

## Genetic Modifications of *Saccharomyces cerevisiae* for Ethanol Production from Starch Fermentation: A Review

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### Abstract

There is a huge demand for developing new technologies for alternative energy sources due to the elevated costs of petroleum and its by-products, depletion of nonrenewable fuel sources, and to eliminate the disadvantages of geopolitical location and environmental pollution caused by high levels of carbon dioxide release. Science is striving to meet this demand and as molecular biology techniques have progressed, genetic engineering tools have been presented as promising future solutions in the form of optimizing the fermentation process to increase the ethanol yield from different carbon sources such as starch. As *Saccharomyces cerevisiae* is not naturally able to ferment starch, it can be genetically manipulated and modulated to improve the fuel production from starchy materials and the amount of cost that is required to produce ethanol would be decreased with these manipulations. General modifications in *S. cerevisiae* include specific gene expressions to gain new properties or improve existing pathways. This review aims to elicit the current status of ethanol production thorough alternative techniques from starch using current genetic engineering applications and to give further directions for high-throughput fermentations using genetically modified *S. cerevisiae* strains.

**Keywords:** *Saccharomyces cerevisiae*; Ethanol; Fermentation; Genetic engineering; Starch

### Introduction

Global energy consumption is rapidly increasing and it causes an elevation of energy cost and contributes to global warming because of the excessive use of petroleum based energy sources; hence the demand for renewable biomass-derived fuels has increased in recent years. Production of such alternative sources, which could also help establish a sustainable and renewable energy supply, has been popularized. Several countries have already used first generation biofuels, including ethanol, as a primary energy sources such that worldwide biofuel production reached 106 billion liters in 2011 and it is estimated to be 155 billion liters by 2020 [1]. Feedstocks rich in sugars are generally used for ethanol production via fermentation processes. Main sugar sources for ethanol production are presented in Figure 1. Although all of fuel ethanol is obtained from corn glucose in US [2] and sucrose in Brazil [3], starchy materials have been accepted as the major renewable biomass resources for ethanol production due to their low cost and abundance [4]. In this line, corn wheat and tubers from starchy crops are used for ethanol production in North America, Europe, and tropical countries [5].

Manipulation and utilization of starchy resources starts with enzymatic hydrolysis followed by fermentation of sugar molecules and subsequently the elimination of ethanol from culture media. Simultaneous Saccharification and Fermentation (SSF), is an alternative method used for bioethanol production from feedstock and it decreases fermentation costs by reducing equipment requirements since both processes occur in one reactor [6]. Another bonus in the SSF system is that the ethanol production rate is higher than the conventional method [7]. SSF has been applied for ethanol production from starch fermentation and remarkable ethanol yields (0.41 liter ethanol per kg of corn) have been obtained [8]. There have been attempts to increase the fermentation efficiency with immobilization of microorganisms in SSF systems [9-11]. Although SSF has advanced in the field, several points should be optimized to reduce the total cost, provide highly efficient utilization of starch, and maximize ethanol yield. These steps include: maintaining the optimal pH for the growth of fermentative

microorganism, sterility, continuous substrate supplementation, and the establishment of cooling systems for high temperature fermentation systems [5]. Furthermore, several ethanol producing microorganisms used in industry, such as *Saccharomyces cerevisiae*, have strains that are not naturally able to utilize starch and they require high amounts of amylolytic enzymes, which is associated with high cost and impractical ethanol production [12]. Therefore, while ethanol production from starchy materials is racing ahead, the conventional processes used today are not favorable at the economic level; hence, improved methods are desired with microbial strains that enable efficiency and lead to high yield ethanol production in a cost-effective way [4].

