

FUSION BETWEEN SERINE-THREONINE REACH DOMAIN FROM *STAI* GENE WITH *BGLB* GENE OF THE *PAENIBACILLUS POLYMYXA*

Leontina GURGU*, Vasilica BARBU, Clemansa TOFAN

University "Dunărea de Jos", Faculty of Food Science and Engineering, Bioengineering Department, Domnească Street, No. 111, Galați, Romania

Abstract

Beta-glucosidase gene of bacterial origin was cloned in *Saccharomyces cerevisiae* to enable growth on disaccharide, cellobiose. To promote the secretion of β -glucosidase B, the catalytic domain of *bglB* gene was fused with the serine-threonine rich domain of the *STAI* gene and was inserted into an yeast expression vector under control of the CYC-GAL inducible *S. cerevisiae* promoter. Expression of hybrid gene and protein secretion was verified in *Saccharomyces cerevisiae* with p-nitrophenyl- β -D-glucopyranoside as substrate. Genetically stable and regulated expression in *Saccharomyces cerevisiae* of β -glucosidase activity is interesting for the development of strains able to ferment β -glycosidic sugars (i.e. cellobiose and lactose).

Keywords: serine-threonine domain, *STAI* gene, *bglB* gene, *Saccharomyces cerevisiae*

Introduction

The production of fuel ethanol from cheap and renewable biomass, such as plant material or lignocelulose, has become a major challenge for the biotechnology industry (Zaldivar et al., 2001). The two-step conversion of biomass to ethanol involves the enzymatic hydrolysis of cellulosic biomass to produce reducing sugars, and the conversion of the resulting sugars to ethanol. However, this is a very costly process due to the recalcitrance of cellulose, and therefore the low yield and high cost of enzymatic hydrolysis process (Lynd et al., 2002). The development of a yeast strain capable of producing ethanol by fermenting cellulosic substrates has received a great deal of interest over recent years (Zhao and Bai, 2009).

Although *S. cerevisiae* is a good choice, it has a few shortcomings, such as inability to degrade polysaccharides. This yeast is able to ferment some disaccharides such as, maltose and sucrose, that are α -glycosides but cannot ferment cellobiose or lactose that are β -glycosides.

Saccharomyces lacks β -glycosidases and consequently no β -glycoside is fermented by this

yeast (Barnett, 1976, 1981). Enzymatic hydrolysis of cellobiose requires the action of β -glucosidases.

Ideally, the development of *Saccharomyces* strains with the capability to ferment cellobiose and lactose will be done by the expression of heterologous genes subjected to the regulatory mechanisms of the yeast.

Furthermore, the easy way of transforming *S. cerevisiae* with foreign DNA and the well-established fermentation technology devoted to this organism make *S. cerevisiae* a good host for heterologous-protein production. (McBride et al., 2005).

In this study, the main interest in β -glucosidases was to create one that can be secreted by the *S. cerevisiae* into the extracellular medium.

The β -glucosidases encoded by the *B. polymyxa* *bglB* gene is a member of family 1 glycosylhydrolases which includes β -glucosidases and β -galactosidases from all sort of organisms: archaea, bacteria, lower eukaryotes, plants and animals (Gonzales – Candelas et al., 1990; Henrissat, 1991; Henrissat and Bairoch, 1993).

On the other hand, yeast strains belonging to *Saccharomyces cerevisiae* var. *diastaticus* are capable to digest starch by their extracellular glucoamylases, as encoded by *STA* genes. There

* Corresponding author: lili_gurgu@yahoo.com

are three unlinked *STA* genes (*STA1*, *STA2*, *STA3*) which code for the glucoamylase isozymes GAI, GAI and GAIII, respectively (Erratt and Stewart 1978; Tamaki 1978).

The glucoamylase contains two well defined domains: Ser/Thr-rich domain, and N-terminal domain structurally related to bacterial invasins, followed by a characteristic $(\alpha/\alpha)_6$ barrel of family 15 glycoside hydrolases (Adam et al. 2004).

