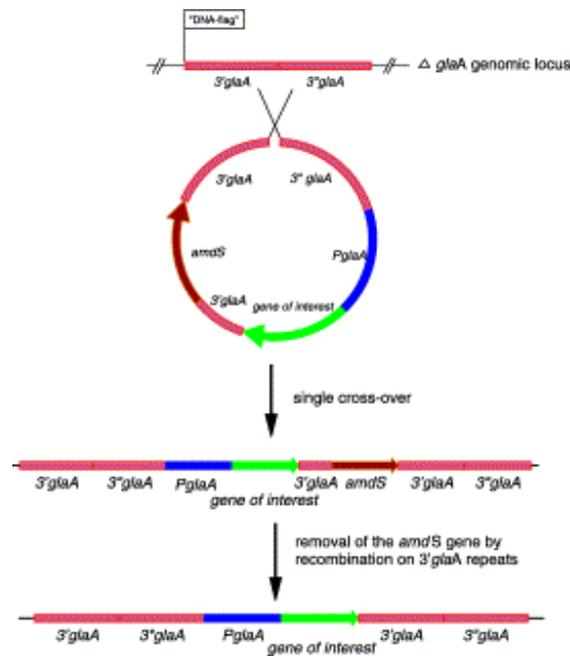


Fig 3.3. Example of the marker-gene free insertion of an expression unit. The expression unit, a linear piece of DNA, is integrated into one of the seven marked loci by homology of the 30 regions of the loci. By varying the conditions of transformation of the expression units multiple copies of the gene of interest arranged in tandem can be integrated in a marked locus. Current practise is that both the gene of interest and the *amdS* selection marker are on different expression units. So the transformation is carried out with a mixture of expression units carrying the gene of interest and expression units carrying the *amdS* marker. By selection on agar plates containing acetamide as sole carbon source the transformants are selected. By counter-selection on agar plates containing fluoro-acetate variants can be selected from these transformants which have lost the *amdS* marker but which have retained (multiple copies of) the gene of interest (van Dijck et al, 2003).