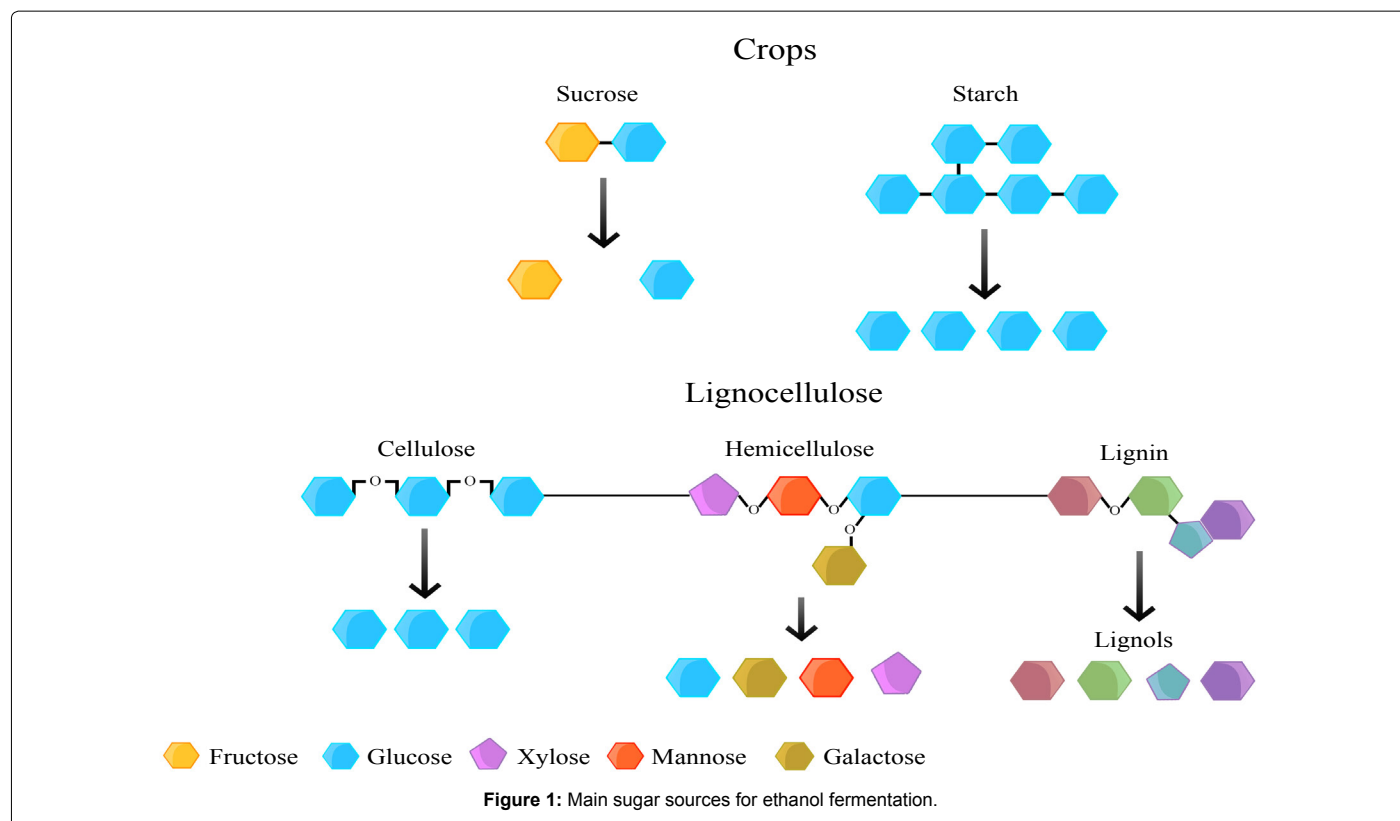
A vast amount of microbial species to obtain ethanol from starch in the fermentation process have been presented in the literature. Regardless of the species, parental microorganisms remain insufficient in conventional ethanol production due to the lack of availability of sugar rich input and low ethanol yields. Over 150 amylolytic yeast strains have been reported to be impractical in industrial use because of limited characteristics [13]. Although *Clostridium* spp. and *Zymomonas mobilis* are popular bacteria for ethanol production [14,15], *S. cerevisiae*, well-known and widely used yeast in alcoholic beverages and bakery industries, is traditionally preferred because it sustains steady-state production, can consume various monomeric sugar molecules, has a high fermentation capacity, ethanol productivity and ethanol tolerance, along with having “generally regarded as safe” (GRAS) status [16]. As

