**PhD Thesis**

# Ethanol production from biomass using consolidated continuous solid-state fermentation system

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## Chapter 1 General introduction

### 1.1 Sustainable energy resources

One of the defining challenges of the 21st century will be shifting our energy supply from fossil to renewable energy. About 90% of our current energy comes from three main fossil fuels; petroleum, coal and natural gas (Sivarkumar et al., 2010). Among these fossil fuels, petroleum is the most widely consumed (35% of the world energy consumption) followed by coal (25%) and natural gas (21%) being used in industrial, commercial, household and transportation sectors (Sriroth et al., 2010). It is convenient to use fossil fuels for energy requirement, but there is a limited supply of fossil fuels on the earth and we are using them much more rapidly than they are being created. The shortage of fossil fuels in the not so distant future could affect the activities of all humankind and impede economic development. The remaining time for utilization of the global energy resources were estimated for petroleum, natural gas, coal, and uranium to be 40.5, 63.3, 147 and 85 years, respectively (Fig. 1.1). Even if there is an unlimited supply of fossil fuels, the burning of fossil fuels release carbon dioxide into the atmosphere. Climate scientists believe it is contributing to a greenhouse effect, resulting in long-term increase in global surface temperature (Searchinger et al., 2008). By this consideration, the world's dependency on fossil fuels must be reduced.

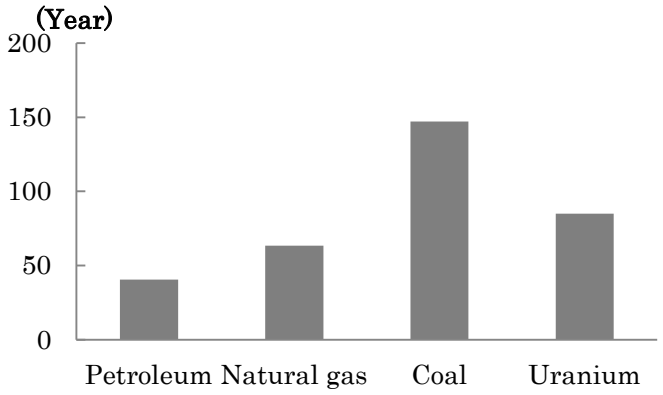


**Fig.1.1** Remaining exploitable global energy resources in terms of years.

(Source: http://www.enecho.meti.go.jp/english/toprunnner/8.9english2008.pdf)

In the last 20-30 years, the utilization of renewable energy has been an important political topic to help us develop energy independence and security. In 2009, the share of renewable energy in the world energy primary mix was 13%, which consists of 77% of bioenergy, 15% of hydropower and 8% of other renewable energy (Fig. 1.2; Bauen et al., 2009) and the contribution of renewable energy tends to increase every year (Beijing international renewable energy conference, 2005). Renewable energy comes from energy sources that are continually replenished by nature (the sun, wind, water, the Earth's heat, and plants) and turn these energy into usable forms, most often electricity, but also heat, chemicals or mechanical power (The National Renewable Energy Laboratory (NREL), 2001). Typical renewable energy sources include the following:

*Bioenergy*

Bioenergy is a common term pertaining to energy related to the exploitation of biomass. Biomass exists in many different forms with different qualities. The most

common area of application for bioenergy is the production of heat. It is also used to produce electric power, liquid biofuel, biogas and hydrogen from biomass.

*Hydropower*

Hydropower generation is the way to convert the energy in flowing water into electricity. The most common form of hydropower uses a dam on a river to retain a large reservoir of water.

*Geothermal energy*

The geothermal energy flows outward from the Earth's core, heating the surrounding area, which can form underground reservoirs of hot water and steam. These reservoirs can be trapped for a variety of uses, such as electricity generation or industrial process heating.

*Solar energy*

Solar technologies directly tap the infinite power of the sun and use that energy to produce heat and electricity.

*Wind energy*

Wind energy has been used for thousands of years for milling grain, pumping water and driving other mechanical devices. This wind power is commonly used in many countries including Germany, Denmark, Spain and United States (Electrical and Mechanical Services Department (EMSD), Hong Kong, 2010).

*Ocean energy*

The ocean can produce two types of energy: thermal energy from the sun's heat and mechanical energy from the tides and waves. Ocean thermal energy can be used for electricity generation from the warm surface water. For the ocean mechanical energy, the electricity conversion of both tidal and wave energy usually involve mechanical devices. Most of the research and development in ocean energy is being done in Europe.

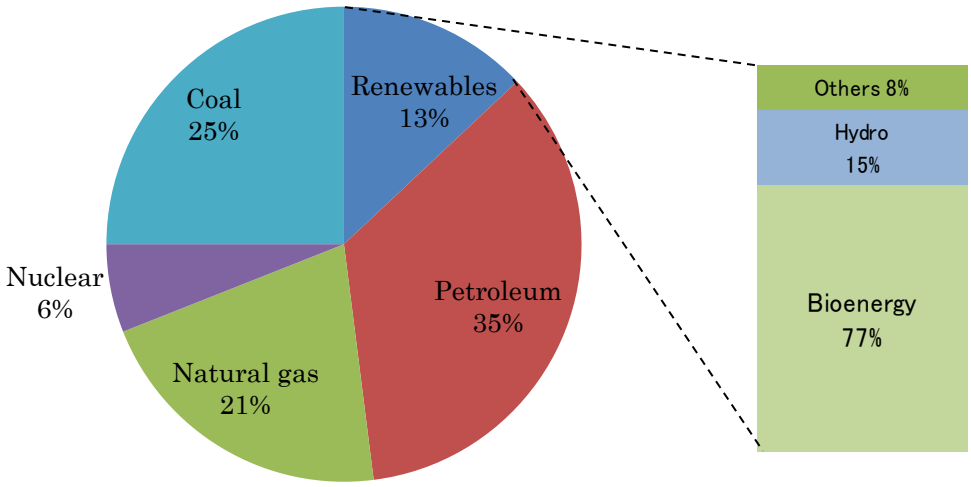


**Fig. 1.2** Share of bioenergy in the world energy primary mix, 2006–2007 (Bauen et al., 2009)

## 1.2 Bioenergy

Among all renewable energies, bioenergy has attracted much interest from a worldwide political point of view. Bioenergy is the fourth largest energy source followed by coal, petroleum and natural gas and can be used to produce different forms (Fig. 1.2).

Typical bioenergies include:

*Biofuel*

Biomass can be converted into liquid fuels, called biofuels. Because biofuels are easy to transport and process high energy density, they are favored to fuel vehicles and sometimes stationary power generation. The most common biofuel is bioethanol and biodiesel. Bioethanol is an alcohol made from the fermentation of biomass with high carbohydrate content. The current largest source of bioethanol is corn which is going to change to lignocellulosic material instead in the future. Ethanol can be used as a gasoline additive to reduce carbon dioxide emission. Biodiesel, which is another major biofuel, can be made from vegetable and animal fats. In 2008, biofuels provide about 0.6% of the global energy consumption (Renewables 2010 global status report, 2010). To increase the available supply of biofuels, researchers are testing crop residues such as cornstalks, wood chips, food waste, grass and even trash as potential biofuel sources.

*Biopower*

Some utilities and power generating companies with coal power plants have found that replacing some coal with biomass is a low-cost option to reduce undesirable emissions. Biomass has less sulfur than coal and thus, less sulfur dioxide, which contributes to acid rain, is released into the air. Biomass can also be heated in the absence of oxygen to chemically convert it into a type of fuel oil, called "pyrolysis" oil. Pyrolysis oil can be used for power generation and as a feedstock for fuels and chemical production.

Since bioenergy has many advantages described above, researchers all over the world have developed technologies for efficient production of bioenergy. In this study, bioethanol, being a representative of bioenergy, is the main focus of research since it is by far the most widely used biofuel for the transportation sector. Bioethanol can be blended with gasoline or used as neat alcohol in dedicated engine, taking advantage of its high octane number and high heat of vaporization.

## 1.3 Bioethanol

Bioethanol has a long history as an alternative transportation fuel. It has been used in Germany and France as early as 1894 by the incipient industry of internal combustion engines (ICEs) (Dermirbas and Karslioglu, 2007). Brazil has utilized bioethanol as a fuel since 1925. By that time, the production of bioethanol was 70 times higher than that of petrol (Lang et al., 2009). The use of bioethanol for fuel was widespread in Europe and the United States until the early 1900s. Because it became more expensive to produce bioethanol than petroleum-based fuel, especially after World War II, the potential of bioethanol was largely ignored until the oil crisis of the 1970s (Demirbas et al., 2009). Since the 1980s, there has been an increased interest in the use of bioethanol as an alternative transportation fuel.

The development of the bioethanol production process is needed to replace petroleum usage for transportation, which is responsible for 60% of the world petroleum consumption (Key world energy statistics, 2008). It accounts for more than 23% of global carbon dioxide ($CO_2$) emissions (Reducing Transport Greenhouse Gas Emission: Trends & Data 2010, 2010). Moreover, the number of cars is projected to increase to 1.3 billion

by 2030 and to over 2 billion vehicles by 2050 (Mobility 2030: Meeting the challenges to sustainability, 2004), which will affect the stability of ecosystems and global climate as well as global oil reserves.

To ensure that ‘‘good” bioethanol is produced, the following demands must be met (Borjesson, 2009):

(1) Bioethanol plants should use biomass and not fossil fuels

(2) Cultivation of feedstock crops should be avoided on land rich in carbon

(3) Carbon compound such as lignin should be efficiently used as energy source

(4) Other element, such as nitrogen, potassium and phosphorus, should be recycled to the agricultural land where biomass is harvested

Using bioethanol as transportation fuel can also help in reducing $CO_2$ buildup in two important ways: by displacing the use of fossil fuel, and by recycling the $CO_2$ that is released when it is combusted as fuel. By using bioethanol instead of fossil fuel, the emissions resulting from fossil fuel use are avoided, and the $CO_2$ content of fossil fuels is allowed to remain in storage. Currently, bioethanol is one of the main biofuel used in the world and its use is becoming increasingly widespread. The worldwide prospects for expansion of the production of ethanol are shown in Fig. 1.3. USA and Brazil are the main producers of bioethanol in the world. In 2009, those two countries produced over six billion gallons of bioethanol which are several folds higher than by other countries such as Europe, China and Canada.

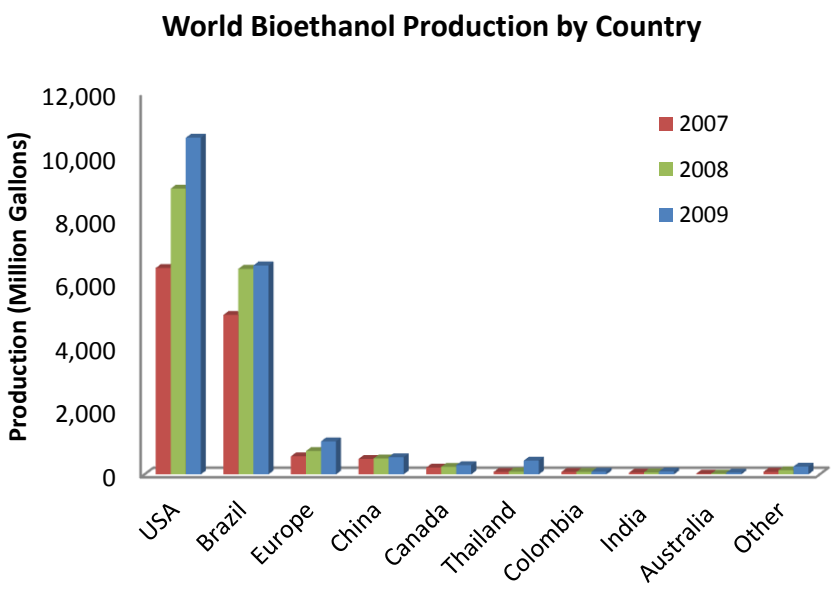


**Fig. 1.3** World bioethanol productions by country during 2007 and 2009 (Source: www.afdc.energy.gov/afdc/data/)

Bioethanol is also used to produce ethylene monomer. This monomer is in turn used in the production of a number of commercial grades of bio-polyethylene at a very competitive cost using standard polymerization technologies. These polymers can be then transformed into various products, with the same processing equipment used now in plastics industry and with the same performance characteristics that customers expect from polyethylene.