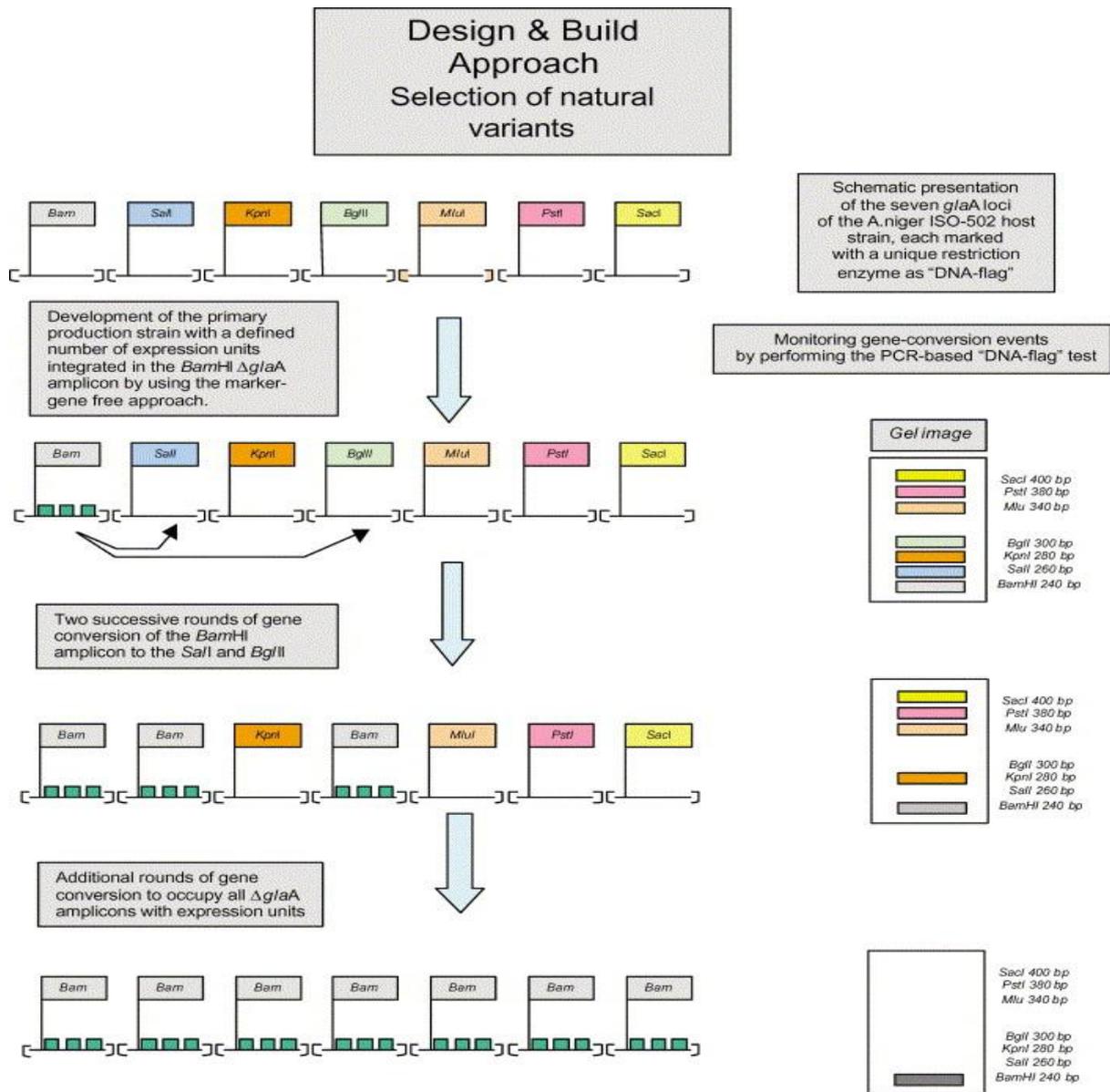


Figure 3.4. Selection of natural variants. The natural process of gene conversion is used to select for variants that have acquired a set of multiple gene copies of the gene of interest in any the remaining empty marked loci. The filling-up process in the several selection rounds can be followed by analysis in a gel electrophoretic system (van Dijck et al, 2003).

(Mota et al., 2020).

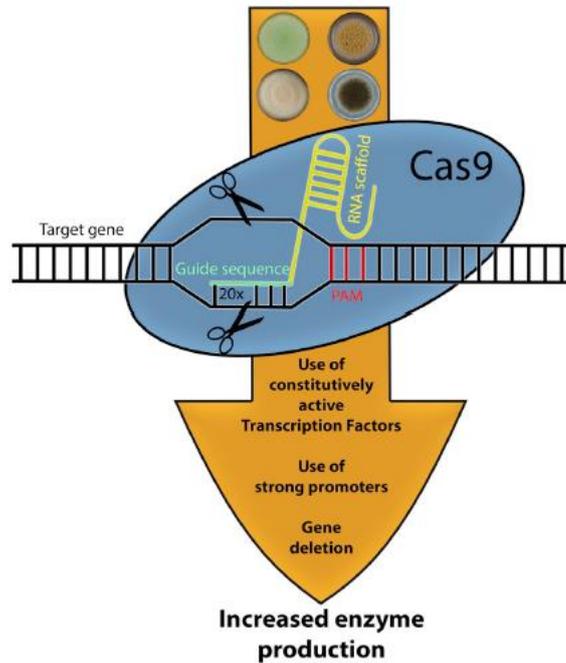


Figure 3.5. Utilization of the CRISPR/Cas9 system in filamentous fungi for increased enzyme production. The artificial sgRNA is composed of a guide sequence (in green) and a scaffold sequence (in yellow), which are the corresponding parts of the naturally occurring crRNA and tracrRNA, respectively. The sgRNA forms a complex with the Cas9 nuclease (in blue), which will be directed to a targeted locus of a gene of interest. In case there is sequence homology between the guide sequence and the target sequence upstream of a PAM sequence (in red), a double strand break (DSB) will be performed. The DSB can be repaired in a targeted manner using repair templates, which can result in precise gene editing (Kun et al., 2019).