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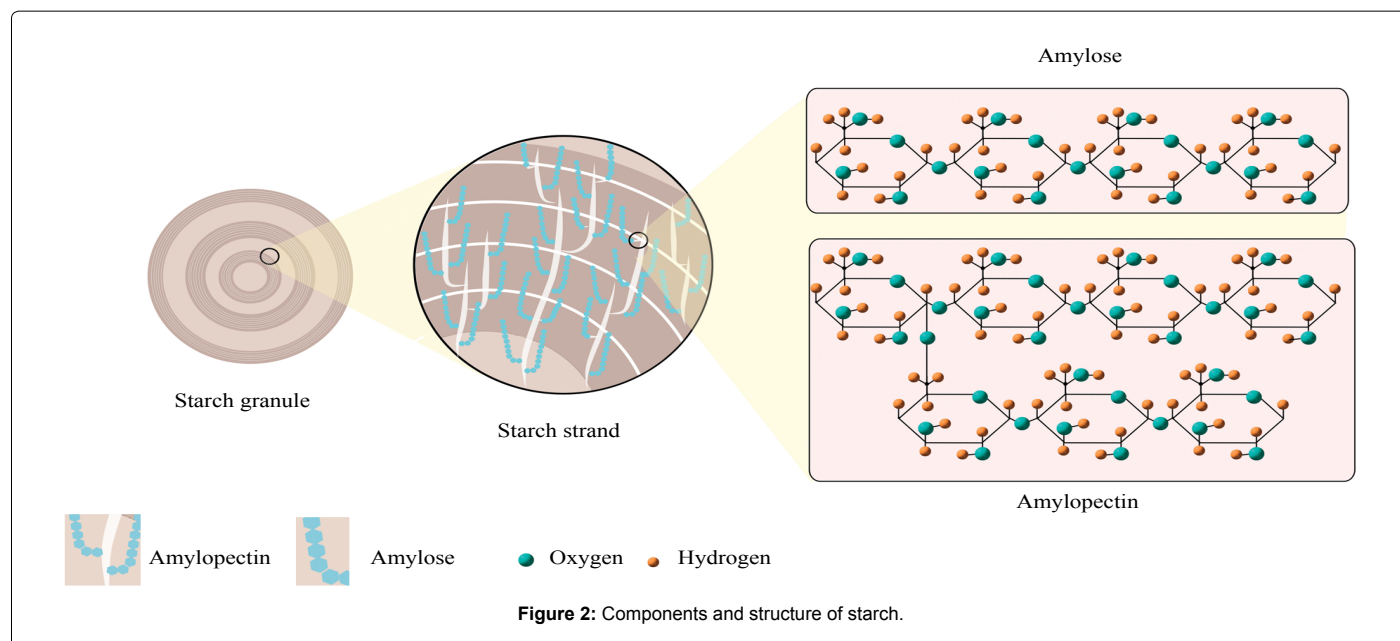
*S. cerevisiae* is not naturally able to ferment starch, the development of genetically modified *S. cerevisiae* strains displaying starch utilizing enzymes ( $\alpha$ -amylase and glucoamylase), leading to saccharification and fermentation of starch at the same time has been an area of interest. In this review the genetic engineering of *S. cerevisiae* to allow it to utilize starchy sources resulting in a decreased total cost of fermentation and the realization of high ethanol levels will be discussed in detail.

## Starch Fermentation

Starch is a cheap, renewable, and fermentable carbon source [17] found in all green plants in various amounts. There is approximately a starch content of 70% in corn, triticale and cassava [18]. Starch molecules are generally associated with lipids, proteins and fatty acids, and the type of association determines the pre-treatment process to be applied before fermentation [19]. Starch consists of glucose monomers joined by glycosidic bonds ( $\alpha$ -1,4 glucan-linked D-glucopyranose chains) [18]. It is mainly composed of the linear glucose polymer amylose (20-30%) and highly branched amylopectin (70-80%) (Figure 2) [20]. Amylose has mostly  $\alpha$ -1,4-linked D-glucopyranose and sparse  $\alpha$ -1,6-linked D-glucopyranose chains (about 0.3% to 0.5%), and forms a flexible molecule leading to interaction with fatty acids, alcohols, and iodine [21]. Amylopectin is a highly branched helix like structured molecule consisting of  $\alpha$ -1,4-D-glucopyranose chains [22]. Amylose and amylopectin, found in the starch structure at different rates, form an interconnected structure which directly determines the chemical characteristic of biomass and fermentation yield [23].

Fermentation of starch commonly involves two stages; i) starch hydrolysis by amylolytic enzymes (liquefaction) and saccharification, ii) fermentation of glucose into ethanol [24]. For an efficient starch fermentation, both  $\alpha$ -1,4 and  $\alpha$ -1,6-debranching hydrolases, with amylases and glucoamylases, and  $\alpha$ -glucosidases displaying both