About 95% of bioethanol produced in the world is from agricultural products (Rossillo-Celle and Walter, 2006). Bioethanol production from sugar crops such as sugarcane and sugar beet account for about 40% of the total bioethanol produced and nearly 60% are from starch crops (Mussatto et al., 2010). Generally, the carbohydrate sources for bioethanol production can be classified into three main groups: 1) Simple

sugars such as sugarcane (Leite et al., 2009; Macedo et al., 2008), sugar beet (Icos et al., 2009; Ogbonna et al., 2001), sorghum (Yu et al., 2008; Prasad et al., 2007a; Mamma et al., 1995), whey (Dragone et al., 2009; Silveira et al., 2005; Gnansounou et al., 2005; Domningue et al., 2001), and molasses (Roukas, 1996), 2) Starchy carbohydrates such as corn (Persson et al., 2009; Gaspar et al., 2007), wheat (Nigam, 2001), and cassava (Kosugi et al., 2009; Rattanachomsri et al., 2009; Amutha and Gunasekaran, 2001), and, 3) Lignocellulose such as woody materials (Ballesteros et al., 2004), straws (Silva et al., 2010; Huang et al., 2009), agricultural wastes (Lin and Tanaka, 2006) and crop residues (Hahn-Hagerdal et al., 2006).

In the short-term, the production of bioethanol as a vehicle fuel is almost entirely dependent on sugars and starch from existing food crop (Smith, 2008). The drawback in producing bioethanol from sugar or starch is that the biomass tends to become more expensive and in high demand for other applications as well (Enguidanos et al., 2002). Lignocellulosic biomass is envisaged to provide a significant portion of the raw materials for bioethanol production in the medium and long-term due to its low cost and high availability. Even though there is an extra processing step in the hydrolysis of lignocellulose to glucose, lignocellulose is highly abundant and diverse in terms of availability. Besides, it does not require intensive agricultural practices as compared to bioethanol produced from food crops where the productivity of crops is maximized to cater to the growing demand of biomass for biofuels. For this reason, the cost of biomass is lower for lignocellulose compared to food crops, in which up to 70% of the total cost for bioethanol from food crop. The production cost of bioethanol is more competitive

compared to fossil fuels such as gasoline or diesel when licnocellulosic materials are used as feedstock (Tan et al., 2008).

## 1.4 Bioethanol production process

In order to expand the use of bioethanol in the world, it is essential to develop the bioethanol production processes. The conventional production system can be classified into three types according to biomass. As shown in Fig. 1.4, starch-based biomass requires a saccharification process prior to fermentation, while simple sugar from sugar cane and sugar beet can be converted directly to ethanol. In the case of lignocelluloses, a delignification process that removes lignin from biomass is required in addition to the saccharification process.

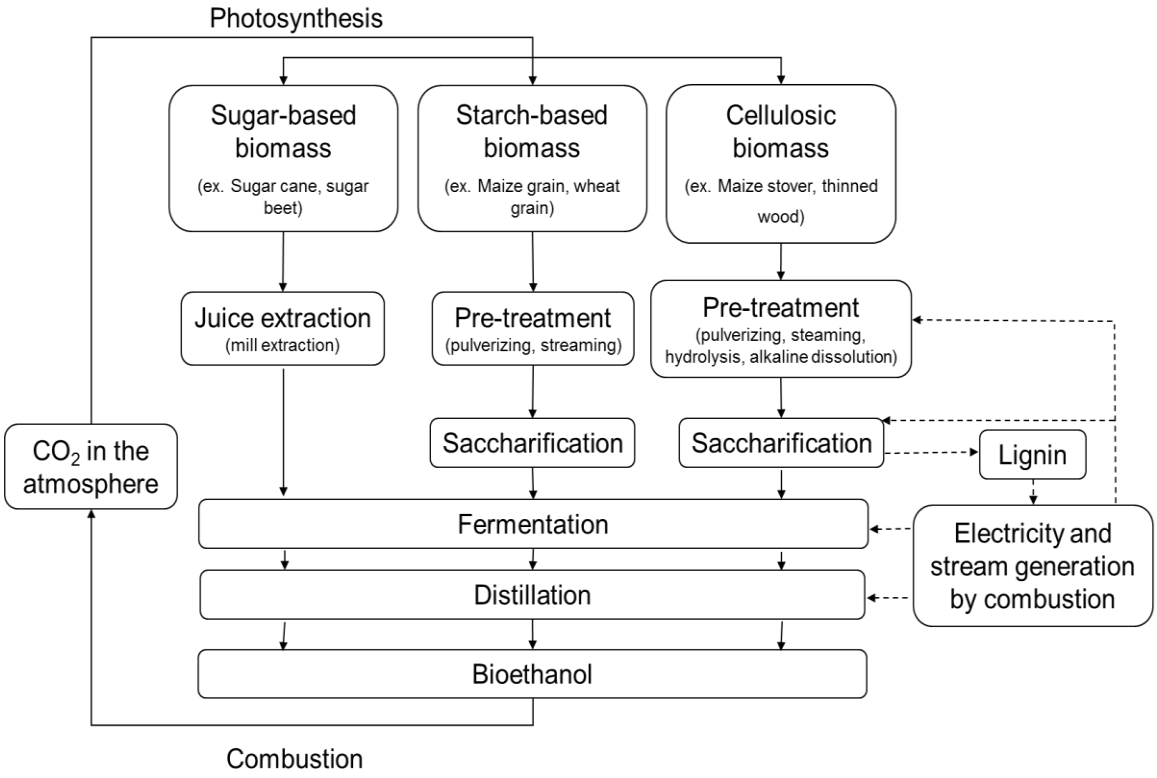


**Fig. 1.4** Production processes of bioethanol from three types of raw materials (Hattori and Morita, 2010)

*Raw material procurement*

Bioethanol is produced from various raw materials. The materials are characteristic of the region, being sugarcane and molasses in Central and South America, cassava in Asia and corn in North America. In Europe, the main raw materials are wheat, rye, barley, wheat bran/middlings or sugar beet juice and sugar beet molasses (Schubert, 2006). Due to the increasing demand for bioethanol every year, various methods come out to increase the production of biomass, e.g. mixtures of various crop species which has been suggested to improve biomass yield per area. Engineered crops that are high in

biomass, low in lignin, and crops that can produce their own cellulases are being tested for their potential to serve as improved lignocellulosic materials (Schubert, 2006).

Currently, bioethanol from biomass is increasingly being produced from biodegradable municipal solid wastes (BMSW) rather than from food crops because the latter competes for land and water with food crops that are already in high demand. The use of food crops such as corn and sugarcane to produce biofuels is increasingly being discouraged due to the current worldwide rise in food prices. In order to minimize food-feed-fuel conflicts, it is necessary to integrate all kinds of bio-waste into a biomass economy (Mahro and Timm, 2007). BMSW, which typically include paper, kitchen waste, garden waste, textiles, fines, and miscellaneous, have been investigated for their potential to produce bioethanol (Li et al., 2007), especially in Japan where the amount of natural biomasses are insufficient. Development of technologies for efficient utilization of BMSW would be beneficial to not only Japan but also many countries that are witnessing a tremendous increase in BMSW owing to rapid population growth and economic development (Banerjee, 2009).

*Pre-treatment (Delignification)*

A pre-treatment step is carried out to release the cellulose portion (and subsequently glucose) from tightly woven lignocellulosic structure. Lignocellulose is composed of cellulose, hemicellulose and lignin which tightly bound to the carbohydrate polymer. Physical (milling and grinding), physico-chemical (stream explosion/

autohydrolysis, hydrothermolysis and wet oxidation), chemical (acid, alkaline, oxidizing agents and organic solvents), and/or biological processes (white or brown rod fungi) have been used for the pre-treatment of lignocellulosic materials in order to make the pre-treated biomass more amenable to subsequent cellulose hydrolysis (Banerjee, 2009; Perez et al., 2002; Balat, 2011).

*Hydrolysis (Saccharification)*

The starchy or lignocellulose polymers need to be converted to simple sugars before fermentation, through a process called hydrolysis (Taherzadeh and Karimi, 2007). Various methods for the hydrolysis of raw materials have recently been described. The most commonly applied methods can be classified into two groups: chemical hydrolysis (concentrated sulfuric acid) and enzymatic hydrolysis (Balat, 2011).

The process of chemical hydrolysis involves exposure of materials to a chemical (concentrated acid) for a period of time at a specific temperature, and result in sugar monomers (Taherzadeh and Karimi, 2007). This method generates high concentrations of inhibitors which affect the fermentative activity of microorganisms such as furans, organic acids and phenolics (Klinke et al., 2004). Moreover, it causes environmental problems and the high cost of acid consumption and recovery which are major barriers to economic success (Yu et al., 2008).

Enzymatic hydrolysis of lignocellulose generates little inhibitors because the enzymes are very specific for carbohydrates. For the hydrolysis of lignocellulose, cellulases are used. Commercial cellulases are a mixture of at least three different

enzymes: (1) endoglucanase (EG, endo-1, 4-$_D$-glucanohydrolase, EC 3.2.1.4.) which attacks amorphous regions of the cellulose fiber, creating free chain; (2) exoglucanase or cellobiohydrolase (CBHI, CBHII, 1, 4-β-$_D$-glucan cellobiohydrolase, EC 3.2.1.91) which degrades the crystal region of cellulose by removing cellobiose units from free chains-ends; and (3) β–Glucosidase (EC 3.2.1.21) which hydrolyzes cellobiose and cello-oligosaccharide to produce glucose (San and Chen, 2003).

Lignocellulose also contains hemicellulose which is a complex of heteropolymers consisting of pentoses (xylose and arabinose), hexoses (mannose, glucose and galactose), and sugar acids. Because xylose is the second most abundant sugar found in hardwood and agricultural residues, the xylan hydrolysis process is described. Enzymatic hydrolysis of xylan involves a multiple system, including endoxylanase, exoxylanase, β-xylosidase, α-arabinofuranosidase, α-glucoronisidase, acetyl xylan esterase, and ferulic acid esterase (Saha, 2004).

While enzymatic hydrolysis is environment friendly, it requires a longer time for digestion compared with chemical hydrolysis. Although it is possible to reduce the time for digestion by increasing the amount of enzyme, it results in an increase in the cost for the enzymes. Moreover, in the case that substrate is saturated with enzyme, the time for digestion cannot be reduced even when one puts an excess amount of enzyme. Thus, many researchers try to increase the specific activity of the enzyme and to reduce the cost for production of the enzymes.

*Fermentation*

*Saccharomyces cerevisiae* is the most commonly used microbial species for industrial ethanol production from sugar- and starch-based raw materials. It produces ethanol at high yields, tolerates a wide spectrum of inhibitors and elevated osmotic pressure (Hahn-Hagerdal et al., 2007). *Zymomonas mobilis* also has been projected as the future ethanologen due to its high ethanol tolerance (up to 14% v/v), energy efficiency, high ethanol yield (up to 97% of theoretical), and high ethanol productivity (1.15 g $g^{-1}$ $h^{-1}$) (Herrero,1983).

Generally, the native *S. cerevisiae* is capable of fermenting only hexoses, and cannot utilize pentoses like xylose, which is the main component of the hemicellulosic fraction and can contribute to as much as 30% of the total biomass. Similarly, *Z. mobilis* can only utilize glucose, fructose, and sucrose. Expanding the substrate range of ethanol producer will greatly contribute to the economic feasibility of bioethanol production from renewable biomass. Therefore, the search for pentose utilizing strains is necessary. Two groups of microorganisms, such as enteric bacteria and some yeasts, are able to ferment pentoses but with low ethanol yields. Although some yeast species such as *Pachysolen tannophilus*, *Candida shehatae*, and *Pichia stipitis* are capable of fermenting pentose, they are not tolerant to high concentrations of ethanol (≥40 g/l) (du Preez et al., 1989; Skoog et al., 1992). Furthermore, they are sensitive to the inhibitors and low pH (du Preez et al., 1986). Therefore, new microorganisms that satisfy the requirements such as tolerance to ethanol and ability of pentose fermentation, need to be developed. Metabolic engineering, by virtue of the recent molecular biology tools, has generated recombinant organisms displaying attractive features for the bioconversion of lignocellulose to ethanol.