Although the function of the Ser/Thr domain has not been clearly established, the presence of this domain in many other *S. cerevisiae* extracellular proteins suggests that it helps the secretion of the enzyme.

The present study describes some of the work carried out to induce the expression of *B. polymyxa* *bglB* gene in *Saccharomyces cerevisiae* by a fusion with Ser/Thr domain of *STA1* gene.

Materials and methods

Strains

Bacillus subtilis CECT-12/ATCC 7050 was used for pS2 vector isolation and *Escherichia coli* DH5 α was used for pLGBGB (Arrizubieta and Polaina, 2000) plasmid isolation.

Saccharomyces cerevisiae 52 (MAT α *ura3 trp1 ilv2-11*) (Adam and Polaina, 1991) was utilised for cloning the *B. polymyxa* *bglB* gene placed under

control of the galactose-inducible promoter, *CYC-GAL*.

Culture media and conditions

Ampiciline for selecting and proliferating resistant *E. coli* bacteria was added in *Luria –Bertani* medium to a final concentration of 100 $\mu\text{g ml}^{-1}$ (Sambrook et al., 1989). Also, *Luria-Bertani* liquid medium was supplemented with 1% starch for selecting *Bacillus subtilis* CECT-12/ATCC 7050 strain.

Cultures were grown at 37°C. Liquid cultures were aerated by agitation on an orbital shaker.

Standard media for *Saccharomyces*: complete, YPD and minimal, SD supplemented with auxotrophic requirements of the strain used (tryptophan [20 mg L^{-1}] isoleucine [60 mg L^{-1}] and valyne [150 mg L^{-1}], were prepared according to Sherman et al. (1986). Complete and minimal media were used for the induction of β -glucosidase activity. Induction of *bglB* gene was achieved by the addition of galactose at a final concentration of 0.5%.

Plasmids

pLGBGB (Arrizubieta and Polaina, 2000) vector was used as *bglB* gene source. Shuttle cloning vector able to replicate in *E. coli* and *S. cerevisiae*, pS2 (Garcia et al., 2005), was used for the construction of recombinant plasmid (Fig.1).

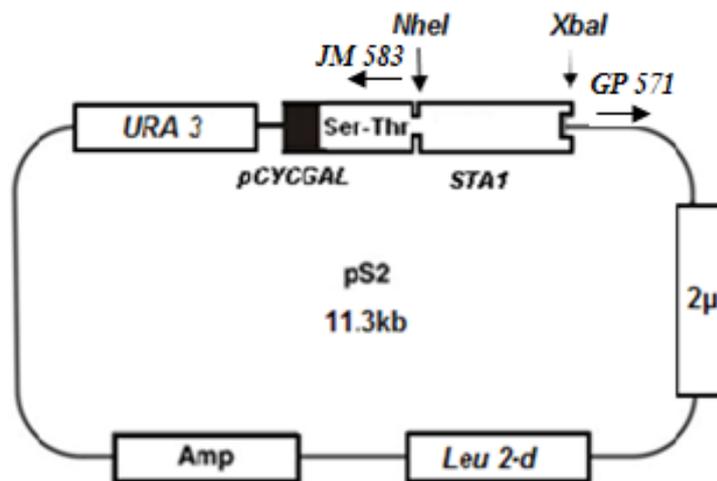


Fig.1. Schematic summary of the pS2 vector (Garcia et al., 2005). *JM583* and *GP571*, are the primers used for pS2 amplification. The catalytic domain of the *STA1* gene was cleaved by *NheI* and *XbaI*, restriction enzymes.

PCR amplification

The sequence-specific primers used to isolate the *bglB* gene and also used to amplify the pS2 vector, are presented in Table 1. The PCR reactions were performed on a Gene Amp PCR system 2400 (Applied Biosystems), using a three-

step thermocycling profile. The reaction mixture (50 μL) was as follows: 200 ng template, 100 pmol of each primer, 0.2 mM each deoxynucleoside triphosphate, the reaction buffer supplied by the manufacturer and 3.5 U of PfuUltra® II fusion HS DNA polymerase (Stratagene). Then, the amplified

products were purified using High Pure PCR Product Purification Kit (Roche).