$\alpha$ -1,4 and  $\alpha$ -1,6-debranching activities are required [25]. As starch itself is not readily utilizable by *S. cerevisiae*, it has to be hydrolyzed by acid treatment and saccharified by enzyme (amylase and/or glucoamylase) treatment before the main fermentation process [7]. This is a relatively expensive application as 30-40% of the total cost for the fermentation process is spent for the liquefaction and saccharification necessary for this popular microbe to be used [26]. Moreover, adding caustic soda, lime and sulphuric acid to maintain optimum pH levels for the enzymes, also increases the total cost [27]. Cold starch hydrolysis is an alternative method to decrease the total energy input. However, total enzyme requirement is much higher in cold hydrolysis than starch hydrolysis at high temperatures [28,29]. Although elevated temperatures in the initial step is advantageous to prevent bacterial contamination, high cooling cost result and become a major problem for fermentation because the temperature for industrially available yeasts should be stabilized to 30-37°C. Co-culture systems (amylolytic microorganisms and yeast) have emerged in the SSF process to avoid pure enzyme requirements [30-32]. However, the process is still expensive and the ethanol yield is not satisfactory because of the high starch content consumed for the growth of amylolytic microorganisms. Consolidated Bioprocessing (CBP), hydrolysis and fermentation of sugar molecules in a single step using microorganisms with fermentation capability, is the most effective method for ethanol production from starch fermentation. In this concept, if a microorganism does not naturally express amylolytic enzymes, genetic engineering tools offer a solution for starch fermentation by non-amylolytic microorganisms, e.g. *S. cerevisiae* as explained in this review [18]. The optimization of temperature requirements for saccharification and fermentation processes and the development of genetically modified *S. cerevisiae* strains that do not need the addition of exogenous enzymes to achieve the full potential for starch fermentation in a single step, are highly



warranted to enhance ethanol production [16,33]. Therefore, genetic manipulations in *S. cerevisiae* for a direct conversion of starch to ethanol is a promising process and it would save the feedstock spent for the growth of amylolytic microorganisms and reduce the total cost along with providing high ethanol yield.

### Glucoamylase expressing *S. cerevisiae*

Construction of a yeast strain that can utilize raw starch has been studied since the 1980s [34,35]. In the beginning of genetically engineered *S. cerevisiae*, fashioned for starch fermentation, 13% (v/v) ethanol yield was obtained from direct hydrolysis of starch via using glucoamylase expressing yeast [36]. The first step of the process began by constructing recombinant strains that express amylolytic enzymes ( $\alpha$ -amylase or glucoamylase) for the liquefaction and saccharification of the starchy biomass. Glucoamylase (1,4- $\alpha$ -D-glucan glucohydrolase; EC 3.2.1.3) is recognized as the most important enzyme which is responsible for the progressive hydrolysis of starch from non-reducing ends to release  $\beta$ -D-glucose units and saccharification of the polymers [37]. The glucoamylase cDNA gene (*glu*) from *Aspergillus awamori* has been successfully incorporated into *S. cerevisiae* genome for the utilization of starch and recombinant strains have been found to be stable for 50 generations without applying any selective pressure [38]. In another study, the glucoamylase enzyme coding sequence was transferred to *S. cerevisiae* genome and hydrolyzation of unprocessed and soluble starch was attained at high performance levels ( $0.23 \text{ g.l}^{-1}.\text{h}^{-1}$ ) [39]. The same research group also increased the enzyme activity of the *A. awamori* glucoamylase by codon-optimizing, compared to native glucoamylase (791 nkat and 591 nkat per gram dry cell weight, respectively) and transformed the recombinant gene to industrial *S. cerevisiae* (27P) strain for a direct starch fermentation [27]. Although co-culture of *S. cerevisiae* with an amylolytic microorganism is a conventional option, Nakamura et al. have reported that a recombinant *S. cerevisiae*, SR93, modified to express glucoamylase has produced more ethanol ( $24.9 \text{ g.l}^{-1}$ ) as compared to a co-culture of *A. awamori* and wild type *S. cerevisiae* system ( $22.0 \text{ g.l}^{-1}$ ), because SR93 has saved the internal starch amount consumed for the growth of the amylolytic microorganism, *A. awamori* [7]. However, SR93 could not degrade