Three most common microbial species that have been improved by metabolic engineering are *S. cerevisiae*, *Z. mobilis* and *Escherichia coli.* To enable *S. cerevisiae* to ferment xylose, three main strategies have been approached: the insertion of bacterial xylose isomerase gene, the insertion of pentose utilization genes from *P. stipitis*; and the improvement of xylulose consumption. For *Z. mobilis* to expand its substrate spectrum, strategies such as insertion of the genes for xylose and arabinose utilization have been applied (Zaldivar+ et al., 2007). Since *E. coli* naturally possesses a broad substrate-utilization range, and produce ethanol, lactate, acetate and formate equally, the main strategy to increase ethanol production and make it suitable for processes with lignocellulose as raw material was to redirect the carbon flux towards ethanol production (Zaldivar et al., 2007; Chu and Lee, 2007). So far, however, the production of ethanol from lignocellulosic materials using these strains has not reached a level sufficient for commercial application. For this reason, *Zymobacter palmae*, given its broad range of carbohydrate substrates and its ability to efficiently produce ethanol, have been considered. However, this organism could not ferment cellulose or its degradation product, cellooligosaccharides and cellobiose, directly. The strategy to breed a strain of *Z. palmae* that can produce ethanol from cellulosic materials has been studied (Okamoto et al., 1993; Yanase et al., 2007).

*Ethanol recovery*

Since ethanol is more volatile than water, recovery by distillation is often the technology of choice. It is desirable to increase the final ethanol concentration of the

fermentation to as high as possible because the energy and capital cost for distillation to obtain a unit of ethanol is almost proportional to the volume of the fermentation broth. However, fermentation slows down under high concentrations of ethanol, resulting in an increase in capital costs for fermentation. An alternative technique that recovers ethanol from fermentation broth during fermentation has been developed to reduce ethanol inhibition. This idea leads to a semi-continuous fermentation that maintains a high fermentative activity of yeast. For the online recovery of ethanol, liquid-liquid extraction (Ishizaki et al., 1999), pervaporation (Liu et al., 2005), membrane distillation (Banat and Al-Shannag, 2000) and gas stripping (Ezeji et al., 2004; Qureshi and Blaschek, 2001) have been reported.

*Wastes and Waste water treatment*

In commercial ethanol production, 10-20 liters of stillage are created for every liter of ethanol produced. This results in a huge volume of wastewater that must be treated. When lignocellulose is used for bioethanol production, the waste produced contains lignin with a high energy content that can be used as fuel. For example, in paper pulp industries, alkaline-solubilized lignin, called black liquor, is utilized efficiently as fuel. It is also possible to use lignin and solid wastes as fuel in the production of ethanol from biomass. In addition, since the wastes also contain elements essential for the growth of plants, such as nitrogen, phosphorus, potassium, magnesium and iron, all wastes from bioethanol processes should be restored to agricultural land as fertilizers to maximize the profit of bioethanol production.

## 1.5 Process integration

In order to reduce the capital costs in bioethanol production, process integration has been established. In the process where hydrolysis and fermentation are carried out separately, cellulose is first hydrolyzed to glucose and then glucose is fermented to ethanol. The advantage of conducting these steps separately is that both hydrolysis and fermentation can be operated at optimum conditions; while the disadvantage is that cellulolytic enzymes are inhibited by the end-product, glucose and cellobiose, resulting in a decrease in the rate of hydrolysis (Feldman et al., 1991). Avoiding product inhibition was the rationale for the development of simultaneous saccharification and fermentation system (Takagi et al., 1997; Kumar et al., 2009). In the integrated process, hydrolysis and fermentation occur simultaneously in the same vessel, and the end-product inhibition of the enzymes can be prevented because the fermenting organism immediately consumes the released sugars. Subsequently, the process leads to the development of an ethanol producer such as arming yeast, a cell surface engineered of yeast displaying saccharifying enzymes on cell surface. The production of saccharifying enzymes, hydrolysis of carbohydrate and sugar fermentation are combined into a single step by utilize arming yeast (Shibasaki et al., 2009; Balat, 2011). This process is attractive in that it reduces the number of reactors, simplifies the operations, and reduces the cost for chemicals (Silverstein et al., 2004). In simultaneous saccharification and fermentation, the influence of the inhibitor in the hydrolysate on the activity of the saccharifying enzymes was reported to be diminished because the fermenting microorganisms were able to detoxify the inhibitor (Tengborg et al., 2001). The integrated process can increase the overall

ethanol productivity, the ethanol concentration and the final ethanol yield (Soderstrom et al., 2005; Wright et al., 1988), although, simultaneous saccharification and fermentation requires compatible conditions, pH and temperature, both for fermentation and saccharification steps (Ballesteros et al., 2004).

Researchers have reported a simplified saccharification and fermentation process by the development of *Saccharomyces* strains capable of displaying saccharifying enzymes on their cell surface (Khaw et al., 2006). This novel yeast strain was able to produce ethanol directly from the carbohydrate source, such as raw corn starch or pre-treated cellulose, at specified conditions without the addition of saccharifying enzymes. Eventually, the complex steps involved in bioethanol production would be reduced.

As technologies for the saccharification and fermentation of biomass approach commercial viability, advantages in technologies for the product recovery are required. Recently, researchers have been interested in integrating the product recovery process into the saccharification and fermentation process in order to reduce the production cost. Although, the integration of product recovery process have been established in the conventional production system (Hashi et al., 2010), the result was not satisfactory. Hence, further developments of process integration will be necessary.

## 1.6 Production of bioethanol in Japan

The transportation sector of Japan is almost 100% dependent on imported oil. In the national energy strategy released in May 2006, the Japanese government articulated

to decrease the dependency on foreign oil to 80% by 2030. Biofuels are considered as an important renewable energy resource to achieve this goal, and bioethanol is one of the most promising biofuels. Although crops such as rice, wheat and sugar beets are cultivated in Japan, these have been used as food. As summarized in Table 1.2, the biomasses that are available for ethanol production are cellulosic materials such as rice straw, logging residue, construction waste timbers etc. (Iijima, 2010). However, the cost for bioethanol production in Japan would be much higher than those in Brazil and the United States.

**Table 1.2** Endowment potential of domestic unutilized biomass (Matsumoto et al., 2009)

| | | Endowment (generated amount) (million tons/year) | Intensity (Place of generation) | Rate of utilization (%) | Utilization potential (million tons/year) | Energy potential (PJ/year:HHV)[a] |
|---|---|---|---|---|---|---|
| Plant | Rice straw, rice husk | 14 | Agriculture land, rice processing facility | 30 | 9.8 (70%) | 147 |
| Woody | Logging residue | 34 | Forest land | 2 | 3.3 (98%) | 50 |
| Woody | Saw mill residue | 4.3 | Factory | 95 | 0.2 (5%) | 3 |
| Woody | Construction waste timbers | 4.7 | Factory | 70 | 1.4 (30%) | 21 |
| Others | Waste paper | 30.63 | Urban area | 91 | 2.79 (9%) | 42 |

Compiled by the Biofuel Technology Innovation Conference based on MAFF, ‘‘Handout at Biomass Nippon Strategy Promotion Conference”, February 2007, for endowment and Saka et al. IPC ‘‘Biomass/Energy/Environment”, July 2001, for waste paper. Energy potentials were converted by the authors.

[a]Conversion rate of 15.0 MJ/kg of biomass for higher heating value (HHV) was used based on the 2007 guideline on calorific value conversion by the Agency for Natural Resources and Energy of Japan.

In Brazil and the Unites States, ethanol has been produced in large scale from sugarcane and corn, respectively. In these countries, large-scale facilities can be operated because they have large tracts of flat agricultural land. In addition, a delignification process is not required and efficient processes for saccharification of starch have already been established. Therefore, it is possible in these countries to produce ethanol at low costs (Fig. 1.5A).

In Japan, in contrast, the bioethanol production processes from rice straw and wood materials are more complicated than those from simple sugar or starchy materials. As shown in Fig. 1.4, the use of cellulosic biomass requires delignification prior to saccharification. In addition, the main carbohydrate of the biomass is cellulose that is not easy to be saccharified compared with starch, which in turn increases the cost for the enzymes and equipment. Furthermore, this type of biomass is bulky (the density of rice straw pressed by roll baler is only 100 $kg/m^3$) and scattered in low density (the yield of rice straw per unit of land is lower than of sugarcane) (Kim and Dale, 2004), making its collection and transportation costly. Therefore, both the production and transportation costs for ethanol production from rice straw are higher than those from sugarcane and corn in Brazil and the United States. Considering these conditions, the total production

cost of bioethanol in Japan is predicted to be a lot higher than in those countries (Fig. 1.5).

With the aim to reduce the total production cost in Japan where biomasses are distributed at low densities, an alternative efficient system that produces ethanol from local raw materials and to consume the resultant ethanol in the region where it is produced, namely "locally-produced-and-locally-consumed", was developed. In conventional ethanol production system, however, the smaller scale facilities result to higher capital costs and energy consumption per unit of bioethanol. Thus, a new geometrically-distributed production system that produces ethanol at a reasonable cost with a low energy consumption even in small scale need to be developed (Fig. 1.5C).

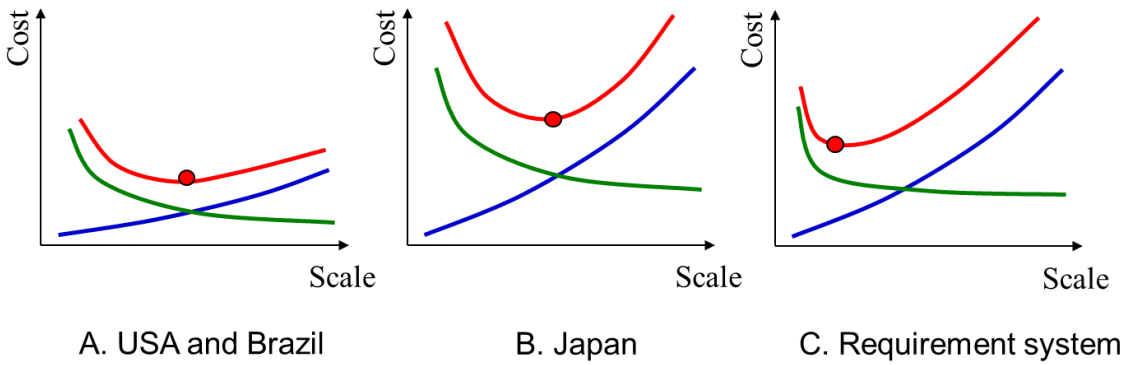


**Fig. 1.5** Cost for bioethanol production. Green, production cost; blue, transportation; red, total cost

## 1.7 Possibility of consolidated continuous solid state fermentation (CCSSF) and scope in this thesis

For practical production of bioethanol, the total energy required for the entire process including the pre- and post-fermentation must be considered, because transportation of biomass and treatment of the waste, require much energy (Luo et al., 2009). In addition, considering agricultural sustainability, nitrogen, phosphorus, potassium and other elements in the waste water and the solid wastes must be recycled into the soil where the biomass is harvested. Otherwise, agricultural land will require further fertilization to compensate for these elements, resulting in an increase in the cost and energy input for fertilizer.

In conventional ethanol production system, about 80-90% of the fermentation mixture is water. After recovery of ethanol, most of the water becomes waste water that need to be treated. In addition, the treatment of solid residues from the process is costly because they contain high moisture. The combination of solid-state fermentation with 'simultaneous saccharification and fermentation' would be the best system to reduce these costs. Solid-state fermentation is defined as the fermentation of microorganisms on moist solid support, either in inert carriers or insoluble substrates that can be used as carbon and energy sources (Mohanty et al., 2009). In the process of bread dough

preparation where the water content is about 50%, it is known that yeast is quite active and produce ethanol and carbon dioxide (Czuchajowska et al., 1989). The application of solid-state fermentation on bioethanol production would reduce the amount of water that is used for fermentation. As a result, the amount of waste water after fermentation will become less and the moisture content of the solid residues low, making it easier to treat. However, fermentation under low moistures can lead to a rapid increase in ethanol content in fermentation mixture, which inhibits the activity of yeast cells. This contradiction would be solved by the continuous recovery of ethanol during fermentation.

The purpose of this thesis is to establish an efficient ethanol production system that consolidates saccharification, fermentation and recovery of ethanol. This thesis will present the establishment of the system called “Consolidated Continuous Solid-State Fermentation (CCSSF)”. The CCSSF system can be operated even in small scale at a low capital cost cost, with little waste water. The details of the system will be discussed in the next chapter.