Table 1. DNA templates and the PCR primers used for their amplification.

DNA template (size)	Overlap primer	Primer sequence
<i>bglB</i> gene	JM 584-sense GP 574 - antisense	5'- GCTGGT GCTAG CGGTATGAGCGAGAATACCTTTATATTTCC- 3' 5'- GTTCTAG ATTATTA AAAACCCGTTCTTCGCCATCATTG -3'
pS2 vector	JM 583- antisense GP 571 - sense	5'-AGCACCG CTAG CAACCTTTTTTCTGTGCGCTGGAGCCAC -3' 5'- GTTCTAG AGTCGACCTGCAGGC-3'

Manipulation of nucleic acid and cloning procedures

All cloning experiments in *E. coli* were carried out with strain *XLI-Blue*. Cloning procedures (plasmid purification, gel electrophoresis, ligation, *E. coli* transformation) were carried out according to the procedure of Sambrook *et al.*, 1989.

Vector construction

The enzymes for DNA cleavage and ligation were purchased from FastDigest® and used as recommended by the supplier. Restriction endonuclease-digested DNA was eluted from agarose gels using the protocol of QIAEX II Agarose Gel Extraction Kit (QIAGEN).

Recombinant plasmid, designated pLG1 was constructed as follows: the *bglB* gene was isolated from pLGBGB and inserted into pS2 at the *NheI-XbaI* site.

Yeast transformation

S. cerevisiae 52 was transformed with the recombinant plasmid (LG1) by lithium acetate/single-stranded carrier DNA/polyethylene glycol, method described by Hinnen *et al.*, (1978) and the transformants were confirmed by PCR.

Enzyme assay

β-glucosidase activity was measured by incubating appropriately diluted cells, cell extracts or supernatant with 5mM of *p*-nitrophenyl-β-D-glucopyranoside (pNPG) in 50mM phosphate buffer, at optimal pH (pH 7.0) and temperature (37°C) for the enzyme. The *p*-nitrophenol released was detected at 405 nm (Beckman DU 530 UV/VIS Spectrophotometer) after adding 1 mL of 2% sodium carbonate to raise the pH and stop the reaction (Adam and Polaina, 1991; Gonzales *et al.*, 1989).

Results and discussion

The plasmid pS2 was isolated from the *Bacillus subtilis* *CECT-12/ATCC 7050* strain and the insertion of the *STA1* gene was verified by double digestion with *SacI* and *XbaI*. These enzymes cut at the corresponding site of the *S. cerevisiae* expression vector pS2. In this way, pS2 vector was opened and the 2.5 kb DNA fragment had been released. Figure 2 shows the pattern of the digestion after migration in 1.5% agarose gel.

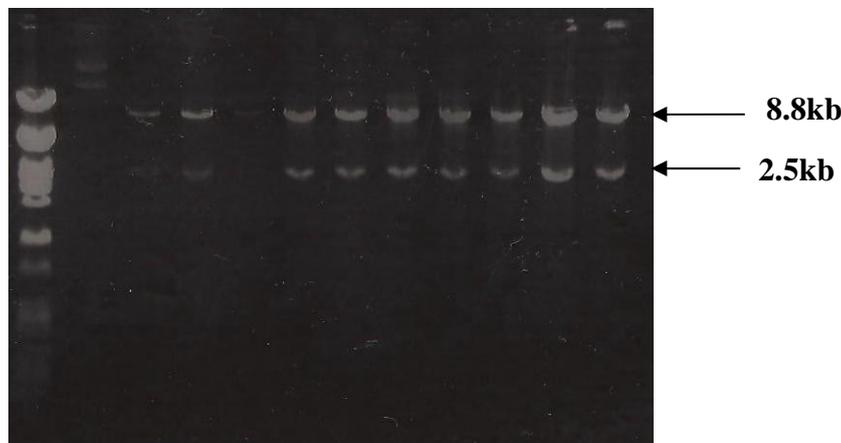


Fig.2. DNA bands separated by agarose gel electrophoresis after pS2 digestion with *SacI* and *XbaI* enzymes

The *bglB* gene encoding β -glucosidase B from *P. polymyxa* (BglB), contained in plasmid pLGBGB (Arrizubieta and Polaina, 2000), was amplified by PCR with PfuUltra® II fusion DNA polymerase. The primers used for isolating the β -glucosidase

gene (JM584/GP574), were based on the sequence data available in Genbank (<http://www.ncbi.nlm.nih.gov/entrez/>). These two primers provide a *NheI* respectively a *XbaI* sites,. The amplified product is shown in figure 3.

