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PRODUCTION OF ETHANOL FROM STARCH BY RECOMBINANT SACCHAROMYCES CEREVISIAE STRAIN EXPRESSING THE ALPHA-AMYLASE GENE

Saeid Ziaei[1], Iraj Nahvi[2], Mohsen Mobini-Dehkordi[3]

[1]Students' Research Committee, Shahid Beheshti University of Medical Sciences, Tehran, Iran.
Corresponding author; Proteomics research center, Faculty of paramedical sciences, Shahid beheshti university of medical sciences, Tehran, Iran. Ziaei.Saeid@hotmail.com, +989133369549
[2]Department of Biotechnology, Faculty of AST, University of Isfahan, Isfahan, Iran.
[3]Department of Genetics, Faculty of Science, University of Shahrekord, Shahrekord, Iran.

ABSTRACT: Shwanniomyces occidentalis possess highly efficient alpha amylase activity that enable this yeast to use raw starch as an carbon source. S.occidentalis alpha amylase gene was cloned into the Saccharomyces cerevisiae under the control of different powerful constitutive promoters, ADH1, GPD and TEF. So that the recombinant strains which carries the same gene under the control of different promoters showed different level of expression. Then, the starch utilization power of these strains compared in aerobic and anaerobic conditions. The final results showed that recombinant strain with Alpha amylase gene under the control of ADH1 promoter could produce up to 2.3% g/l ethanol from 5% starch. The pleasant work demonstrates a new approach for improving ethanol production by regulating the expression level of one enzyme instead of using different enzymes, modified enzymes or combining several enzymes.

Keywords: Amylase, Ethanol, Starch Fermentation.

INTRODUCTION

Ethanol is a renewable, environmentally friendly energy source, which is currently used as an absolute fuel or in combination with other traditional fossil fuels [1, 2]. In general, ethanol production is based on fermentation of simple sugars like glucose and sucrose by microorganisms in anaerobic growth condition [3, 4]. This particular type of ethanol which is produced by biological systems is called Bio-ethanol. Since the key microorganism in bio-ethanol production, Saccharomyces Cerevisiae, use only simple sugars as substrates for production of ethanol, all the traditional ethanol production process is based on sweet ingredients such as sugar cane and beet derivative [5]. However, the limitations of this substrate are the main burden in ethanol production technology and hence finding new substrates as substitution of molasses may prove very important in ethanol biotechnology [5].

Cellulose and starch are the most abundant carbon sources in earth and they can be used as new substrate for ethanol fermentation [6]. Although Cellulose is more abundant than starch, but difficulty of Cellulose degradation made starch as the first choice for ethanol fermentation processes [7].

As described, the main problem in using complex materials is lack of utilization potential of the S.cerevisiae. so before fermentation, the substrate must be hydrolyzed to fermentable sugars like glucose and maltose by pre-fermentation processes [8, 9]. These procedures are extremely energy demanding and time consuming and finding the new way for starch fermentation is very important [10].

Unlike Saccharomyces cerevisiae, some microorganisms are able to utilize starchy materials because of their starch degrading enzymes such as alpha-amylase, beta-amylase and glucoamylase [11]. It has been suggested that, recombinant saccharomyces cerevisiae which expressed recombinant starch degrading enzymes, could perform ethanol fermentation directly from starch [10]. Many researches has been done by cloning of starch

degrading enzymes in Saccharomyces cerevisiae, but still its need more and more endeavor to improve the ethanol production efficiency.

In this study, the direct fermentation of starch to ethanol by genetically recombinant yeasts having alpha-amylase activity under control of different expression platforms was investigated, and the amounts of ethanol produced by each recombinant strains were compared in terms of starch degradation and ethanol production.