The aim of this study is to develop a CCSSF system that enables a geometrically-distributed ethanol production at a reasonable cost and energy-input. This

chapter provided the background information, which describes the concept of this work. Chapter 2 presents the development of CCSSF system, while Chapter 3 will present the initial conditions of CCSSF for preventing contamination of bacteria which leads to reduction of ethanol yield. In Chapter 4, the cost analysis of the CCSSF system will be shown. Based on the sensitivity analysis, important parameters that lead to further reduction of the cost and input energy will be discussed. In the last chapter, the general conclusions of CCSSF and further prospective of the system development will be discussed.

# Chapter 2 Ethanol production from biomass by repetitive solid-state fed-batch fermentation with continuous recovery of ethanol

## 2.1 Introduction

In recent years, the efforts in research and development have been directed toward a reducing the input energy and cost for production of bioethanol as the most promising biofuel.

The processes for ethanol production from biomass consist of delignification, saccharification, fermentation, recovery and purification of ethanol. Many new technologies have been developed, for example, biological delignification (Perez et al. 2002), simultaneous saccharification and fermentation (Kumar et al. 2009), surface display of saccharifying enzymes on yeast cells (Fujita et al. 2002; Shigechi et al. 2004), and membrane separation of ethanol (Nakayama et al. 2008). For practical production, the total energy balance throughout the entire process including the pre- and post-fermentation must be considered, because transportation of biomass and waste water treatment, respectively, require much energy (Luo et al. 2009). In addition, considering agricultural sustainability, nitrogen, phosphorus, potassium and other elements present in waste water and solid wastes must be recycled in the land where the biomass is

harvested. Most of the conventional ethanol production methods involve liquid fermentation and exhaustion of fermentation broth and residue, which require large amounts of energy and cost for treatment. In addition, because the water content of fermentation residues is high, a considerable amount of energy is required to dry them before incinerating or recycling them as fertilizers.

To reduce the amount of waste water in ethanol fermentation, solid-state fermentation is one of the preferable options. In the process of bread dough where the water content is about 50%, it is known that yeast is quite active and produce ethanol and carbon dioxide (Czuchajowska et al. 1989). The application of solid-state fermentation for ethanol production from biomass, however, requires the regulation of both sugar and ethanol contents in the fermentation mixture below suitable levels because a high osmotic pressure and a high ethanol content decrease the fermentative activity of yeast.

In this chapter, an alternative system was demonstrated to maintain high yeast activity and decrease the amount of waste water, the number of process steps and the energy input. Consolidated continuous solid-state fermentation (CCSSF) was developed by a combination of simultaneous saccharification and fermentation with continuous recovery of ethanol in solid-state fermentation.

## 2.2 Materials and Methods

### *Strain and Media*

A heat-tolerant yeast, *Saccharomyces cerevisiae* TJ14 (Benjaphokee et al., 2011), which was developed through a collaboration between Mahidol University in Thailand and Osaka University, was used throughout the experiment. TJ14 is a hybrid strain between a heat-tolerant strain HB8(R1)-3A (*MAT***a** *his3*Δ*1 leu2*Δ*0 ura3*Δ*0*) and an ethanol yeast TISTR5056 (Thailand Institute of Scientific and Technological Research) by spore-to-cell mating. HB8(R1)-3A is a derivative strain from a natural thermo-tolerant yeast isolate (C3723) in Thailand and thermo-sensitive laboratory yeast strain BY4742 (k*MAT***a** *his3*Δ*1 leu2*Δ*0 lys2*Δ*0 ura3*Δ*0*) (Brachmann et al. 1998). A synthetic medium (SD medium) containing 6.7 g $L^{-1}$ yeast nitrogen base without amino acids (Difco, Becton Dickinson, Sparks, MD, USA) supplemented with 0.12 g $L^{-1}$ palmitic acid, 0.08 g $L^{-1}$ $ZnSO_4 \cdot 7H_2O$ and 0.002 g $L^{-1}$ biotin was used.

The yeast was precultivated aerobically in 400 ml of SD medium at 37°C for 12 h with shaking at 200 rpm. The preculture medium was inoculated in a fermentor (model PC-5; Able, Tokyo, Japan) containing 1.6 L of SD medium. The culture was maintained at pH 5.0 by adding sodium hydroxide. The agitation speed and the air flow rate were set at 400 rpm and 1 vvm, respectively. After exhaustion of glucose, the

agitation speed was increased to 600 rpm and the SD medium containing 100 g $L^{-1}$ glucose and two fold concentrations of the supplements was fed using a pump. The flow rate of feed medium, $F$ (L $h^{-1}$), was determined every hour according to the following equation.

$$F = \frac{\mu L_0 I_0}{Y_{X/S} S_F} \exp(\mu t) \quad (2.1)$$

where the initial cell concentration ($I_0$), initial volume ($L_0$), and glucose concentration in feed medium ($S_F$) were set to be 3 g-dry-cell $L^{-1}$, 2 L, and 100 g $L^{-1}$, respectively. The cell yield ($Y_{x/s}$) was determined to be 0.4 g-dry-cell g-glucose$^{-1}$ in an independent experiment. The specific growth rate ($\mu$) was regulated to be 0.25 $h^{-1}$ during the fed batch culture. The fed-batch culture was carried out when the optical density at 660 nm, $OD_{660}$, reached 30. Here, the unity of $OD_{660}$ was estimated to be 0.25 g-dry-cell $L^{-1}$ in an independent experiment. The cells were harvested and washed once with 0.85% NaCl by centrifugation at 2,000×*g* for 10 min.

*CCSSF system*

A schematic diagram of the CCSSF system is shown in Fig. 2.1a. The system consisted of a rotating drum reactor, a Liebig condenser (3.5 cm in diameter × 18 cm in

length) cooled at −10ºC by a refrigerator (Eyela CCA-1111, Tokyo Rikakakai Co., Ltd., Tokyo, Japan), an air pump (Model APN-085LV-1, Iwaki Co., Ltd., Tokyo, Japan), a mass flow control barb (Model RK1200, Kofloc, Tokyo, Japan) and a humidifier containing 0.4 l of water to maintain the temperature at 47 °C.

A plastic cylinder (12 cm in inner diameter×15 cm in length, 1 cm thick) with two screwed disk-shaped lids (1 cm thick) on either side was used as a reactor (Fig. 2.1b). Into the center of each lid, a stainless pipe (8 mm in inner diameter×16 cm in length, 1 mm thick) was attached through a mechanical seal (Model Perfect seal P-100, Sansyo, Tokyo, Japan). The pipes were connected through a stainless bar [(11 cm, diameter of the central part was 8 mm but that of both ends (2 cm from the edge) was 6 mm]. To the bar, a Teflon plate (14.8×1.5 cm, 1 mm thick) was attached through the stainless arms to scrape the mixture from the inner wall of the reactor.

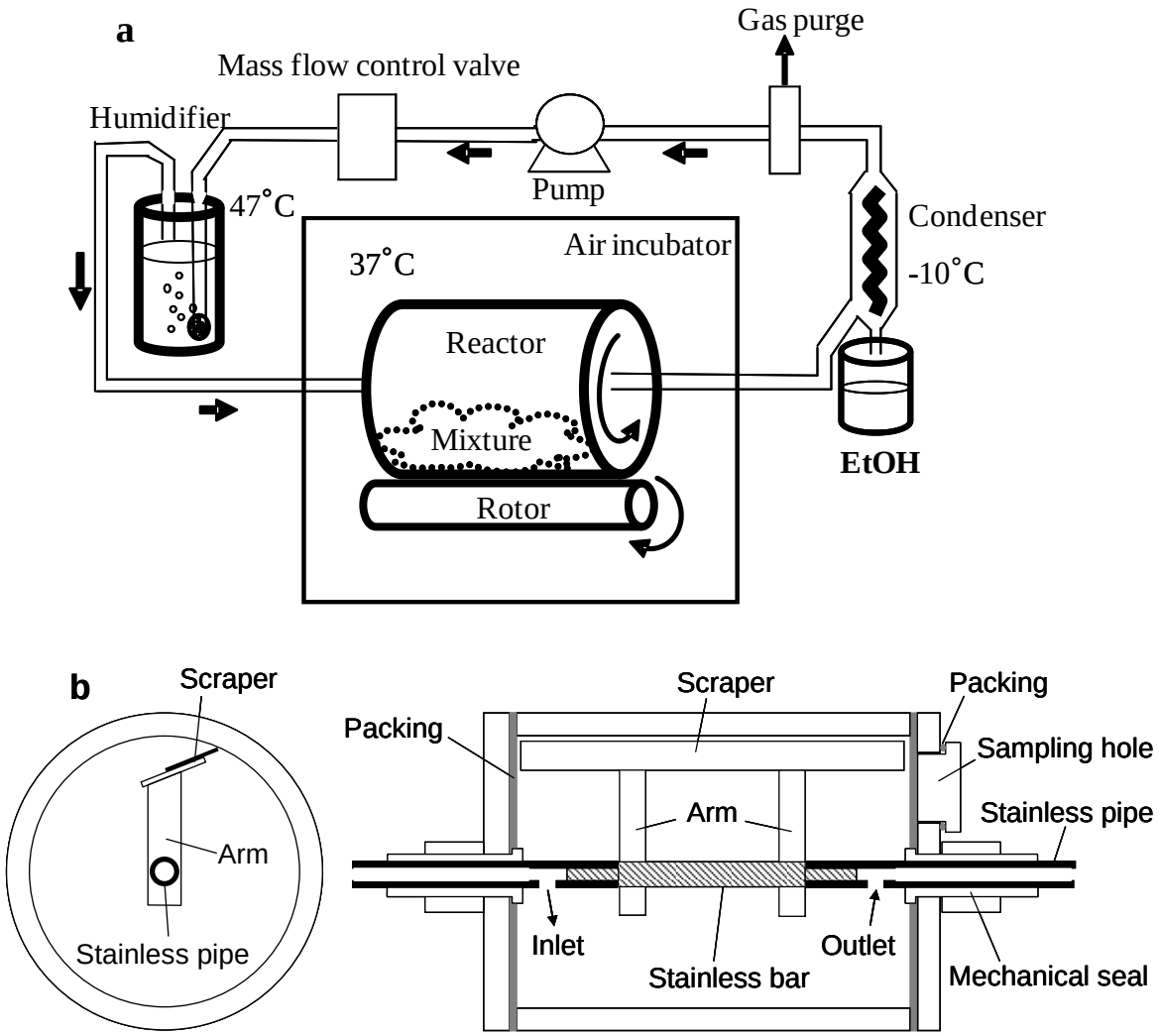


**Fig. 2.1** Schematic diagram of CCSSF system (a) and rotating drum reactor (b)

*Ethanol fermentation*

The fermentation mixture was composed of 30 g of yeast TJ14 (6 g-dry-cell), 65 ml of non-sterile YPS medium (10 g $L^{-1}$ yeast extract (Difco, Becton Dickinson, Sparks, MD, USA), 20 g $L^{-1}$ polypeptone (Nihonseiyaku, Osaka, Japan), 0.5 g $L^{-1}$

potassium disulfite (Wako Pure Chemical Industries, Ltd., Osaka, Japan)), 50 g of raw corn starch (Wako Pure Chemical Industries, Ltd., Osaka, Japan), 2000 units of glucoamylase (from *Aspergillus niger*, Wako, one unit produces 10 mg of glucose from starch for 30 min at pH 4.5, 40°C) and 2000 units of α-amylase (from *Bacillus subtilis*, Wako Pure Chemical Industries, Ltd., one unit produces 1 μmol of maltose from starch in 1 min at pH 6, 25°C). Here, potassium disulfite added to the YPS medium was to prevent contamination by anaerobic bacteria such as lactic acid bacteria. During the fermentation, the pH of the fermentation mixture was maintained at 5.0 by adding 28% ammonium water, and the reactor was rotated at 5 rpm to prevent the sedimentation of starch and cells. When the ethanol content in the fermentation mixture reached a set value (15, 40, 60 or 80 g kg-mixture$^{-1}$), the circulation of the headspace gas to the condenser and the humidifier was initiated and ethanol content was maintained within a range (10–20, 30–50, 50–70 or 75–85 g kg-mixture$^{-1}$, respectively) by changing the flow rate of the pump manually in accordance with the control protocol shown in Fig. 2.2. When flow rate is corresponds to the ethanol production rate obtained in experiment, $P_{recovered}$, against the ethanol production rate obtained by calculation, $P_{theoretical}$. The ethanol production rate obtained in experiment was calculated by

$$P_{recovered} = \frac{(\gamma_{i+1}K - \gamma_i K) + (Con_{i+1}V_{i+1} - Con_i V_i)}{T_{i+1} - T_i} 10^{-3} \quad (2.2)$$

When ethanol content in fermentation mixture, initial weight of fermentation mixture, the ethanol concentration in recovery system and sampling time are γ, *K*, *Con* and *T*, respectively.