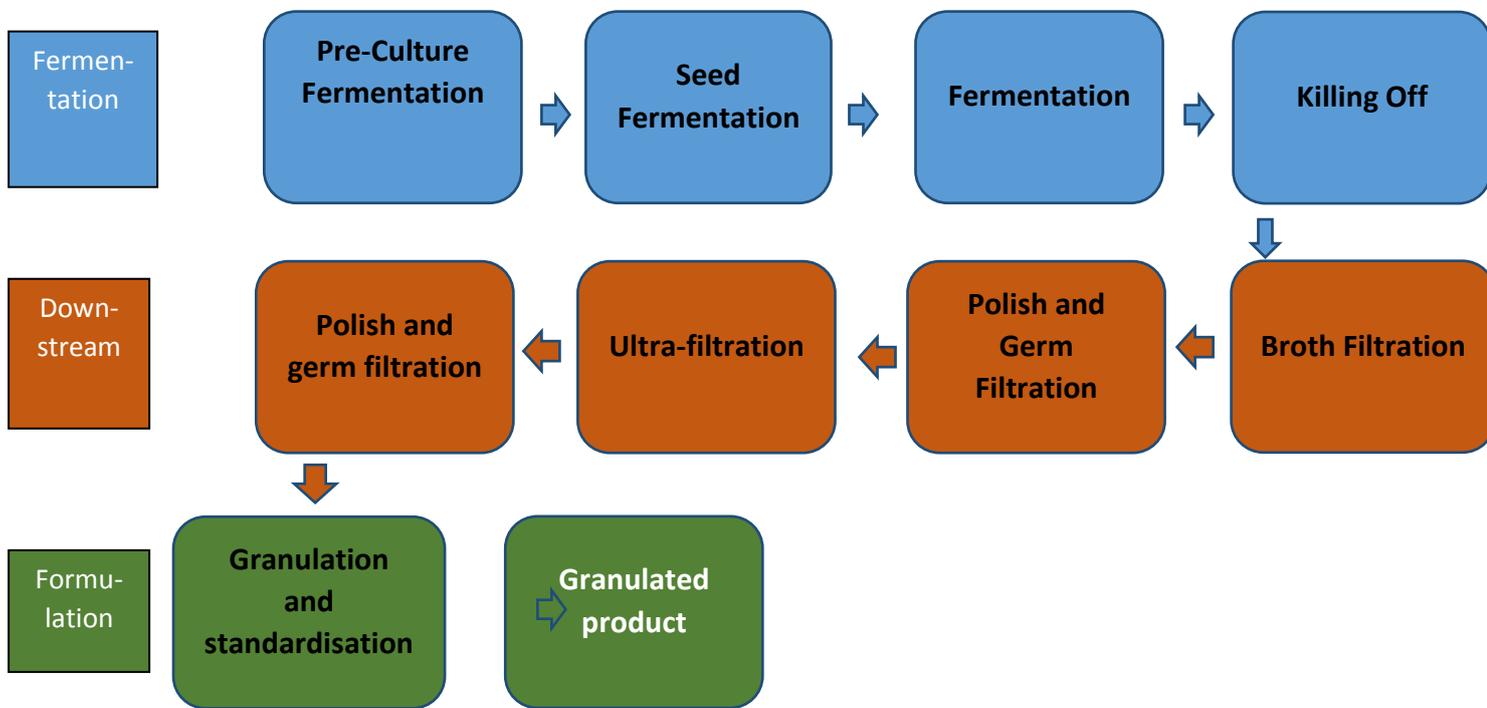


Figure 3.6. Example of a process sheet used for food enzyme production.