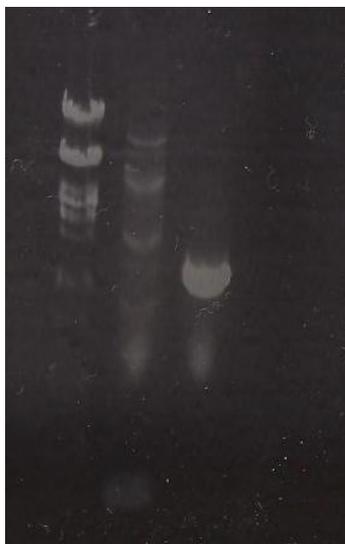


Fig.3. A 1.5% agarose gel electrophoresis of the *bglB* gene amplified with JM584 and GP574 as primers. Lane 1, Lambda DNA/Pst Marker. Lane 2, positive sample

On the other hand, pS2 vector was amplified by PCR using JM583 and GP571 as primers. These primers were designed to amplify the whole vector without *STA1* gene.

In order to reach the right temperature for the pS2 vector amplification it was necessary to use different melting temperatures in the PCR programme. The melting temperature, T_m , of an oligonucleotide is the most critically important value and it is affected by salt concentration, strand

concentration and denaturants presence. It was possible that one of these factors to affect the amplification and for this reason was necessary to try, for pS2 vector amplification, more than one melting temperature in the PCR programme: 48.1°C, 49.9°C, 51.8°C and 52.1°C.

Figure 4-line 4 shows that only $T_m=51.8^\circ\text{C}$ was the optimum temperature for obtaining a good amplification.

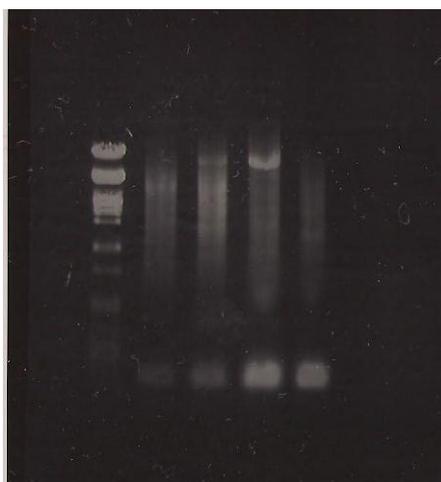


Fig.4. A 1.5% agarose gel electrophoresis of the amplification product obtained from the PCR reaction for pS2 vector using JM583 and GP571 as primers. Lane 1, Lambda DNA/Pst Marker. Lanes 2, 3 and 5, negative samples. Lane 4, positive sample.

The amplified materials, the vector and the *bglB* gene were digested with *NheI/XbaI* and were run on a 0.7% TAE gel with the Fermentas Lambda DNA/*PstI* marker. The two digested DNA fragments which were visible as a single band on

an agarose gel (Fig. 5) were recovered from the gel and were ligated into new plasmid, designed pLG1. This plasmid was used to transform *E. coli* *XLI-Blue*, competent cells.