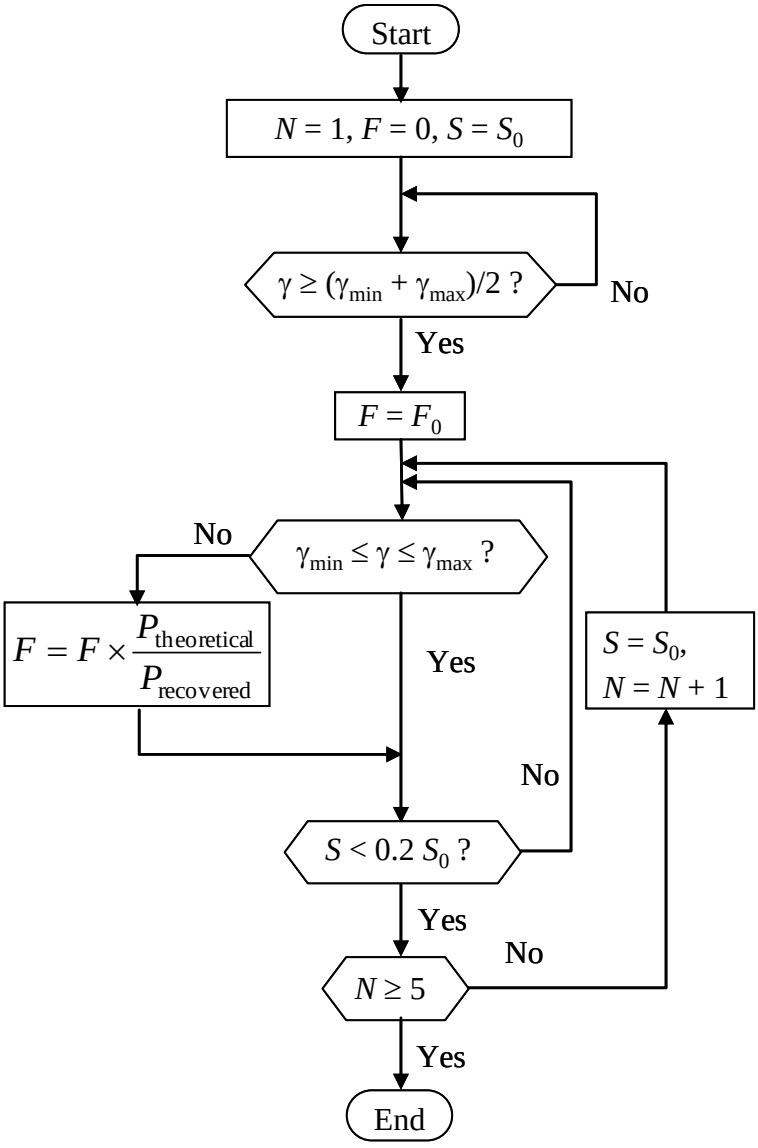


**Fig. 2.2** Algorithm for control of ethanol content in reactor

*Analyses*

Starch concentration was determined by a packed volume method. A sample (0.2 g) was mixed with 0.5% iodine solution (100 μl) and the mixture was centrifuged at 2000×*g* in 1 ml Hopkins centrifuge tube. The amount of starch in the sample was estimated using intact starch as a standard by assuming that the variation in packed volume by changes in the particle size of starch by digestion was negligible. Glucose concentration was determined using a glucose CII kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Glucoamylase and α-amylase activities were determined using a saccharifying ability assay kit and an α-amylase assay kit (Kikkoman Corp., Chiba, Japan), with 4-nitropheyl β-maltoside and 2-chloro-4-nitrophenyl $6^5$-azide-$6^5$-deoxy-β-maltopentaoside as the substrates, respectively. One unit of glucoamylase and α-amylase activities were defined as the amount of enzyme required to release 1 μmol of 2-chloro-4-nitrophenol and *p*-nitrophenol per minute, respectively. Ethanol concentration was determined using a gas chromatograph (model G-3000; Hitachi, Tokyo, Japan) equipped with a flame ionization detector with penthanol as the internal standard under the following conditions: capillary column, 0.53 mm×15 m TC-1 (GL Science Inc., Tokyo, Japan); temperature of column, 50°C; temperature of injector and detector, 230°C; and carrier gas, nitrogen.

Due to the difficulty in estimating of free water amount in the mixture, data on starch, glucose and ethanol were expressed as gram per kilogram fermentation mixture. The specific rate of ethanol production from glucose was determined as follows. The fermentation mixture (about 0.2 g) harvested from the reactor was resuspended in 0.8 ml of water and centrifuged at 2000×*g*. After removal of the supernatant, about half of yeast cells sedimented on starch were recovered and resuspended in 5 ml of YPD medium (10 g $L^{-1}$ yeast extract, 20 g $L^{-1}$ polypeptone and 50 g $L^{-1}$ glucose). After measuring $OD_{660}$, the suspension was transferred to a 15-ml plastic tube equipped with a check valve followed by degassing by an aspirator. The tube was incubated for 80 min at 37°C and the ethanol concentration in the suspension was measured every 20 min. Fermentative activity was expressed as the specific rate of ethanol production from glucose (g g-dry-$cell^{-1}$ $h^{-1}$).

## 2.3 Results

*CCSSF conditions*

Test-tube cultures were used to demonstrate the initial condition of CCSSF. Here, raw corn starch and amylases were used as the substrate model and saccharifying enzymes, respectively. The temperature for CCSSF was set at 37°C. The pH was set at

5 because the optimum pH for α-amylase is 6, as reported previously (Takada and Hirai, 2004), and that for glucoamylase is in the 4.5–5.5 range (from the manufacturer's data sheets); moreover, the fermentative activity of yeast cells were found to be almost constant between pH 4 and 8 (data not shown). As shown in Fig. 2.3, the fermentative activity of yeast cells did not decrease significantly even at a moisture content of 40%. Since it becomes difficult to mix the materials homogeneously at a moisture content less than 50%, the initial moisture content was set at 61% to ensure reproducible sampling. To avoid accumulation of glucose and to prevent bacterial contamination and product inhibition by the saccharifying enzymes, the rate of glucose production by the saccharifying enzymes was set at about 5-fold lower than the rate of glucose consumption by yeast cells. Because preliminary experiments showed that 1 g of starch is digested in 1 h by 1000 units each of α-amylase and glucoamylase under the conditions for CCSSF (37°C, pH 5) and that the specific rate of glucose consumption by yeast cells prepared by fed-batch culture was typically 2.0–2.5 g·g-dry-cell$^{-1}$·h$^{-1}$, 2000 units each of the enzymes and 6 g-dry-cell of the yeast were added to 50 g of starch in the pilot scale.

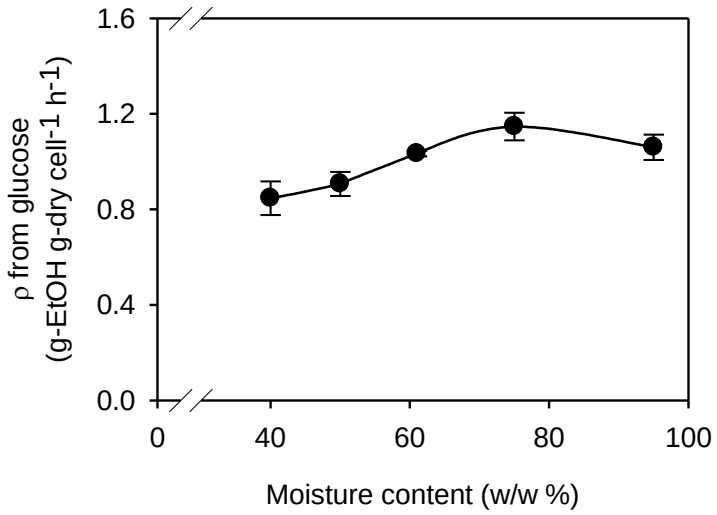


**Fig. 2.3** Effect of moisture content on fermentative activity of yeast TJ14

Starch (5 g) and yeast (3 g-dry-cell) were incubated anaerobically at 37°C in various volumes of YPD medium containing 5% glucose. Fermentation activity was calculated on the basis of ethanol formation rate and dry cell weight in the medium assuming that the cell mass is constant during the fermentation (80 min). Bars indicate standard deviations (SDs: n=3)

*Effect of continuous removal of ethanol on fermentation activity*

During the fermentation stage where ethanol is produced, the inhibitory effect of ethanol on yeast cells and saccharifying enzymes in solid-state fermentation become serious because the absolute ethanol concentration in solid-state fermentation is higher

than that in liquid fermentation. As expected, accumulation of glucose was observed after 6 h of fermentation and ethanol production stopped at 8 h of fermentation (Fig. 2.4a). The amount of ethanol produced in the fermentation mixture was 14 g in the system, which corresponds to 157 g $L^{-1}$ ethanol concentration, considering that the mixture contains 65 ml of YP medium and 24 g of water in wet yeast cells. The limitation of ethanol production would be due to the inhibitory effect of ethanol on fermentation rather than decreases in the activities of the saccharifying enzymes because the decreasing rate of starch content was almost constant.

The continuous removal of ethanol from the fermentation mixture was examined in the ranges of 10–20, 30–50, 50–70 and 75–85 g kg-mixture$^{-1}$. Figures 3b and c show the representative performances of this CCSSF system in maintaining the ethanol content at 30–50 and 75–85 g kg-mixture$^{-1}$. In the case of ethanol content within the range of 30–50 g kg-mixture$^{-1}$, circulation of the headspace gas by an air pump was started at an initial flow rate of 1.7 L $min^{-1}$, when the ethanol content reached 40 g kg-mixture$^{-1}$. Ethanol content was maintained in the range of 30–50 g kg-mixture$^{-1}$ by changing the flow rate of the pump and the produced ethanol was recovered continuously to the condenser. Since no glucose accumulation was observed until all of starch was exhausted, the rate of glucose consumption by yeast cells was maintained

higher than the rate of glucose production by the saccharifying enzymes. On the other hand, when ethanol content was controlled between 75–85 g kg-mixture$^{-1}$, ethanol production rate and starch consumption rate decreased after 6 h of fermentation. Because accumulation of glucose began as in the case without the circulation, the decrease in ethanol production rate was considered to be due to a decrease in the fermentative activity of yeast cells induced by such a high content of ethanol, whereas the activities of saccharifying enzymes were decreased by the accumulated glucose and ethanol.

As shown in Fig. 2.5a, the specific rate of ethanol production from glucose after 15 h of CCSSF deceased markedly at an ethanol content more than 50 g kg-mixture$^{-1}$. On the other hand, the specific rate of ethanol production from starch was maintained in the 0.21–0.23 g g$^{-1}$ h$^{-1}$ range until an ethanol content in the range of 50–70 g kg-mixture$^{-1}$, whereas it decreased to 0.09 g g$^{-1}$ h$^{-1}$ at an ethanol content in the range of 75–85 g kg-mixture$^{-1}$. In addition, the concentration of recovered ethanol was almost proportional to ethanol content (95±3, 226±9, 458±26 and 509±64 g L$^{-1}$ at ethanol contents of 10–20, 30–50, 50–70 and 75–85 g kg-mixture$^{-1}$, respectively). As the results, the ethanol content should be maintained at 30– 50 g kg-mixture$^{-1}$ to keep the fermentative activity and obtain high recovered ethanol concentration.