MATERIALS AND METHODS

Microbial strains and plasmids:

Saccharomyces cerevisiae CEN.PK 113-5D, was used as an standard host for expression of alpha amylase and ethanol production. Saccharomyces cerevsiae CEN.PK 113-7D was used as a control for fermentation of glucose to ethanol in comparison to recombinant cells. Escherichia coli TOP10F' was used routinely for plasmid amplification. P426ADH, P426GPD and P426TEF plasmids was used in this study. All these plasmids contains a 2-micron origin of replication, a constitutive promoter , and a uracil (Ura) selection marker. They contained different constitutive yeast promoters – Alchohol dehydrogenase 1, gelycer aldehyde dehydrogenase and translation elongation factor 1 alpha respectively although each vector backbone contains pMB1 origin of replication and ampicillin resistance gene for propagation and selection in E. coli [12].

The source and relevant genotype of all strains and plasmids used in this study are summarized in table1.

Table 1

Source/reffrence	genotype	Strain and plasmid
Invitrogen	F'{lacIqTn10(tetR)}\, mcrA, Δ(mrr-hsdRMS-mcrBC), φ80lacZΔM15,ΔlacX74, deoR, recA1, araD139, Δ(ara-leu)7697, galU, galK, rpsL(strr), endA1, nupG	TOP10F'
[13]	MATa SUC2 MAL2-8c ura3-52	CEN.PK 113-5D
[13]	MATa SUC2 MAL2-8c	CEN.PK 113-7D
DSMZ 3451	Schwanniomyces occidentalis Kloecker	S.occidentalis
[12]	ApR ADH3pCYC1TURA3	P426ADH
[12]	ApR GPD3pCYC1TURA3	P426GPD
[12]	ApR TEF3pCYC1TURA3	P426TEF

Growth media and culture conditions:

Escherichia coli transformants were grown in luria bertani medium (1% tryptone, 0.5%yeast extracts, 0.5% sodium chloride) containing 100 µg/ml ampicillin [14]. YPD medium (2%peptone, 1%yeast extracts and 2% glucose) was general yeast media for pre transformation culture of yeasts [15]. The synthetic complete (SC) medium without Uracil (0.67% yeast nitrogen base without amino acid, 2% glucose and 0.2% Yeast Synthetic Drop-out powder without uracil) which used for screening of recombinant yeasts [16]. SCS media which share all components with sc except glucose that substituted with starch, which was used for starch degradation analysis and ethanol production process.

Construction of Recombinant plasmids:

Total genomic DNA from Shwanniomyces occidentalis was isolated and prepared as described by Hoffman et. Al. [17]. The amy gene from s.occidentalis was amplified according to previously described sequence using Pfu DNA polymerase. The suitable restriction sites which designed according to P426 vectors in forward and reverse primers was BamH1 and EcoR1 respectively

Amplified gene from PCR was determined by gel electrophoresis, and purified amylase gene was ligated to digested plasmids using t4 DNA ligase according to supplier manual. Transformations of recombinant vectors - which named P426ADH-amy, p426GPD-amy and P426TEF-amy- into E. coli Top10f' cells was performed with chemical transformation according to the protocol described by Seidman CE (1989) [18] and plasmid harboring bacteria was selected on LB-Amp medium and introduction of amylase gene was tested by colony PCR. Plasmid DNA was prepared from E.coli by the alkaline lysis miniprep method [19]. The resulted plasmids were

transformed into S. cerevisiae CEN.PK 113-5D according to standard lithium acetate procedure described by Becker et. Al. [20]. Final yeast Transformants selection was performed on SC-Ura medium.

Starch degradation:

Functionality of expression of recombinant amylase gene was tested on SCS-ura medium and Degradation of Starch by recombinant yeasts which cultivated on SCS-ura, revealed by iodine staining with iodine vapor [21]. Starch composition of fermentation media in each day was measured by using micro plate format of starch–iodine spectrophotometric assay in 96-well microplates measured at 580 nm [22].

Fermentation:

Ethanol fermentation was done in SCS medium in anaerobic shaking flask, which is designed especially for ethanol production. Our designed system is composed of an Erlenmeyer with robber stopper, air vent and sampling probe. Air vent tube allowed the exhausting gases from flask but does not allow the air to entrance. Sampling was done every 24h by gamma sterilized polystyrene syringe, and transferred to sterile vials for subsequent analysis.