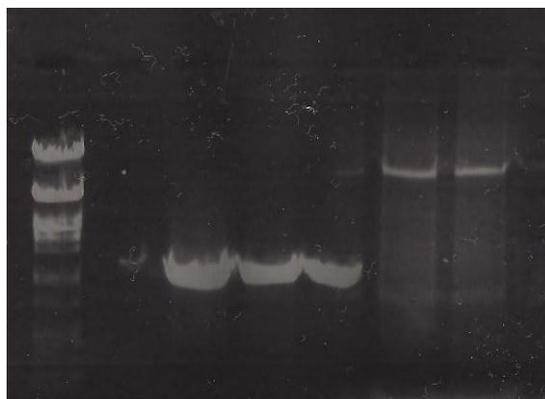


Fig. 5. A 0.7% agarose gel electrophoresis of the *XbaI/NheI* digested plasmid DNA (lanes 5 and 6) and *bglB* gene (lanes 2 and 4). Lane 1 is Lambda DNA/*PstI* marker

In order to check which colony has the pLG1, plasmidial DNA from twenty colonies was isolated and was digested with *XbaI* and *BamHI* enzymes. The last enzyme cut into seryne-threonine domain at 121pb site and *XbaI* enzyme cut into pS2 vector at 4802pb site. So, after digestion with these two

enzymes, the expected sizes of the released fragments were: 8.9 kb for the vector and 2.3 kb for the gene.

Figure 6 shows that, after the digestion with these two enzymes, only one *E. coli* colony had the recombinant plasmid.

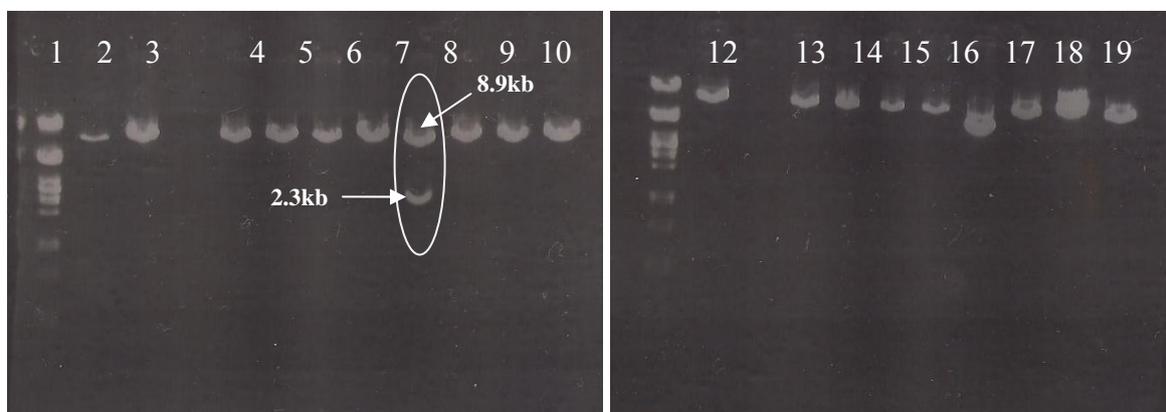


Fig.6. Bands separated by agarose gel electrophoresis after plasmidial DNA digestion with *XbaI* and *BamHI*, enzymes

In order to be sure about the right insertion of the *BglB* gene into pLG1, other three digestions were being made with the following enzymes: *SacI/XbaI*, *NheI/XbaI* and *HindIII/NheI*.

The double digestion *SacI/XbaI* opened the recombinant plasmid and released a 2.4kb fragment which was associated with the *bglB* gene and seryne-threonine domain.

This remark was sustained by the second digestion with *NheI* and *XbaI* enzymes that cut the vector

and released a 1.5 kb fragment that corresponded to the expected size of *bglB* gene.

The recombinant plasmid was used to transform *S. cerevisiae* 52 strain in order to check if *bglB*, with seryne-threonine domain, under control of a galactose inducible promoter, can be expressed in this strain.

Plasmid pLG1 contains three essential elements for transformation, the *bglB* gene under control of a galactose inducible promoter (Latorre-Garcia et al, 2005), the promoter-defective *leu2-d* gene, and

the *URA3* gene, respectively. After transformation, the *Ura*⁺ transformants, carrying pLG1, were first selected on SD plates containing the auxotrophic requirements and then were grown in YPD liquid medium with 0.5% galactose. The β -glucosidase activity was checked by using *p*-nitrophenyl- β -D-glucopyranoside (chromogenic analog of cellobiose) substrate.