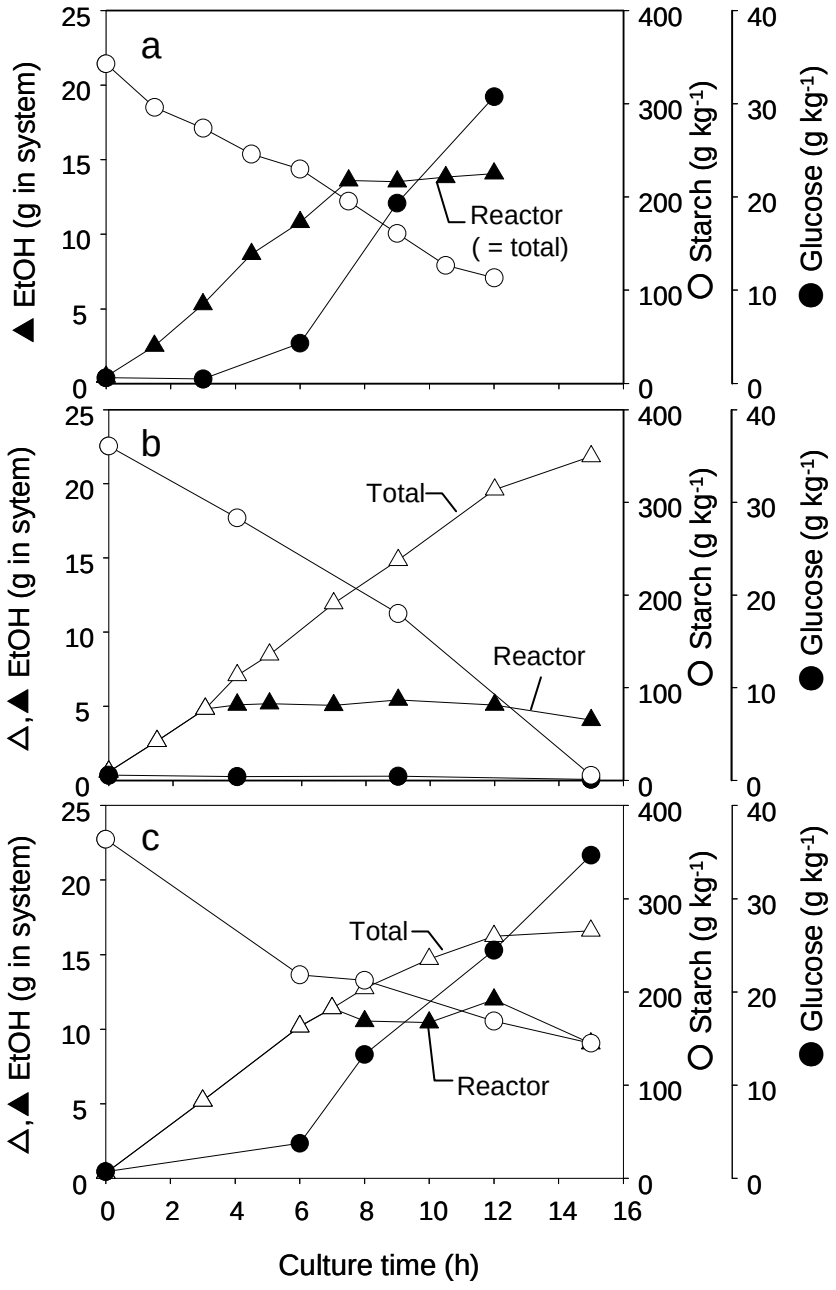


**Fig. 2.4** Time courses of solid-state fermentation.

a, without removal of ethanol; b and c, ethanol content was controlled in the range of 30–50 and 75–85 g $kg^{-1}$, respectively. Arrows indicate the timing when circulation of the headspace gas was initiated.

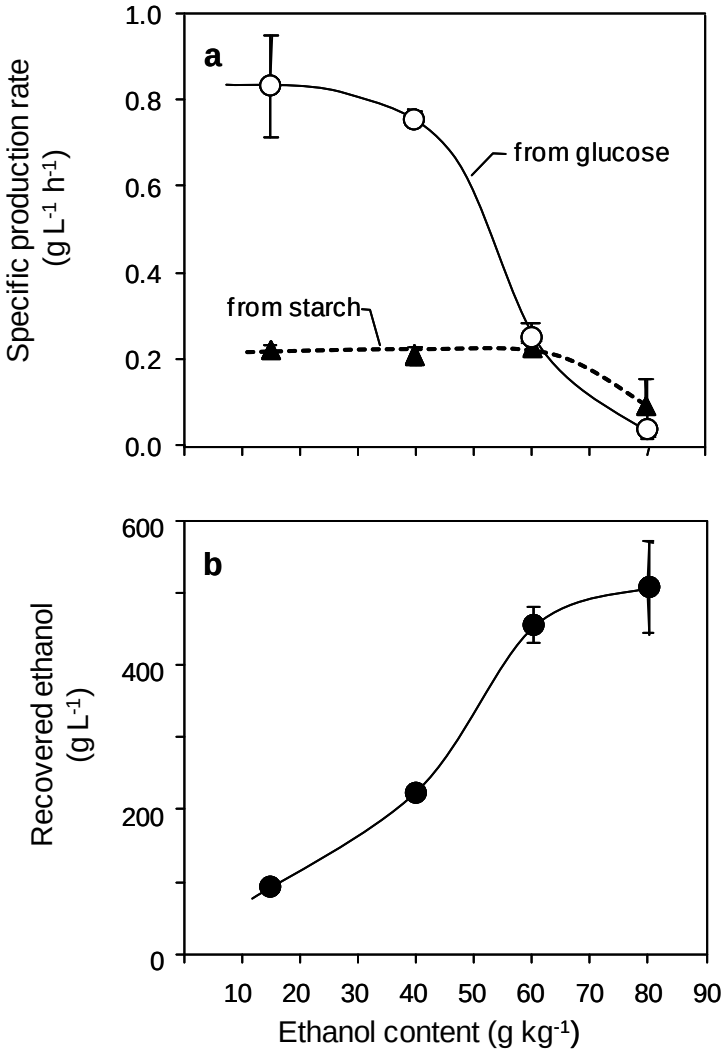


**Fig. 2.5** Influence of ethanol content on ethanol productivity and recovery in CCSSF

Each symbol is plotted at a set ethanol content (15, 40, 60 and 80 g $kg^{-1}$) and the ranges of ethanol content (10–20, 30–50, 50–70 and 75–85 g $kg^{-1}$, respectively) induced by arrows on the horizontal axes. The specific rate of ethanol production from glucose was measured for yeast cells after 15 h of CCSSF by the method shown in Materials and

Methods, whereas that from starch was calculated on the basis of ethanol production rate from 0 h to 15 h assuming that the cell mass is constant. Bar represents the SDs (n=3).

*Repetitive fermentation*

Since one of the advantages of the CCSSF system is that the operating cost for saccharifying enzymes and yeast can be reduced by repetitive addition of delignified biomass, repetitive fermentation was conducted to examine the performance of this system. Ethanol content was maintained in the range of 30–50g kg-mixture$^{-1}$ because the fermentative activity of yeast decreased markedly at an ethanol content above 50 g kg-mixture$^{-1}$ whereas the concentration of recovered ethanol increased. When 80% of initial starch was consumed, 40 g of starch was added to the reactor to continue the fermentation. Fig. 2.6 shows a representative time course of 3 independent fermentations. Ethanol was recovered continuously and the ethanol yield was 93% (87 g of ethanol was recovered from 165 g of consumed starch). The yeast cells did not grow during repetitive fermentation. Even after the third addition of starch, no glucose accumulation was observed, indicating that the rate of glucose consumption by yeast cells could be maintained higher than the rate of glucose production by the

saccharifying enzymes. Ethanol production rate decreased gradually with the progress of fermentation. This decrease would be due to decreases in the activities of the saccharifying enzymes, which is in accordance with a decrease in starch consumption rate (see the top panel of Fig. 2.6).

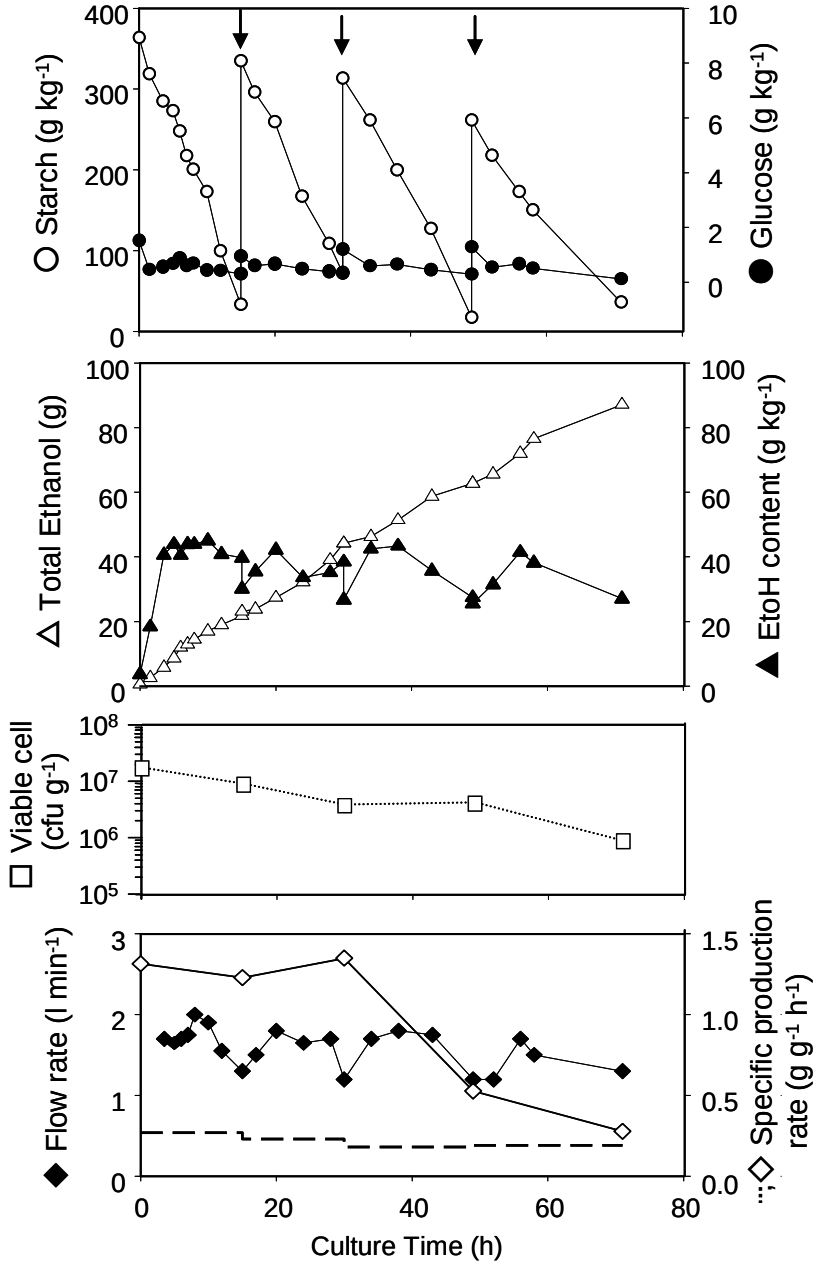


**Fig. 2.6** Representative time course of repetitive CCSSF

Arrows represent the time when 40 g of starch was added. The specific rate of ethanol production from starch represented as a broken line was calculated on the basis of average ethanol production rate during each period.

## 2.4 Discussions

*Effect of moisture content on fermentation*

Yeast is tolerant to low-moisture conditions compared with other microorganisms. The yeast strain used in the present study maintains a sufficient fermentative activity for CCSSF even at a moisture content of 40% (Fig. 2.3). Although it is possible to perform CCSSF at a moisture content less than 40%, it would become difficult to mix the fermentation mixture homogenously, resulting in an inhomogeneous ethanol content that leads to further decrease in fermentative activity. Therefore, CCSSF should be performed at a moisture content of approximately 50% for homogeneous mixing, whereas the present CCSSF was conducted at 61% moisture content to ensure reproducible sampling.

To maintain a suitable moisture content for CCSSF, water must be added to the fermentation mixture to compensate for the removal of not only ethanol but also water by circulating the headspace gas to the condenser. Although it is possible to compensate for water loss by spraying liquid water directly to the fermentation mixture in large–scale CCSSF, it is difficult practically to compensate for water loss continuously and homogeneously in small laboratory-scale CCSSF. In the present study, therefore, water was replenished in the vapor for equipping a humidifier with which the temperature of

water was maintained higher than the reactor. As a result, although a slight increase in moisture content was observed after CCSSF (up to 70%), this would not affect any conclusions in the present study because the water content was maintained above 40% at which the fermentative activity of the yeast strain is constant (Fig. 2.3). In industrial-scale CCSSF, however, the desired moisture content should be maintained by an appropriate method that makes the treatment of residual waste easy.