Ethanol assay:

Ethanol assay was done by gas chromatography (GC) method on a on a. Hewlett Packard (HP) –INNOWax (cross-linked Poly Ethylene Glycol), 0.25mm * 15m * 0.25um – by flame ionization detector [23]. Column temperature was held at 50˚C for 2 min, increasing to 250˚C at 20˚C/min, and then maintained at 250˚C for 5 min. Helium was used as carrier gas at a flow rate of 2.9 ml/min. inlet temperature was 225˚C, split ratio of injection was 1:30 and Detector temperature was 285˚C [24].

RESULTS

Three plasmids containing alpha amylase drove from shwanniomyces occidentalis were constructed (fig.1). Each constructs different only in expression promoter and all three promoters have constitutive activity in Saccharomyces cerevisiae. The plasmids were transformed into yeast saccharomyces cerevisiae and recombinant yeasts are named according to the promoter whom they contained, 5D-ADH-Amy, 5D-GPD-Amy and 5D-TEF-Amy.

Expression of amylase in recombinant yeasts was shown with halo formation in SCS-agar medium, and the resulting halo diameter measured. (table1).

Hallow diameter	Strain name
2.5	5D-ADH-amy
5	5D-GPD-amy
4	5D-TEF-amy

According to reference literature, expression level of GPD promoter is higher than TEF and ADH promoter. The data showed that, starch degradation power of the recombinant yeasts in agar plate culture in presence of selective marker have direct relationship with promoter activity and 5d-GPD-amy showed maximum hallo formation in these conditions.

Utilization of starch and ethanol production by transformants in fermentation media was measured in small scale batch fermentation (150ml) during three-day fermentation processes, with soluble starch as the only carbon source. Starch composition of medium and ethanol concentration was measured each day.

Original media was containing 5 percent (G/L) starch at first. After 24-hour maximum starch in fermentation media was seen in 5D-GPD-amy strain. After 48 h, the amounts of starch in 5D-TEF-amy and 5D-ADH-amy was negligible but 1 percent starch was detected in the last fermentation media. After 72 h, all the starches were consumed.

time	5D-TEF-amy	5D-GPD-amy	5D-ADH-amy
24	0.42	1.53	0.61
48	0.1	1	0.08
72	0	0.1	0

96	0	0	0

The result of this stage was not the same of the results of hallo fermentation stage, even in more repetitions. We are suggested that this happen due to anaerobic pressure in the absence of selection marker and may be related to plasmid stability. Ethanol production results validate the starch concentration results. Maximum ethanol production was seen in 5D-ADH-amy strain.

In first 24h, the only strain which is produce ethanol was Cen-PK-7d in 5% glucose medium with 2.08 % ethanol (g/L). In the first-day strains which is growing on starch, does not showing notable ethanol production. In second day (48 h), ethanol concentration of 5D-ADH-amy and 5D-TEF-amy culture was 1.8% and 1.5% respectively. Maximum ethanol production in a complete period of fermentation was achieved after 72h (three days) with CEN-PK-5d-amy about 2.36 % which is higher than production of the 7d strain in glucose containing media.

time	Cen-PK-7d	5D-TEF-amy	5D-GPD-amy	5D-ADH-amy
24	2.07	0.07	0.01	0.04
48	2.08	1.53	0.08	1. 8
72	2.25	1.94	0.73	2.36
96	2.35	2.22	1.61	2.17

DISCUSSION

By these experiments, we investigated the new theory regarding the effects of expression balance on ethanol production by different recombinant strain under anaerobic conditions.

Although the expression power of GPD promoter is higher than ADH promoter, but side effects of the very high expression of exotic protein on metabolic pathways of host cell, prevent the cell from normal fermentation. We are suggested that metabolic burden which is resulted from these conditions may be having effects on plasmid stability and growth rate. Confirmation tests emphasize that Cen-PK-ADH-amy have very higher plasmid stability and colony count in third day from CEN-PK-GPD-amy. (Data not showed)

The pleasant work demonstrates a new approach that the expression level of the recombinant enzyme is the key point in this experiment and we should find the equilibrium point in the enzyme expression and metabolic pressure to find optimum strain for production of the desired product.

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