Chapter

Simultaneous Saccharification and Fermentation and Factors Influencing Ethanol Production in SSF Process

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Abstract

Ethanol production from agricultural products mainly corn, wheat, sweat potato and residue are gaining importance and requires an industrially viable novel technology namely simultaneous saccharification and fermentation process. This process has an advantage of carrying out saccharification using enzyme and fermentation using yeast in a single fermenter. The investment cost for industrial ethanol production using cheap agricultural residues can be well achieved using SSF process. The success of SSF process greatly depends upon the pretreatment methods using different enzymes to break the complex carbohydrates to simple sugars. Optimization of key process variables is essential to maximize the ethanol yield from suitable substrates. The key process variables affecting the SSF process are pH, temperature, fermentation time, enzyme concentration and substrate concentration. The medium components are to be screened for effective nitrogen, potassium and phosphorous sources to increase the ethanol yield.

Keywords: simultaneous saccharification and fermentation, pretreatment, enzymes, ethanol, yeast pH and temperature

1. Introduction

The raw materials for ethanol production can be classified based on the type of carbohydrates they contain, i.e., sugar, starch, or cellulose by fermentation. Sucrose, glucose, or fructose for ethanol production for simultaneous saccharification and fermentation process are derived from any of the two classes of raw materials namely, starchy and cellulosic materials [1].

Ethanol production from simple sugars derived from sugarcane molasses, beet sugar is commercially well established. The yeast or bacterial cells can metabolize the simple sugars directly without the necessity of pretreatment step. The starch and cellulose polymers must be hydrolyzed to simple sugars before they can be fermented by yeast or bacteria [2–4]. Although cellulosic materials are available in plenty than starchy and sugar-containing raw materials, the process of conversion of it to fermentable sugars is often a very expensive pretreatment step using

enzymes [5, 6]. Starch-containing substrates must be hydrolyzed by enzymes or acid to simple sugars and can be used for the production of ethanol. The carbon, hydrogen, and oxygen are normally provided by a complex carbohydrate source such as cane or beet molasses in industries. Vitamins and minerals may be added as additional nutrients. The sources of nitrogen are generally ammonium sulfate and urea, but they require biotin for effective utilization [7]. Other cheaper raw materials such as spent sulfite liquors, and whey also are sources of fermentable sugars. The sugar concentration in the above-mentioned industrial effluents is very much lower than in usual starchy and cellulosic substrates. Spent sulfite liquors contain 20–30 gL^{-1} of hexose while whey contains 40–50 gL^{-1} of lactose. Cellulosic raw materials on acid or enzyme hydrolysis give a maximum sugar concentration of around 40–60 gL^{-1} [8]. Ammonium or potassium phosphate provides the potassium and phosphorous required for growth of yeast. The magnesium sulfate, chloride and biotin can be provided as additional supplements [8, 9]. In a study by Qureshi and Manderson [10] four renewable agricultural resources were considered, namely wood, molasses, whey permeate, and starch. He reported that molasses sugars were cheaper than sugars derived from the other raw materials.

The simultaneous saccharification and fermentation (SSF) process was conceptualized in the late 1970s by Wright et al., Takagi et al., and Blotkamp et al. [11, 12]. This process employs fermentative microorganisms in combination with amylolytic enzymes in a single fermenter. Sugar accumulation in the fermenter is minimized in this process that favors increased hydrolysis and ethanol yield when compared to separate hydrolysis and fermentation. The main advantage process over separate hydrolysis and fermentation is that high substrate concentration, long residence time and high enzyme concentration can be used in same reactor. Optimization of process variables namely substrate concentration, enzyme concentration, pH and temperature are important to maximize the ethanol yield.

Starches that can be used for ethanol production by fermentation, includes grains, cassava (manioc, tapioca), sweet potato, sweet sorghum, and Jerusalem artichoke, corn, wheat, rice, potatoes, and sugar beets are the mostly used feedstocks in Europe and North America, sugarcane, molasses, cassava, babassu nuts, and sweet potatoes appear to provide the most promising feed for ethanol for countries such as Brazil.