*Influences of ethanol content on fermentation*

The concentration of recovered ethanol becomes higher at a higher ethanol content at the fermentation mixture. As a result, the fermentation system can save energy for not only recovery but also dehydration of ethanol because the amount of water evaporated together with ethanol decreases. However, when the ethanol content was maintained at 75–85 g kg-mixture$^{-1}$, it was impossible to perform repetitive fermentation because the fermentative activity of yeast decreased markedly, whereas ethanol solution at 509 g L$^{-1}$ was recovered. If fermentative activity cannot be maintained, the system should be required to supply yeast cells in every batch of fermentation, resulting in an increase in energy and cost for preparation of yeast cells.

To determine the optimum ethanol content, fermentation temperature should also be considered because damage of yeast by ethanol becomes serious with increasing temperature. In the present study, the ethanol content in repetitive fermentation was controlled at 30-50 g kg-mixture$^{-1}$ at 37°C, whereas the average concentration of recovered ethanol was 233 g L$^{-1}$. When CCSSF is performed at a lower temperature, it is possible to maintain fermentative activity for a longer period even at higher ethanol content. However, CCSSF at a low temperature would result in an increase in energy for condensation of ethanol solution because the efficiency of the condensation depends on the difference in temperature (saturation level of ethanol vapor) between the reactor and the condenser.

*Optimum conditions for CCSSF*

The optimum conditions (temperature, ethanol content and moisture) for CCSSF of biomass should be determined by considering the energy and cost for not only the preparation of yeast and saccharifying enzymes but also for the recovery and dehydration of ethanol. The temperature of CCSSF affects the activities of saccharifying enzymes, yeast cells and efficiency of ethanol recovery. Ethanol content affects the activity of yeast cells, efficiency of recovery and dehydration, and activities of

saccharifying enzyme. In addition, moisture content in the fermentation mixture affects the cost for treatment of fermentation residues. These optimum conditions would vary with both type of biomass used and method of pretreatment.

In the cases of using starchy biomasses with high carbohydrate contents, because it is possible to repeat CCSSF several times until the reactor full with residues, the lifetime of yeast cells should be considered first. In the case of using lignocellulosic biomasses such as rice straw, in addition to the influences of ethanol content and temperature (Aldiguier et al., 2004), a synergistic influence of inhibitory materials formed in the pretreatment process such as furfural and phenolic compounds should be considered (Klinke et al., 2004), whereas a short lifetime of yeast would be acceptable because the reactor would become full with residues of fermentation even after a few additions of delignified biomasses.

In CCSSF system, since the amount of water is minimized, the size of the reactor becomes half. In addition, waste water is very little, whereas 80 to 90% of the fermentation broth becomes waste water in conventional liquid fermentation. Furthermore, moisture content of the residues is lower than that from conventional systems, this can save energy and cost for recycling of residue to agricultural land.

Moreover, the cost of yeast and saccharifying enzymes can be saved because of the repetitive fermentation. The derived ethanol solution can be dehydrated by energy-saving zeolite membrane.

## 2.5 Conclusions

To save the cost and input energy for bioethanol production, the CCSSF was performed. The CCSSF system consists of two parts, ethanol conversion and product recovery. Since the CCSSF is the solid-state fermentation that minimizes water, the content of ethanol increases rapidly during the fermentation. Ethanol produced by simultaneous saccharification and fermentation is continuously recovered as vapor from the headspace of the reactor while the humidifier compensates for water loss. The concentration of the recovered ethanol was proportional to the ethanol content in the reactor. However, when the ethanol content was maintained at 75–85 g kg-mixture$^{-1}$, it was impossible to perform the repetitive fermentation because the fermentative activity of yeast decreased markedly, even when ethanol solution at 509 g L$^{-1}$ was recovered. In the present study, therefore, the ethanol content in repetitive fermentation was controlled at 30-50 g kg-mixture$^{-1}$ at 37°C, whereas the average concentration of the recovered ethanol was 233 g L$^{-1}$.

From the prospective, the CCSSF could be accomplished the primary purpose of biomass utilization that is to save petroleum resources. In the production of bioethanol, however, it is necessary to minimize the total energy required for not only pretreatment, saccharification, fermentation, recovery and dehydration but also cultivation, harvesting and transportation of biomass, treatment of waste and recycle of residues to the harvesting place. In general, the larger the production scale, the lower the energy and cost for production per unit of ethanol, but the higher the energy and cost for transportation of biomass, particularly in the cases of lignocelluloses such as rice straw in which the amount of carbohydrates harvested per unit of land is lower than that in corn or sugar cane. The CCSSF system will solve this trade-off and enable a geometrically distributed production of ethanol that can save the total energy and cost.

**Nomenclature**

| | |
|---|---|
| *Con* | ethanol concentration in recovery system, g $L^{-1}$ |
| *F* | flow rate, L $min^{-1}$ |
| $F_0$ | initial flow rate, L $min^{-1}$ |
| $I_0$ | initial cell concentration, g-dry-cell $L^{-1}$ |
| *K* | initial weight of fermentation mixture, g-mixture |

| | |
|---|---|
| $L_0$ | initial volume of the microbial culture, L |
| $N$ | batch number or number of substrate addition |
| $P_{theoretical}$ | ethanol production rate obtained by calculation, g $h^{-1}$ |
| $P_{recovered}$ | ethanol production rate obtained in experiment, g $h^{-1}$ |
| $S$ | substrate amount, g |
| $S_0$ | initial substrate amount, g |
| $S_F$ | glucose concentrate in feed medium, g $L^{-1}$ |
| $T$ | sampling time, h |
| $t$ | culture time, h |
| $V$ | volume of recovered ethanol, ml |
| $Y_{X/S}$ | cell yield, g-dry-cell g-glucose$^{-1}$ |

Greek letters

| | |
|---|---|
| $\gamma$ | ethanol content in fermentation mixture, (g kg-mixture$^{-1}$) |
| $\gamma_{max}$ | maximum ethanol content in fermentation mixture, (g kg-mixture$^{-1}$) |
| $\gamma_{min}$ | minimum ethanol content in fermentation mixture, (g kg-mixture$^{-1}$) |
| $\mu$ | specific grow rate, $h^{-1}$ |

# Chapter 3 A strategy for preventing bacterial contamination by addition of external ethanol in solid-state bioethanol production

## 3.1 Introduction

In chapter 2, we developed a consolidated continuous solid-state fermentation (CCSSF) system composed of a rotating drum reactor, a humidifier and a condenser. The mixing of biomass, saccharifying enzymes, yeast and a minimum amount of water in the reactor for simultaneous saccharification and fermentation, and the circulation of the head space gas in the reactor to the condenser enable continuous ethanol production. This continuous ethanol production was performed at moderate ethanol content in the reactor without any loss of yeast activity.

In further development of industrial bioethanol production, bacterial contamination is one of the most serious problems (Muthaiyan and Ricke, 2010; Schell et al., 2007; Makanjuola et al., 1992). Lactic acid bacteria (LAB) such as *Lactobacillus plantarum*, *L. paracasei* and *L. fermentum* are the major contaminants in ethanol fermentation (Narendranath and Power, 2004; Narendranath and Power, 2005). These bacteria consume saccharide, thus decreasing in ethanol yield. In addition, lactate that produced by LAB, has been reported to be a strong inhibitor of ethanol production by

yeast cells (Watanabe et al., 2008). There are several preventive methods for bacterial contamination, including addition of antiseptics and antibiotics (Watanabe et al,.2008; Saithong et al., 2009; Bischoff et al., 2009). However, addition of these reagents to the fermentation mixture is costly and not environmentally friendly because the wastes of bioethanol production could be recycled as fertilizer. In particular, the remaining antibiotics in the wastes would lead to the generation of drug-resistant microorganisms. Therefore, alternative methods with low environmental burden are required for bioethanol production. In this chapter, we propose a simple method of preventing bacterial contamination by the addition of exogenous ethanol at the start of fermentation in a practical application of our CCSSF system for bioethanol production.

## 3.2 Materials and Methods

### *Strains and medi*a

*L. plantarum* NRIC1067, a contaminant LAB model, was cultivated in MRS broth (Difco, Becton Dickinson, Sparks, MD, USA) at 30ºC. *Saccharomyces cerevisiae* TJ14 as the ethanol-producing yeast was prepared as described in chapter 2.

*Ethanol fermentation*

The solid-state fermentation in test tubes was conducted at various initial contents of exogenous ethanol in the fermentation mixture, which consists of 2.5 g of corn starch (Wako Pure Chemicals, Tokyo, Japan) and 3.9 ml of YP medium [1% yeast extract (Difco, Becton Dickinson, Sparks, MD, USA), 2% peptone (Difco, Becton Dickinson, Sparks, MD, USA)] containing 4% glucose and $7\times10^8$ colony forming units (CFU) of the yeast or $7\times10^6$ CFU of the LAB. The viable cell numbers at 0 h ($Q_{t=0}$) and 4.5 h ($Q_{t=4.5}$) in anaerobic fermentation at 37°C were determined using YPD plates (1% yeast extract, 2% peptone, 2% glucose, 2% agar) or MRS plates (5.5% MRS broth, 10 μg $ml^{-1}$ cycloheximide, 2% agar) for the yeast or the LAB, respectively.

To demonstrate the repression of contamination, the yeasts and LAB were cocultured in the CCSSF system at various contents of ethanol. In a drum-shaped reactor (10 cmϕ × 15 cm), 50 g of corn starch, $2\times10^{10}$ CFU of the yeast (30 g-wet cell), $1\times10^7$ CFU (0.02 g-dry cell) of LAB, 65 ml of YP medium, 700 units of gluocoamylase (Wako Pure Chemicals, Tokyo, Japan) and 700 units of α-amylase (Wako Pure Chemicals, Tokyo, Japan) were placed. The reactor was rotated at 5 rpm and the temperatures of the reactor, humidifier and condenser were set at 37, 47 and −10°C, respectively, as described in chapter 2. Gas circulation was started at an initial rate of

0.5 l $min^{-1}$ and ethanol content was maintained by changing circulation rate.

*Analysis methods*

The contents of ethanol, glucose and lactate were determined using a biosensor (Biosensor BF5, Oji Scientific Instruments Co., Ltd., Hyogo, Japan). For the detection of L-lactate, an L-lactic acid enzyme electrode was used, and for the detection of D-lactate, a D-lactic acid enzyme electrode and D-lactic acid kits were used in accordance with the manufacturer's instructions. The content of starch was determined by the packed volume method.

## 3.3 Results

*Effect of ethanol content on growth of yeast and L. plantarum*

As shown in Fig. 3.1, cell viability defined as the ratio of $Q_{t=4.5}$ to $Q_{t=0}$ for the LAB decreased with increasing ethanol content, being almost unity at 41 g $kg^{-1}$ ethanol content. In contrast, yeast cell viability remained nearly constant at an ethanol content of 47 g $kg^{-1}$, whereas it decreased markedly at 62 g $kg^{-1}$.

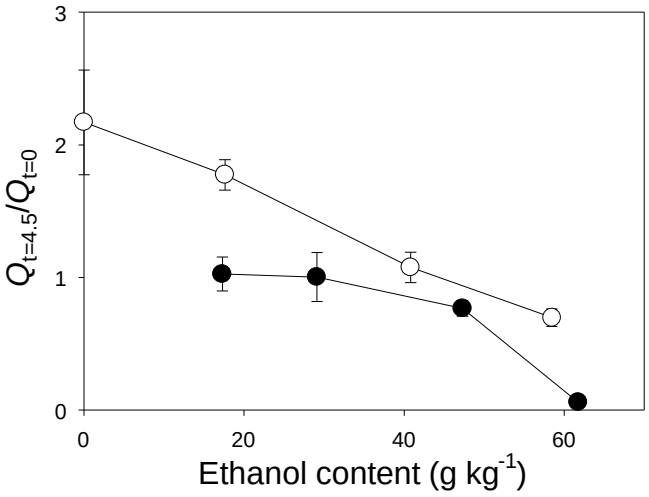


**Fig. 3.1** Effect of initial ethanol content on growth of LAB and yeast cells

Open circles, LAB; closed circles, yeast. The ethanol contents are average of the initial and final ones of the fermentation.