all the starch content efficiently because recombinant glucoamylase enzyme originated from *Saccharomyces cerevisiae* var. *diastaticus* was not able to degrade  $\alpha$ -1,6 glycoside bond of amylopectin units. It has been recognized that this glucoamylase enzyme, coded by the STA1 gene, lacked a starch-binding domain which made fermentation and ethanol production unsatisfactory [40,41]. Therefore, the starch-binding domain of the *Aspergillus niger* glucoamylase gene has been fused with STA1 gene resulting in a remarkable hydrolysis and utilization of insoluble starch [42]. Instead of using a starch binding domain, the gene for glucoamylase of *Rhizopus oryzae*, capable of breaking down both  $\alpha$ -1,4- and  $\alpha$ -1,6-glycosidic bond efficiently, has been transferred to *S. cerevisiae* and approximately 80% of starch content was utilized in a 100h fermentation period [43]. As a long fermentation period ( $\sim 150\text{h}$ ) is required for sufficient starch fermentation by glucoamylase secreting *S. cerevisiae*, in anaerobic or minimal aerobic conditions [7,34,35,44], a recombinant strain of *S. cerevisiae* (YF207/pGA11) expressing cell surface anchored *R. oryzae* glucoamylase has been tested for ethanol production under aerobic conditions (dissolved oxygen was  $2.0 \text{ ppm}$ ), using soluble starch [4]. A high ethanol production rate ( $0.71 \text{ g.h}^{-1}.\text{l}^{-1}$ ) was achieved and the fermentation process has been completed in seven repeated fermentations over 300 h without losing modified gene stability. As the same enzyme from various sources would provide different outcomes due to their divergent kinetic properties and activities in different experimental conditions, determining optimal enzyme source to be transferred is one of the most critical issues. For instance, *R. arrhizus* glucoamylase gene has been transferred to *S. cerevisiae* and up to 5% ethanol and  $2400 \text{ U.l}^{-1}$  enzyme activity (one of the highest level reported to date) has been obtained in a flask ferment experiment [45]. *S. cerevisiae* has been modified by *Aspergillus oryzae* glucoamylases, encoded by *glaA* and *glaB*, and *R. oryzae* glucoamylase separately on the cell surface and compared with each other for their starch fermentation ability [46]. The highest ethanol yield has been obtained from *glaA* glucoamylase expressing yeast ( $15 \text{ g.l}^{-1}$  in 24 h); although *R. oryzae* glucoamylase exhibited the highest glucoamylase activity ( $9 \times 10^{-9} \text{ U/cell}$ ). From a different point of view, as raw and naive starch is not favorable from a commercial perspective, Kosugi et al. have designed an experiment to produce ethanol from cassava pulp rich in starch (up to 60%) [47]. A high production rate of ethanol from



5% cassava pulp (91% of theoretical value) has been achieved using *S. cerevisiae* displaying *R. oryzae* glucoamylase enzyme.

### **$\alpha$ -amylase expressing *S. cerevisiae***

Apart from transforming glucoamylase into *S. cerevisiae* for starch hydrolysis and saccharification,  $\alpha$ -amylase modification is another important option in recombinant technology for starch fermentation. The  $\alpha$ -amylases (EC 3.2.1.1) breakdown starch molecules from the internal  $\alpha$ -1,4-bonds of amylose and amylopectin and release dextrans (10-20 glucose units in length), free glucose and maltose units [37]. Wheat  $\alpha$ -amylase has been cloned into *S. cerevisiae* and found secreted into medium successfully as early as 1987 [48]. Since then, amylase enzymes from different sources such as bacterial [49], yeast [50,51], mold [52], barley [53] and rice [54] have been cloned into *S. cerevisiae*. From the beginning of these studies, optimization of cell growth and enzyme activity has been determined as the key factors for an efficient ethanol production. The selection of the source for the enzyme is a critical issue as the nature and activity of the enzyme should be compatible with the fermentation conditions. It has been shown that decrease in pH, from 5.5 to 4.5 due to yeast growth, in fermentation resulted in a dramatic reduction in *Bacillus subtilis*  $\alpha$ -amylase activity, whereas there was no effect, even a slight increase, on barley  $\alpha$ -amylase activity [55]. *S. cerevisiae* strains expressing recombinant  $\alpha$ -amylase genes (*LKA1* and *LKA2*) obtained from *Lipomyces kononenkoae* have been proven to convert starch directly into ethanol [56]. However, the rate of ethanol production has been found to be low (17.2 g.l<sup>-1</sup> in 200 h fermentation period) since the capacity of starch hydrolysis by recombinant  $\alpha$ -amylase was inadequate. Similarly, Ramachandran et al. have reported *LKA1* expressing flocculent and non-flocculent *S. cerevisiae* strains for ethanol production from raw starch [57]. Genetically modified flocculent strains produced higher ethanol levels compared to non-flocculent counterparts (4.61 kg.l<sup>-1</sup> and 5.1 g.l<sup>-1</sup>, respectively) in a 90 h fermentation period. However, the general accepted concept is that *S. cerevisiae* should express a high amount of  $\alpha$ -amylase under aerobic conditions for effective starch fermentation [56,58]. As a different strategy, apart from genetically engineered *S. cerevisiae* that expresses recombinant  $\alpha$ -amylase, ethanol production rate sometimes increased by addition of exogenous glucoamylase enzyme [53,59]. Although this is a preferable option for a high starch fermentation rate, it is not economically favorable from an industrial perspective due to the high price of pure enzymes. In this line, scientists have focused on both  $\alpha$ -amylase and glucoamylase expressing *S. cerevisiae* to increase ethanol yield and decrease total cost.