1.1 Substrates for ethanol production using SSF process

1.1.1 Corn

According to Miranowski [13], corn is the most viable feedstock for ethanol production. The main factors are high yield, broad geographical cultivation range and available at cheaper cost. Annual production of corn biomass is about 300×10^6 tons (dry basis), about 40% of which are residues which is suitable for ethanol production. Extremely efficient systems are already in place for corn production from seed at very low cost. In evaluating the potential of corn (and any other food crop) for the production of energy, the moral issue of food vs. fuel must be considered. Approximately 66% of the grain produced consumed as food. The proportion of grain that are unsuitable for food production is about 5% of the annual grain production and it is suitable for alcohol production. In many countries corn is used as a raw material. The suitability of corn for ethanol production using SSF process depends on the contents of starch. A high content of horny endosperm leads to problems in ethanol production using SSF processes. The starch isolated from horny endosperm is difficult to gelatinize, and has low swelling, swelling value, and α-amylase digestibility is very less when compared to floury endosperm. Pretreatment of horny endosperm is difficult and requires more enzyme concentration.

1.1.2 Wheat

Wheat is mostly used in distilleries, because it yields a mild and smooth distillate. The starch content of wheat is usually about 60%. Wheat containing more than 13% raw protein causes problems in fermentation. Wheat mashes with high protein forms foam during fermentation and the use of antifoam agent (e.g., silicone anti-foam) is necessary. **Table 1** shows the composition of key components in wheat grain and **Table 2** shows the average composition of wheat.

Components	Protein	Ash	Carbohydrates	Fat
Seed coat	7–12	5–6	80–85	1.0
Aleurone layer	24–26	10–12	52–58	1.8
Endosperm	4–6	0.4–0.6	80–84	8–10

Table 1.
Composition of wheat components in % dry solids.

Components	Composition in g/100 g of flour
water	13.2
Crude protein	11.7
Crude fat	2.0
Starch	69.3
Crude fiber	2.0
Ash	1.8

Table 2.
Average composition of wheat.

1.1.3 Cassava

Cassava (*Manihot esculenta*), is cultivated widely in many tropical countries and used as food in African countries. Brazil, Indonesia, and Zaire are the major producers in the world. Cassava roots have 20–35 wt.% starch and 1–2 wt.% protein [14]. The composition of cassava is shown in **Table 3**. At a productivity level of 30 tons ha^{-1} of Cassava with 25 wt.% starch, 70% conversion to ethanol has been reported [15].

Components	Composition in g/100 g of flour
Reducing sugars	0.1
protein	2.1
Fat	0.2
Starch	80
Crude fiber	2.0
Ash	0.9
Total sugars	3.6

Table 3.
Average composition of cassava.

1.1.4 Sweet potato

Sweet potato (*Ipomoea batatas*) represents a fuel crop of significant potential [16] and has a starch content of 64.4% on a dry basis. SSF process is used to get a maximum ethanol yield from sweet potato tubers and stalk using combination of enzymes and microbes in a single reactor.

1.1.5 Sweet sorghum

Sweet sorghum (*Sorghum saccharatum*) is a valuable energy crop containing both starches and sugars. More than 17,000 types of sorghum are known to exist in world. Ethanol production of 3500 L ha^{-1} can be obtained from the fermentable sugars alone. An additional 1600–1900 L is derived from stalk fibers using SSF process. With hybrid strains, the yield may be increased 30% above present levels [17].

The adaptability to the majority of the world's agricultural regions, its resistance to draught, and its efficient utilization of nutrients make it as a viable raw material for ethanol production using SSF process [18].