No activity was detected in the reference strain. Also the culture supernatant or the cells did not yield B-glucosidase activity for any strains (data not shown).

These results suggest that serine-threonine domain did not help *BglB* to be secreted in *Saccharomyces cerevisiae* laboratory strain.

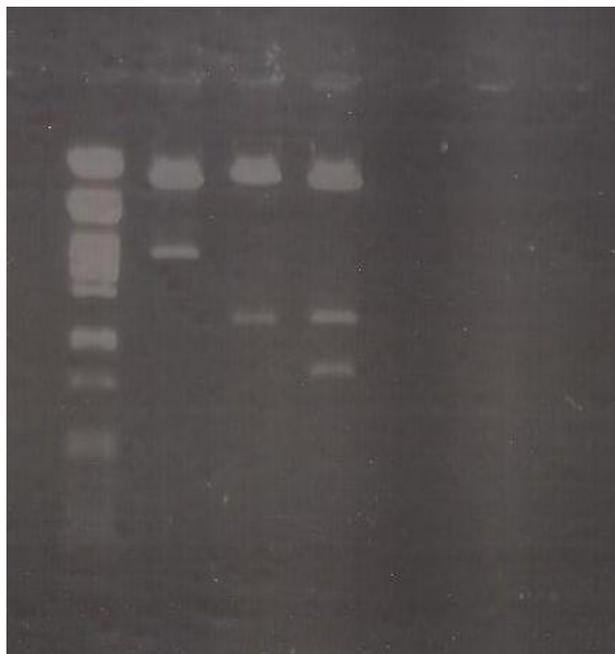


Fig.7. DNA bands separated by agarose gel electrophoresis after pLG1 digestion with *SacI/XbaI* (lane 2), *NheI/XbaI* (lane 3) and *HindIII/NheI* (lane 4)

To construct *Saccharomyces* strains with a good capability to ferment cellobiose and lactose, in addition to stable and regulated expression of a high level of β -glycosidase activity, it is necessary to overcome the inability of the yeast to permeate these sugars. Two alternative solutions to this problem are the expression in *Saccharomyces* of a permease for β -glycosides, or to make the enzyme secretable by *Saccharomyces* (Adam et al., 1995). Also, Adam and coworkers (1991) have reported the successful expression of β -glucosidase encoded by *bglA* gene from *Bacillus polymyxa* under control of the CYC-GAL-inducible yeast promoter.

The experiments reported here revealed that the other gene, *bglB*, from *Bacillus polymyxa*, could not be expressed in *Saccharomyces* under control of the same promoter.

From the practical side, the construction of cellobiose-fermenting strains is relevant in connection to the production of fuel and chemicals from cellulosic substrates.

From another point of view, the serine-threonine domain can be a useful tool to address questions related to heterologous gene expression in yeast.

Conclusions

Aiming to obtain a yeast strain with good glucosidase activity, useful for the conversion of cellobiose into ethanol and other products, *BglB* gene from *Paenibacillus polymyxa* with the serine-threonine reach domain from *STAI* gene was cloned in a *S. cerevisiae* laboratory strain.

Recombinant *S. cerevisiae* strain was generated by cloning *bglB* gene as a 1.5kb fragment into *NheI* and *XbaI* sites of pS2 to yield pLG1. The electrophoresis on agarose gel after digestion with *SacI/XbaI*, *NheI/XbaI* and *HindIII/NheI* enzymes showed that the *bglB* gene was inserted on the right position into recombinant plasmid, pLG1.

Beta-glucosidase assay with *p*-nitrophenyl- β -D-glucopyranoside reveals no β -glucosidase activity in the culture supernatant, cells or cells extract of recombinant *S. cerevisiae* strain.

According to these results presented, it can be concluded that the serine-threonine domain was

not capable to produce expression of β -glucosidase, encoded by *bgIB* gene from *Bacillus polymyxa*, under control of the CYC-GAL-inducible yeast promoter in *Saccharomyces cerevisiae*.