### **Co-expression of $\alpha$ -amylase and Glucoamylase**

In order to increase the rate of ethanol production from starch fermentation by *S. cerevisiae*, additional genetic manipulations such as co-expressing of  $\alpha$ -amylase and glucoamylase have emerged as a latter strategy. Glucoamylase and  $\alpha$ -amylase enzymes synergistically enhance the rate of corn and wheat starch hydrolysis with respect to their individual performances [51,60-62]. Hence, scientists have been working on constructing *S. cerevisiae* strains that express both enzymes. The glucoamylase gene of *A. awamori* (*GA1*), *Debaryomyces occidentalis* glucoamylase (*GAM1*), and  $\alpha$ -amylase (*AMY*) encoding plasmids have been transformed into industrial *S. cerevisiae* strain for a direct conversion of starch to ethanol [63]. Yeast containing *GAM1*, *GA1* and *AMY* genes have exhibited the highest glucoamylase (required for debranching of starch molecules) activity (1020 U.l<sup>-1</sup>) compared to only *GAM1* or *GA1* transformed strains (790 U.l<sup>-1</sup> and 560 U.l<sup>-1</sup>, respectively); indicating synergistic activity. Altıntaş et al.

have also transformed *S. cerevisiae* with a bifunctional fusion protein that contained both the *B. subtilis*  $\alpha$ -amylase and the *A. awamori* glucoamylase, but they have found the biomass (3.86-6.24 g.l<sup>-1</sup>) and ethanol production (18.4-23.2 g.l<sup>-1</sup>) insufficient in the experimental model of intermittent starch feeding system [64]. In a different study, three different recombinant strains of *S. cerevisiae* have been used for the comparison of their ethanol production capabilities [44]. YPG/AB strain expresses *B. subtilis*  $\alpha$ -amylase and the *A. awamori* glucoamylase separately, but YPB-G strain expresses both enzymes as a fusion protein. One last strain YPG/MM expresses mouse  $\alpha$ -amylase and *A. awamori* glucoamylase. YPG/AB strains were found to be superior in ethanol production (43.8 g.l<sup>-1</sup>) than YPB-G (35.2 g.l<sup>-1</sup>) and YPG/MM (24.3 g.l<sup>-1</sup>) strains. Although the glucoamylase activity in YPG/AB and YPB-G strains were similar (1053 U.l<sup>-1</sup> and 1100 U.l<sup>-1</sup>, respectively),  $\alpha$ -amylase of YPG/AB showed 2.2 fold higher activity than the fusion protein group which was the possible explanation for the higher rate of ethanol production. In addition, low levels of ethanol produced by YPG/MM strain have been attributed to negligible glucoamylase activity. From a different point of view, expressing amylase and glucoamylase genes together with bacterial pullulanase in *S. cerevisiae* resulted in complete (99%) utilization of the initial starch [65]. However, as it was mentioned in the previous section, using laboratory strains and raw starch resources are not economically favorable and do not always reflect real conditions. In this sense, scientists have tried to optimize starch fermentation using unprocessed biomass and industrial yeast strains. In a high-yielding brown rice fermentation, yeast strain expressing  $\alpha$ -amylase and glucoamylase exhibited acceptable ethanol production rate (1.1 g.l<sup>-1</sup>.h<sup>-1</sup>) [66]. Industrial yeast strains have also been genetically modified for starch hydrolysis due to their high ethanol production rate and tolerance to harsh conditions. Viktor et al. have tested ethanol production capacities of *Aspergillus tubingensis* T8.4  $\alpha$ -amylase and glucoamylase expressing laboratory strain, *S. cerevisiae* Y294, and the semi-industrial strain, *S. cerevisiae* Mnuu1. Y294 and Mnuu1 strains have produced 9.03 and 6.67 g.l<sup>-1</sup> ethanol, respectively, from a substrate load of 200 g.l<sup>-1</sup> raw corn starch after 10 days fermentation period without any heat treatment [37]. Industrial strains of *S. cerevisiae* have been transformed with amylase and glucoamylase genes separately and co-cultured for an efficient one-step starch utilization [67]. Activities for glucoamylase and  $\alpha$ -amylase have been determined as 920 U.l<sup>-1</sup> and 7960 U.l<sup>-1</sup>, respectively.