1.1.6 Barley

Barley is mostly used as malting grain in ethanol production. It is also an interesting raw material in ethanol production using SSF process. The disadvantages of barley as a feed stock in distilleries are the husks surrounding the kernels and the content of glucans that leads to high viscosities in mashes. Therefore, special pretreatment step before SSF process is necessary in preparing mashes from barley. **Table 4** shows an average analysis of barley. Barley with 55% starch is also a major feedstock for beer production. Potable distillates produced from barley are smooth, but they have a more powerful grain taste.

Components	Composition in g/100 g of flour
Protein	11.8
Fat	2.3
Starch	63.2
Crude fiber	5.3
Ash	2.8

Table 4.
Average composition of barley.

1.2 Pre-treatment of substrates used in SSF process

1.2.1 Enzymatic liquefaction of starch in SSF process

It is essential to liquefy the starch as a pretreatment step before using the substrate SSF process. Liquefying enzymes are virtually all α-amylases (α-l, 4-glucane 4-glucanohydroase, E.C. 3.2.1.1) that split α-1,4 bonds in amylose and amylopectin that are basically derived from plants, bacteria and fungi. Liquefying enzymes may be classified as endo-acting enzymes and exo-acting enzymes. The α-1,6 glycosidic bonds are not hydrolyzed by alpha amylase since they are endo-acting enzymes. The enzyme activity of α-amylase is majorly dependent on the type of microorganisms

or plants from which it is synthesized. α-Amylases rapidly decrease the viscosity due to its endo-acting nature and is used in simultaneous saccharification and fermentation process for pretreatment.

1.2.2 Treatment with α-amylase of Bacillus licheniformis (TBA)

The optimum conditions of pH for enzyme hydrolysis of starch using TBA is between 6 and 7 and the optimum temperature is in the range of 85–90°C [18]. The hydrolysis of corn starch with TBA, mainly produces maltotriose, maltopentaose, and maltohexaose. TBA enzyme is highly unstable and degrade at temperatures above 65°C in absence of Calcium ions and substrate. Senn [19] established an optimum pH range from 6.2 to 7.5, and pH values below 5.6 lead to a rapid decrease in enzyme activity. Enzyme activity is influenced greatly by the proportion of horny to floury endosperm present in the corn feed stock. Liquefaction of corn mashes using TBA yields mainly starch fragments with a maltotriose as well as maltose and glucose.

1.2.3 Treatment with α-amylase of Bacillus subtilis (BAA)

BAA synthesized using *Bacillus subtilis* is found to have an optimum pH value between 5.3 and 6.4, and an optimum temperature of 50°C [20]. Fogarty and Kelly [21] reported that with starch as substrate BAA produces limit dextrins. BAA enzyme produces limit dextrins that cannot be hydrolyzed using glucoamylase obtained from mold *A. niger* and starch degradation often remains incomplete BAA is unsuitable for SSF process which mainly uses glucoamylase enzyme. The BAA enzyme activity reaches a maximum for a pH between 5.8 and 6.8 and a temperature of 55–60°C, when corn is used a substrate [22, 23].

1.2.4 Treatment with α-amylase expressed by Bacillus licheniformis (BAB)

BAB, a new technical enzyme produced with a genetically engineered strain of *B. licheniformis* (Liquozyme, NOVO Nordisk, Denmark) [24] for its tolerant even at low pH values down to 4.8–5. But BAB is used to liquefy cereal mashes and is very effective. This enzyme express it activity up to 90°C and is used in pretreatment step for liquefying substrate in SSF process.

1.2.5 Treatment with fungal α-amylase of Aspergillus oryzae (FAA)

Fogarty and Kelly [21], reported that FAA contains only a few amino acid residues and is highly stable in acidic pH. The enzyme activity is maximum in a pH between 5.5–5.9 and at a temperature of 40°C. FAA can hydrolyze starch granules at a pH of 7.2 and temperature of 37°C and only 40% of starch was dextrinized in pretreatment step after 60 hour. The optimum pH ranges from 5.0 to 6.0 while corn is used as a substrate. The optimum temperature is reported between 50 and 57°C. FAA reduces the viscosity which is desirable for saccharification and is more effective in producing dextrins.