This study will be helpful in applying the fusion between serine-threonine reach domain with other genes for which an effective expression is not available in *Saccharomyces cerevisiae* strains.

Acknowledgements

We thank Dr. Julio Polaina strains, plasmids and helpful discussions. This work was supported by the project "Probiotic lactic acid bacteria for functional food on basis of germinated cereals", PN II IDEI cod CNCISIS 658, no. 315/1.10.2007.

References

Adam AC, Latorre-Garcia L, Polaina J (2004). Structural analysis of glucoamylase encoded by the *STAI* gene of *Saccharomyces cerevisiae* (var. *diastaticus*). *Yeast* 21:379-388. doi: 10.1002/yea.1102.

Adam, A. C. and Polaina, J (1991). Construction of a *Saccharomyces cerevisiae* strain able to ferment cellobiose. *Curr. Genet.* 20, 5-8.

Adam AC, Rubio-Teixeira M, Polaina J (1995). Induced Expression of bacterial β -glucosidase activity in *Saccharomyces*. *Yeast*, 11: 395-406.

Arrizubieta M.J. and Polaina J (2000). Increased Thermal Resistance and Modification of the Catalytic Properties of a β -Glucosidase by Random Mutagenesis and *in Vitro* Recombination. *Journal of Biological Chemistry.* 275, 28843–28848

Barnett J. A. (1976). The utilization of sugars by yeasts. *Adv. Carbohydr. Chem. Biochem.* 32, 125-234.

Barnett J. A. (1981). The utilization of disaccharides and some other sugars by yeast. *Adv. Carbohydr. Chem. Biochem.* 39, 347-405.

Erratt, J. A. and Stewart, G. G. (1978) Genetic and biochemical studies on yeast strains able to utilize dextrin. *Journal of the American Society of Brewing Chemists* 36, 151-161.

Gonzales – Candelas, L., Aristoy, M. C., Polaina, J. And Flors, A. (1989). Cloning and characterization of two genes from *Bacillus polymyxa* expressing β -glucosidase activity in *Escherichia coli*. *Appl. Environ. Microbiol.* 55, 3173-3177.

Gonzales – Candelas, L., Ramon, D. and Polaina, J. (1990). Sequences and homology analysis of two genes encoding β -glucosidases from *Bacillus polymyxa*. *Gene* 95, 31-38.

Henrissat, B. (1991). A classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochem. J.* 280, 309-316.

Henrissat, B. and Bairoch, A (1993). New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochem. J.* 293, 781-788.

Hinnen, A., Hicks, J. B. and Fink, G. R., (1978). Transformation of yeast. *Proc. Natl. Acad. Sci. USA*, 75, 1929–1933.

Latorre-Garcia L, Adam AC, Manzanares P, Polaina J (2005). Improving the amylolytic activity of *Saccharomyces cerevisiae* glucoamylase by the addition of a starch binding domain. *J. Biotechnol* 118:167-176. Doi: 10.1016/j.jbiotec.2005.03.019.

Lynd, L.R., Weimer, P.J., Van Zyl, W.H., Pretorius, I.S. (2002). Microbial cellulose utilization: fundamentals and biotechnology. *Microbiol. Mol. Biol. Rev.* 66, 506-577.

McBride, J.E., Zietsman, J.J., Van Zyl, L.R. (2005). Utilization of cellobiose by recombinant β -glucosidase expressing strains of *Saccharomyces cerevisiae*: characterization and evaluation of the sufficiency of expression. *Enzyme Microbiol. Technol.* 37, 93-101.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Tamaki H. (1978). Genetics studies of ability to ferment starch in *Saccharomyces*: gene polymorphism. *Mol Gen Genet* 164, 205-209.

Zaldivar, J., Nielsen, J., Olsson, L. (2001). Fuel ethanol production from lignocellulose: a challenge for metabolic engineering and process integration. *Appl. Microbiol. Biotechnol.* 56, 17-34.

Zhao, X.Q. and Bai, F.W. (2009). Mechanisms of yeast stress tolerance and its manipulation for efficient fuel ethanol production. *J. Biotechnol.* 144: 23-30.