### **Cell surface anchored enzyme expressing *S. cerevisiae* strains**

Co-expressing of starch utilizing enzymes has also been studied by anchoring enzymes on the cell wall of yeast to provide long term durability. Expressing on the cell surface and secreting to the medium has various advantages and disadvantages. Secreting of the enzymes to the fermentation environment increases the rate and amount of ethanol by providing high possibility of interaction between starch molecules and enzymes [68]. On the other hand, secreting to the environment is not favorable because of the loss of stability in the early stage of fermentation. An additional consideration for secreted enzymes is including extra ingredients such as metal ions or surfactants in the fermentation medium to provide enzyme stability for repeated large-scale productions. For example, calcium ions have been claimed to be protective for  $\alpha$ -amylase during repeated 10-cycles of raw starch fermentation [69]. However, cell surface engineering of yeast for starch utilizing enzymes is suitable for long term stability and repeated large-scale production without the necessity of adding additional reagents. In a recent study, *S. cerevisiae* strain co-expressing glucoamylase

and  $\alpha$ -amylase on the cell surface has been used in 23 continuous cycles of ethanol fermentation without losing enzyme activity [70]. Recombinant yeast strains, glucoamylase and  $\alpha$ -amylase anchored on the cell wall (or secreted to the medium), have been developed for a direct starch utilization [71]. Cell surface glucoamylase and  $\alpha$ -amylase expressing *S. cerevisiae* strain has produced more ethanol (60 g.l<sup>-1</sup> in a 100 h fermentation period) and a higher starch degradation rate than the only cell surface glucoamylase expressing strain (50 g.l<sup>-1</sup> in 120 h fermentation). In another study, *S. cerevisiae* co-displaying glucoamylase of *R. oryzae* and  $\alpha$ -amylase of *Streptococcus bovis* anchored to cell membrane via C-terminal-half region of  $\alpha$ -agglutinin and the flocculation functional domain of Flo1p, respectively, has been effective (61.8 g.l<sup>-1</sup> ethanol in a 72 h fermentation period) in raw corn starch hydrolysis [12]. A recent study has brought a different point of view to starch conversion into ethanol by *S. cerevisiae*. A recombinant strain of yeast which expressed cell surface engineered aspartic protease has been constructed for the consuming of complex nitrogenous materials, other than starchy molecules found in crops, that resulted in high ethanol yield and shorter fermentation duration ( $3.8 \pm 0.15\%$  improvement in the final ethanol concentration compared to parental strain in 72 h fermentation period) [72].

### Stable Starch Fermenting *S. cerevisiae* Strains

Although genetic manipulation is a relatively easy process by using episomal vectors which do not require integrative sites, the stability of the episomal plasmid is a serious problem for long-term and repeated fermentation such that plasmid leakage is generally observed in long-term incubation [7,73]. Gene integration into the microbial genome has been undertaken in many studies to provide long-term enzyme activity [38,74,75]. The  $\delta$ -sequences of the Ty retrotransposon, or rDNA sequence, of *S. cerevisiae* are generally used elements for chromosomal integration of a recombinant gene. Enzyme activities could be increased 20-fold by means of  $\delta$ -integration sequence with respect to the conventional transformation [76]. In another study, it has been shown that 90% of the initial starch content was fermented by recombinant *S. cerevisiae* that co-expressed glucoamylase and  $\alpha$ -amylase transformed via  $\delta$ -integration [77]. Although, there has been a 2-fold decrease in

ethanol production and cell mass in *S. cerevisiae* strain transformed with episomal vector after 7-repeated fermentation process, the yeast strain transformed with  $\delta$ -integration sequence containing plasmid has exhibited long term stability of enzyme activity up to 10 cycles [69,78] and a high ethanol production rate up to 23 cycles [70]. Ribosomal DNA sequence of yeast is another effective option for chromosomal integration via homologous recombination. An  $\alpha$ -amylase gene has been integrated into yeast chromosome and exhibited a 2-fold increase in starch consumption compared to the episomal vector cloned strain [79]. Targeting ribosomal DNA sequence (150-200 copy in yeast genome) via homologous recombination has resulted in stable high copy number of recombinant gene [59,80]. Multiple copy integrated genes via rDNA homologous recombination displayed a higher quantity of starch consumption and ethanol production (19.2 g.l<sup>-1</sup>) [81]. As a novel strategy, rDNA and  $\delta$ -integration combination could be used by targeting two separate DNA sites for efficient cloning of two or more genes [63]. Cell fusion technique is also used to make diploid and tetraploid *S. cerevisiae* strains to obtain high level of biomass and ethanol production. Diploid and tetraploid strains have proliferated and grown faster, fermented starch more efficiently in comparison with parental strains. At the end of 72h fermentation process, haploid, diploid and tetraploid strains have produced 0.55, 0.72 and 0.93 g.l<sup>-1</sup>.h<sup>-1</sup> ethanol, respectively [76].