1.2.6 Enzymes for starch saccharification in SSF process

Glucoamylase (EC 3.2.1.3) enzyme, hydrolyzes α-1,4, α-1,6, and α-1,3 glycosidic linkages of starch molecules. Hydrolysis rate of starch is based upon the size and structure of the molecules [21].

1.2.7 Treatment with glucoamylase of Aspergillus niger (GAA)

Glucoamylases from *Aspergillus niger*, have been characterized by Fogarty and Kelly, 1979 The suitable pH for GAA is found to be in the range of 4.5–5.0 and an optimum temperature of GAA is 60°C. When corn mash was used as substrate, the optimum range of pH value reaches from 5.0 down to 3.4 [22, 25]. Thus, GAA is stable during fermentation. GAA was stable up to 70°C with an optimum at 65°C.

1.2.8 Treatment with glucoamylase of Rhizopus sp. (GAR)

GAR enzyme shows a maximum activity at temperature of 40°C and a pH value of 4.5–6.3 [21]. Glucoamylase 1 exhibits maximum debranching activity and totally degrades starchy materials to fermentable sugars in SSF process. Saccharification using GAR was carried out in a temperature range of 55–60°C and a pH of 4.4–5.4 [23]; GAR was also stable in acidic pH while corn is used as substrate.

1.2.9 Enzyme combinations in saccharification process

Single enzymes are rarely used for saccharification process. Enzymes may be combined successfully in mashing processes and fermentation. As reported by [24], different combinations of technical enzymes may exhibit either complementary or inhibitory effects. "OPTIMALT" is an industrially used enzyme combination off GAR GAA and FAA [28]. The concentration of fermentable sugars in mashes rises rapidly when enzyme combination is used in SSF process.

1.3 Microorganisms for ethanol production using SSF process

The yeast species mainly *S. cerevisiae, S. uvarum, Schizosaccharomyces pombe, Kluyveromyces marxianus and Candida utilis* are used for industrial alcohol production using SSF process [29]. *Saccharomyces cerevisiae* is the common microbe used for industrial ethanol production owing to its use for long time food industry. *Kluyveromyces marxianus* yeast grows well even up to 40°C [30]. This species is mainly used for production of alcohol from cellulosic, starch and saccharine substrates using SSF process. The activity of the yeast is very high at high temperatures and results in high ethanol production in less fermentation time.

Yeasts can utilize a variety of substrates. In general, they are able to grow and efficiently ferment in a pH between 3.5–6.0 and temperature in the range of 28–35°C. The overall productivity of the fermentation was less due to ethanol product inhibition and substrate inhibition [26]. This drawback of substrate inhibition can be overcome in SSF process where simultaneous utilization of substrate by microbes and synthesis of glucose by enzymes at faster rates.

Yeast, under anaerobic conditions, converts glucose to ethanol by the Embden-Meyerhof pathway and is shown in **Figure 1**. 2 mol of ethanol, CO_2, and ATP per mol of glucose fermented were synthesized in this pathway with a yield coefficient of 0.51 g alcohol [27].

1.4 Simultaneous saccharification and fermentation (SSF) process and key variables

Simultaneous saccharification and fermentation SSF is a process in which sugars from the liquefied substrates are saccharified and fermented in a single fermenter using enzyme and yeast. The drawback of SSF of cellulose using enzymes is

feedback inhibition by the product. Separate Hydrolysis and Fermentation uses different temperature for hydrolysis and fermentation but the main disadvantage is the end product inhibition of glucose that accumulates in the hydrolysis step [31]. SSF process overcomes this difficulty of accumulation of sugars inside the fermenter by simultaneous fermentation of sugar by suitable yeast [32, 34]. The flow sheet of the SSF process using corn starch is shown in **Figure 2**.

Verma et al. [35] studied the conversion of starch to ethanol in a SSF process using co culture of amylolytic yeast and *S. cerevisiae*. The optimum temperature was reported as 30°C. Banerjee et al. [36] reported an optimum temperature of 37°C for *S. diastaticus* using soluble starch as a substrate. Saha and Ueda et al. [37] reported that 38°C gave a maximum ethanol yield by *S. cerevisiae* in a fermentation of glucoamylase treated starch. Bandaru et al. [38] had optimized the operating variables of fermentation for the production of ethanol using sago starch using co-immobilized glucoamylase and *Z. mobilis* and he reported an optimum temperature of 32.4°C and desirable pH at 4.93.

Amutha et al. [39] studied the ethanol from pretreated cassava starch by co-immobilized cells of *Z. mobilis* and *S. diastaticus* in batch and continuous fermentation. Pretreatment of substrate was carried out using BAB at 75°C for 1 hour. The

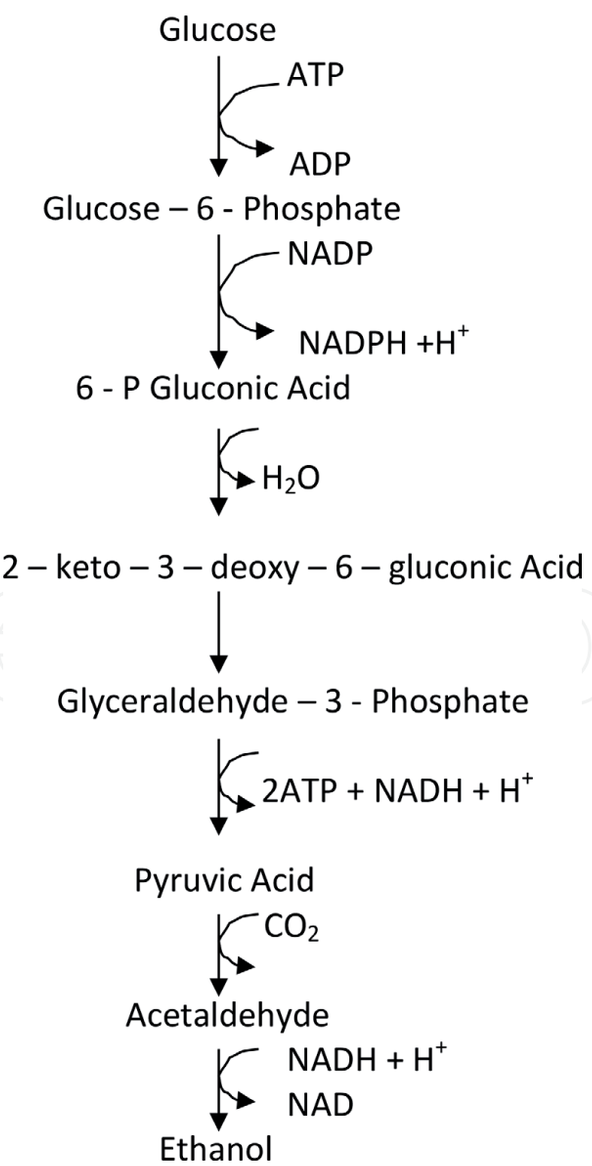

Figure 1.
EMP pathway for glucose to ethanol.