### Conclusion and Future Perspectives

Energy prices are growing fast, mainly because of depletion of petroleum sources. Therefore, alternative energy sources obtained from sun, wind and biomass have become attractive recently. Ethanol, produced by fermentation from biomass, as a promising alternative energy is a transportable and economically favorable source. In addition, ethanol production from various feedstocks such as starch maintains a considerable potential due to their availability, accessibility and relatively low cost in comparison to sucrose and glucose based feedstocks. Recombinant DNA technology offers a valuable opportunity for consolidated bioprocessing processes of the biomass fermentation. To date however, transforming new pathways and overexpression of a single or group of enzymes have

| Engineered Enzyme  | Gene Source  | Cultivation Time (h) | Ethanol Yield (g.l <sup>-1</sup> .h <sup>-1</sup> ) | Enzyme Activity             | Starch Source                     | Reference |
|--|--|----------------------|---|-----------------------------|-----------------------------------|-----------|
| $\alpha$ -amylase  | <i>Lipomyces kononenkoae</i>   | 90                   | 0.05  | 87 U.l <sup>-1</sup>        | Raw corn starch                   | [57]      |
| Glucoamylase   | <i>Aspergillus awamori</i>   | NA*                  | ND#   | 162 U.l <sup>-1</sup>       | Soluble starch                    | [38]      |
| Glucoamylase   | <i>Aspergillus awamori</i>   | 50                   | 0.23  | 624 U.l <sup>-1</sup>       | Soluble starch                    | [39]      |
| Glucoamylase   | <i>Saccharomyces diastaticus</i> (var. <i>diastaticus</i> )                                      | 200                  | 0.66  | ND                          | Soluble starch                    | [7]       |
| Glucoamylase   | <i>Rhizopus oryzae</i>   | 168                  | 0.77  | 60.2 U/g of wet cells       | Cassava pulp rich in starch (60%) | [47]      |
| Glucoamylase   | <i>Aspergillus awamori</i>   | 80                   | 0.175   | ND                          | Soluble starch                    | [58]      |
| Glucoamylase/<br>$\alpha$ -amylase   | <i>Rhizopus oryzae</i> / <i>Streptococcus bovis</i>  | 72                   | 0.85  | 57/114 U/g of wet cells     | Raw corn starch                   | [12]      |
| Glucoamylase/<br>$\alpha$ -amylase   | <i>Saccharomycopsis fibuligera</i> /<br><i>Lipomyces kononenkoae</i>                             | 120                  | 0.178   | 1340 /<30 U.l <sup>-1</sup> | Soluble starch                    | [56]      |
| Glucoamylase/<br>$\alpha$ -amylase   | <i>Rhizopus oryzae</i> / <i>Streptococcus bovis</i>  | 120                  | 0.74  | 790/1306 U.l <sup>-1</sup>  | Raw corn starch                   | [70]      |
| Glucoamylase/<br>$\alpha$ -amylase   | <i>Rhizopus oryzae</i> / <i>Streptococcus bovis</i>  | 24                   | 1.2   | 4700/1800 U.l <sup>-1</sup> | High-yielding rice                | [66]      |
| $\alpha$ -amylase/ glucoamylase/<br>glucoamylase with debranching activity | <i>Debaryomyces occidentalis</i> / <i>Aspergillus awamori</i> / <i>Debaryomyces occidentalis</i> | 168                  | 0.45  | 5940/1020 U.l <sup>-1</sup> | Soluble starch                    | [63]      |
| Glucoamylase/<br>isoamylase  | <i>Aspergillus awamori</i> /<br><i>Pseudomonas amyloclavata</i>                                  | 140                  | 0.137   | ND                          | Soluble starch                    | [81]      |

NA: Not available,  
#ND: Not determined

**Table 1:** Genetic modifications on *S. cerevisiae* for high efficient ethanol fermentation.

not taken biomass fermentation via *S. cerevisiae* to a satisfactory level for industrial arena. Although there are hundreds of studies claiming higher ethanol production from starchy materials, most of these have been tested at laboratory scale only and not at large-scale (Table 1). Several laboratory yeast strains have been proven to exhibit remarkable starch fermenting capacity and produce high amounts of ethanol, but transferring engineering technology to industrial *S. cerevisiae* strains has remained insufficient. They are not convenient for repeated large scale applications because of low stability of the modification, high cost of the process, low yield of ethanol production and fermentation rate. Although using exogenous amylase and glucoamylase seems to increase total yield, developing yeast strains sufficient alone for the whole fermentation process is desirable. New amylase and glucoamylase from different sources should be presented and investigated for their activity (alone or in combination) and compatibility with industrial yeast strains. Researchers should continue to conduct studies on two main areas; efficient hydrolysis and fermentation of biomass, which result in high ethanol production rate and combination of improved bioprocess applications with genetic engineering tools in the view of productivity and large scale processes.

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