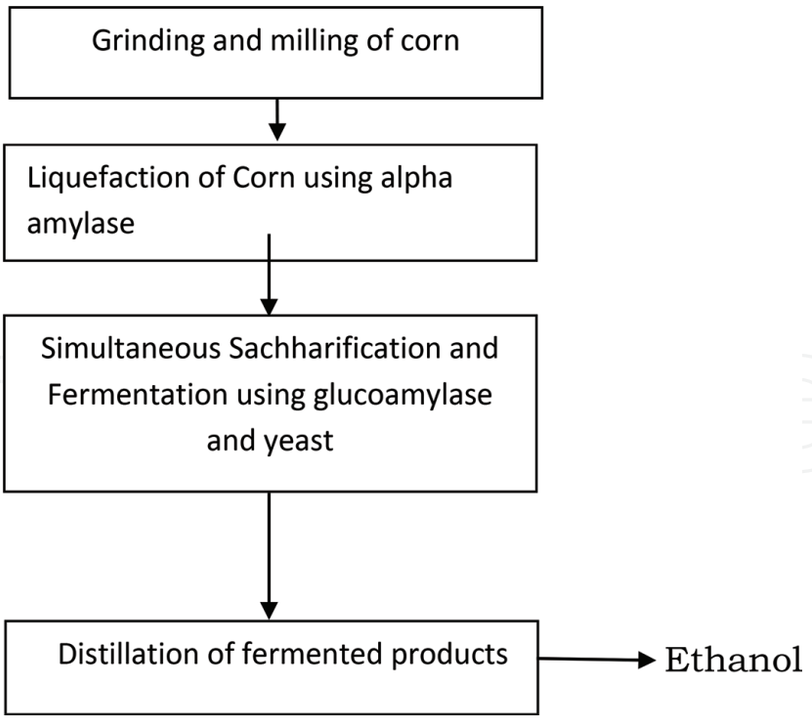

Figure 2.
Flow sheet for simultaneous saccharification and fermentation.

batch fermentation was carried out at a temperature of 30°C and at an initial pH of 6.0. 37.5 gL^{-1} of ethanol production was reported using free cells in mixed culture fermentation and 46.7 gL^{-1} using co-immobilized cells in batch fermentation.

Neves et al. [40] studied the ethanol production from wheat flour by SSF process. SSF process was conducted at 5°C and a controlled pH of 4.5 using glucoamylase 200 U/g of flour and *S. cerevisiae* in a batch fermenter. The fermentation time was 72 hour. 38.76 gL^{-1} of ethanol production was reported.

Davis et al. [41] studied the production of ethanol using waste starch stream by SSF process using *Z. mobilis* and *S. cerevisiae*. The operating conditions for SSF process were a controlled pH of 5.0 and temperature at 30°C. A maximum ethanol production of 39 gL^{-1} was reported.

Nakumara et al 1997 [42] studied the production from raw wheat flour using glucoamylase and *S. cerevisiae*. The pre-treatment of starch was carried out by adding 0.02 g of Termamyl/kg of starch and at a temperature of 95°C for 2 hour. Ethanol concentration of 67 gL^{-1} was reported using SSF process at a controlled temperature of 35°C and controlled pH of 4.5. The alcoholic fermentation of whey using K. marxianus yeast immobilized on delignified cellulose material. The optimum pH value was reported as 4.5. The optimum temperature for fermentation was reported as 37°C.

Pavla et al. [43] had studied the SSF process using wheat bran as substrate. Wheat bran was pre-treated with FAA followed by saccharification using glucoamylase. Pre-treatment temperature for FAA was 55°C and pH 6.0 for 4 hour and saccharification at 55°C for 48 hour to ensure the total hydrolysis of starch. The fermentation of filtrates resulting from pre-treatment using *S. cerevisiae* was carried out with initial pH of 5.5 and 30°C. The ethanol yield reported was 0.41 g/g of glucose fermented.

Reddy et al. [44] had studied the direct fermentation of potato starch to ethanol by co culture of *A. niger* and *S. cerevisiae*. The optimum pH for maximum ethanol production was reported as 5 to 6. The temperature of the fermentation medium was controlled at 30°C.

SSF process using maize starch as substrate by glucoamylase and *S. cerevisiae* at 35°C with the initial pH 5.5 was carried out. A maximum ethanol productivity of 1.23 $gL^{-1}h^{-1}$ was reported.

Kadam and Newman [33] evaluated several industrially available nutrient sources for their effectiveness in the SSF of pretreated starch with *Saccharomyces cerevisiae* D5A. Ethanol production was found to increase for a combination of 0.3% CSL and 2.5 mM $MgSO_4.7H_2O$ Hence, it is more industrially relevant medium than the medium containing rich nutrients.

The pH and temperature of the medium plays a vital role in all types of fermentation processes. As temperature increases the rate of biological reactions also increases upto a certain temperature and further increase in temperature may result in lesser product formation. That temperature was always chosen as the optimum temperature for the fermentation. This characteristic is similar to chemical reaction. This increase in rate of biological reaction may be due to more production of required enzymes at the faster rate. The ethanol producing microorganisms such as *S. cerevisiae, K. marxianus, S. diastaticus* prefer to grow best at 30°C [47]. Most of the microorganisms prefer to grow at neutral pH and hence we have more contamination at that pH. Ethanol producing yeast prefer to grow and metabolize in the pH

Culture	Source of starch	Process and fermentation conditions	Ethanol concentration gL^{-1}	Ethanol productivity $gL^{-1}h^{-1}$	Ethanol yield g/g of starch	Reference
Glucoamylase + yeast	cassava	batch fermentation	16.5	0.14	0.49	Ueda et al. [37]
Co-immobilized *Aspergillus niger*, and yeast	Rice	Mini jar fermenter	40	0.18	0.48	Lee et al. [45]
A. niger and *S. cerevisiae*	Potato	SSF pH—5.5, T—30°C, S—100 gL^{-1}	13.5	0.18	0.135	Reddy et al. [44]
Glucoamylase + *S. cerevisiae*	Raw wheat flour	SSF process pH—4.5, T—35°C, S—150 gL^{-1}	60	9.5	0.40	Nakumara et al 1997 [42]
Co-immobilized *Z. mobilis* + *S. diastaticus*	liquefied cassava	continuous fermentation pH—6.0, T—30°C, S—150 gL^{-1}	69.6	0.99	0.46	Amutha et al. [39]
A. awamori and *S. cerevisiae*	Cassava	SSF pH—5.5, T—30°C	90	0.5	0.45	Roble et al., (2002)
Glucoamylase + *S. cerevisiae*	Raw starch	Fed-batch fermentation pH—5.0, T—30°C	20–30	0.60	0.35	Konda et al. [2]
Mutant *A. niger* + *S. cerevisiae*	Raw starch	SSF pH—5.5, T—35°C, S—150 gL^{-1}	50	1.42	0.33	Rajoka et al [46]
Co-immobilized glucoamylase + *Z. mobilis*	Sago starch	SSF pH—4.9, T—32°C, S—150 gL^{-1}	55.3	0.98	0.36	Bantaru et al. [38]

Table 5.
Production of ethanol from starch sources using SSF process.

5–6 and a controlled pH environment is always preferred for maximum ethanol production. Very low pH is also not preferred as the rate of growth was very less. Hence an optimum pH of 5–6 must be maintained in the medium. In addition to that the medium should have optimum mineral concentration which provides more biomass and in turn more ethanol yield **Table 5**.

2. Conclusion

SSF process is found to be a promising technology for industrial ethanol production from cheaper substrates like cellulose and starchy substrates. The success of the SSF process depends mainly on pre-treatment step using suitable enzymes for cellulose hydrolysis and starch hydrolysis. Starchy substrates can be easily liquefied using low cost commercially available alpha amylase enzymes at optimum conditions and can be utilized in SSF process. But the pre-treatment steps in cellulosic materials are more challenging because of the presence of lignin and hemicelluloses. A suitable pre-treatment steps to separate cellulose from naturally occurring lignin and hemicelluloses substrates involves energy intensive process. Furthermore, presence of inhibitory end products from hemicelluloses may hinder the SSF process. SSF process using starch substrates are more promising and also commercial industrial production is feasible in many countries. The advantages of the process are reduction in investment by having single fermenter for both saccharification and fermentation. The feedback inhibition of sugars is greatly reduced. The fermentation time is very less in SSF process.

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