*Effect of premixing of external ethanol on CCSSF*

CCSSF with the addition of exogenous ethanol at the start of fermentation was performed at various ethanol contents of 40, 50 and 60 g kg$^{-1}$ (runs 1, 2 and 3, respectively). As shown in Fig. 3.2, the viabilities of yeast and LAB cells remained constant. Lactate content, however, increased with time ($t$), which was 0.66 g kg$^{-1}$ at the end of the culture (run 1 at $t$=18 h). In the case of run 2, similar profiles of cell viabilities of the yeast and LAB were obtained; however, the lactate content was lower than that in run 1. In addition, a higher initial ethanol content of 60 g kg$^{-1}$ (run 3) caused

lower viabilities of yeast and LAB cells, although a lactate content of zero was achieved. To further understand the significance of adding exogenous ethanol at start of fermentation, CCSSF without the addition of exogenous ethanol was performed (run 4 in Fig. 2). Ethanol content increased with the progress of fermentation. When the ethanol content reached 50 g $kg^{-1}$ at $t$=6 h, the content was controlled by change flow rate. The viability of yeast cells remained constant. However, the viability of LAB increased to $1\times10^6$ CFU $g^{-1}$ at $t$=18 h. In addition, lactate content increased and the final lactate content at $t$=18 h reached 1.07 g $kg^{-1}$, which was 3.6 times higher than that in CCSSF with the initial addition of exogenous ethanol (run 2).

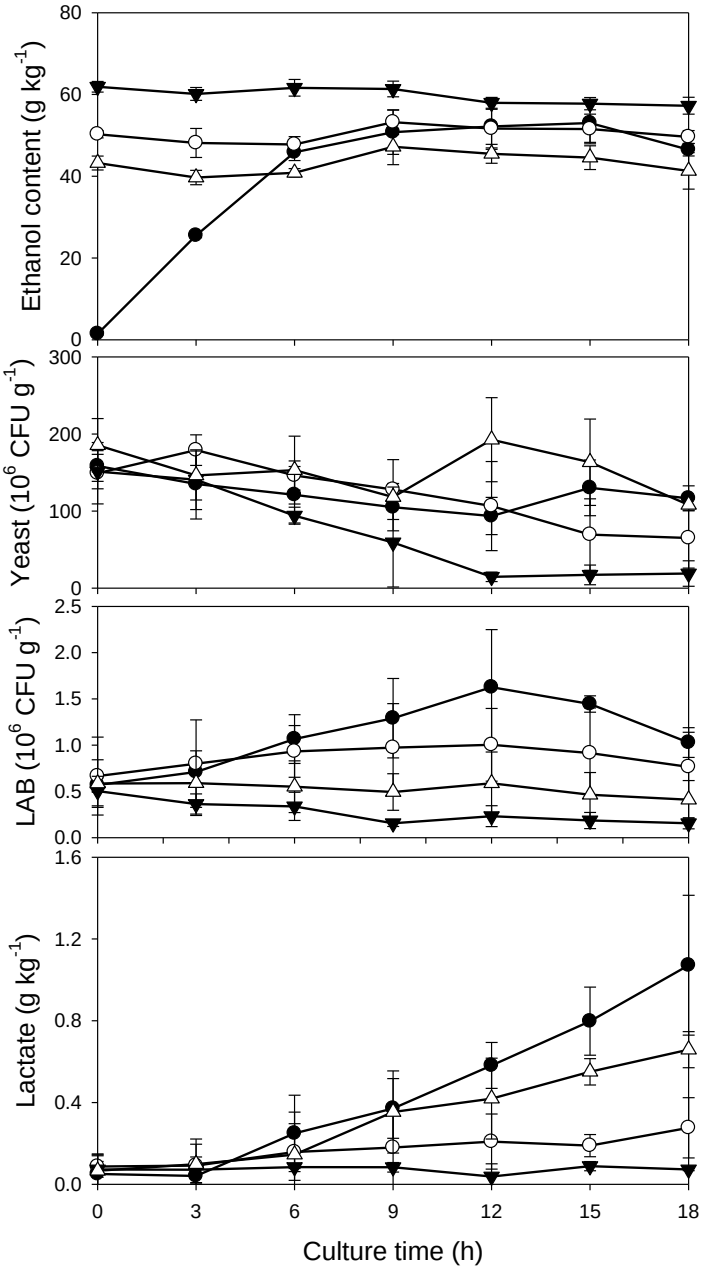


**Fig. 3.2** Representative culture performances with and without initial addition of exogenous ethanol at various ethanol contents

Reverse triangles, 60 g $kg^{-1}$; closed circles, without premixing with exogenous ethanol; open circles, 50 g $kg^{-1}$; triangles, 40 g $kg^{-1}$

## 3.4 Discussions

Bacterial contamination in ethanol production processes is unavoidable. In the present study, we used *L. plantarum* as a contaminant model. When it was added at $7\times10^4$ CFU $g^{-1}$ to the fermentation mixture, LAB cells multiplied and produced a significant amount of lactate, which decreased the ethanol yield to 0.39 g $g^{-1}$ (run 4 in Table 1), compared with the theoretical yield of 0.51 g $g^{-1}$. In contrast, when exogenous ethanol was premixed with the fermentation mixture and ethanol content was maintained, ethanol yield increased with an increase in ethanol content and reached 0.50 g $g^{-1}$ at an initial ethanol content of 60 g $kg^{-1}$ (run 3), which is almost the same as the theoretical yield. However, 32.2 g $kg^{-1}$ glucose accumulated at 18 h and the ethanol productivity was 0.52 g $h^{-1}$, suggesting a lower viability of yeast cells at an ethanol content that the cells cannot tolerate as shown in Fig. 3.1. The moderate ethanol content of 50 g $kg^{-1}$ (run 2) led to a low amount of glucose accumulated and an ethanol yield of 0.45 g $g^{-1}$ and the fermentative activity of the yeast remained high. In practical production of ethanol, the addition of exogenous ethanol would be one of the most convenient methods to prevent yield loss by repression of contaminant viability in CCSSF.

Actual ethanol production processes would be contaminated by bacteria that may be more tolerant to ethanol than the contaminant model used here. In addition, the adaptation of a contaminant to ethanol might enhance its tolerance. Indeed, the LAB produced lactate even after the ethanol content reached 50 g $kg^{-1}$ (run 4) and the LAB started to produce lactate after 6 h even in the presence of 40 g $kg^{-1}$ ethanol (run 4), suggesting the capability of adaptation to ethanol at a certain level. It would be necessary to enhance the tolerance of yeast to ethanol and add exogenous ethanol at higher concentrations.

**Table 3.1** Effects of exogenous ethanol on CCSSF

| Run No. | Ethanol content (g $kg^{-1}$) | | Lactate content at *t*=18 h (g $kg^{-1}$) | Glucose content at *t*=18 h (g $kg^{-1}$) | Productivity[a)] (g $h^{-1}$ $L^{-1}$) | Ethanol yield (g $g^{-1}$) |
|---|---|---|---|---|---|---|
| | Initial | During ethanol recovery | | | | |
| 1 | 40 | 40 | 0.66±0.09 | n.d. | 0.72±0.05 | 0.41±0.03 |
| 2 | 50 | 50 | 0.28±0.15 | 0.04±0.05 | 0.77±0.06 | 0.45±0.03 |
| 3 | 60 | 60 | 0.07±0.01 | 32.2±14.5 | 0.52±0.08 | 0.50±0.02 |
| 4[b)] | 0 | 50 | 1.07±0.34 | n.d. | 0.66±0.05 | 0.39±0.01 |

Average values and standard deviation of three independent fermentations are shown. Ethanol yield was calculated on the basis of the amount of consumed starch and residual

glucose assuming that the consumed starch was converted completely to glucose by glucoamylase. n.d., not detectable.

a) The average ethanol production rate when 50 g starch was converted in the reactor with an inner volume of 1.28 L

b) When the ethanol content reached 50 g $kg^{-1}$, the ethanol content was controlled at 50 g $kg^{-1}$.

## 3.5 Conclusions

In conventional methods to repress contamination in ethanol production, an antibiotic or an antiseptic is added. However, these reagents are costly and not safe for the environment. In the present method, in contrast, ethanol as the additive for preventing contamination can be recovered. That is, by recycling a portion of produced ethanol in the next batch of CCSSF, it is possible to repress contamination without additional cost. By adding ethanol to the materials such as delignified biomasses and food wastes, it is possible to prevent their putrefaction during transportation and storage. The combination of CCSSF and the present method will lead to the realization of one of the most ideal bioethanol production process that is cost-saving and environmentally friendly.

# Chapter 4 Simulation studies of the production cost for CCSSF system

## 4.1 Introduction

In recent years, research and development efforts on bioethanol production are focusing on the commercial production with the aim of reducing the cost. Generally, the production cost consists of a capital cost, operating cost and other costs. The capital cost for bioethanol is the sum of costs for facilities of pretreatment, saccharification, fermentation, distillation, and waste water treatment. The operating cost is the total costs for raw materials including transportation and storage costs, saccharifying enzymes and yeast. The other costs are the sum of the cost for utilities (electricity, steam and water), maintenance, labor, waste water treatment, and overhead (general expenses, tax, insurance and so on).

Although, both capital and operating costs for ethanol production by the CCSSF system are expected to be lower than those for conventional systems as described in chapter 2, further reduction of the costs is necessary to expand the use of the CCSSF system. In this chapter, the bioethanol production costs using CCSSF system will be estimated and way to reduce the costs will be discussed based on the results of sensitivity analysis.

Three biodegradable municipal solid wastes (BMSW) were chosen as the representative biomass, considering that the CCSSF system is applicable for herbal and woody biomasses after lignin is removed by pretreatment. The first is food wastes that contain relatively high amount of starch.

In Japan, 11 and 6.5 million tons of food wastes are produced from households and food factories, respectively. Some food factories generate wastes such as breads, noodles and snacks that have relatively high starch contents, whereas the starch content of household wastes is not so high. The second is off-spec rice such as cadmium- or mold-contaminated rice that is incinerated in Japan at present. The off-spec rice is one of the best biomass because the starch content is very high and starch is easy to be saccharified; however, the amount generated is not so much (several thousand tons per year). The third is waste cotton. Whereas waste cotton is also one of the best biomass because the cellulose content is over 90% (Taherzadeh and Jeihanipour, 2009), but cellulose is more difficult to be saccharified compared with starch. In addition, the available amount of waste cotton is estimated to be 0.9 million tons per year in Japan. For these three typical cases (relatively high starch content, very high starch content and high cellulose content), the costs of bioethanol production were estimated.

## 4.2 Materials and Methods

*The amount of ethanol produced by a unit of CCSSF system*

For estimation of the production cost, it is necessary to know the amount of ethanol that can be produced by one unit of CCSSF system in a year. In a CCSSF system, the amount of ethanol, $E$ (kL year$^{-1}$), that can be produced by one unit of CCSSF system in a year is given as:

$$E = M \times \frac{D}{d} \times X \times \frac{2 \times Mw_e}{Mw_g} \times Y \times \frac{1}{\rho_e} \quad (4.1)$$

where $Mw_g$ and $Mw_e$ are the molecular weight of glucose unit in carbohydrate and ethanol corresponding to 162 g-carbohydrate mol$^{-1}$ and 46 g-ethanol mol$^{-1}$, respectively; $\rho_e$ is the specific gravity of ethanol equivalent to 0.79 kg L$^{-1}$; $M$ is capacity of CCSSF system ($10^3$ kg batch$^{-1}$); $D$ is operation period (day year$^{-1}$); $d$ is fermentation time (day batch$^{-1}$); $X$ is the carbohydrate content of the material (g-carbohydrate g-raw-material$^{-1}$); and $Y$ is ethanol yield (against theoretical yield).

To calculate the amount of ethanol produced by a unit of CCSSF system, the capacity of the CCSSF system, $M$; the operation period per year, $D$; the time required for a batch of fermentation, $d$; the carbohydrate content, $X$, and the ethanol yield, $Y$, are estimated as summarized in Table 4.1.

**Table 4.1** Estimation values for the parameters used in the present study

| Parameter | Food waste | Off-spec rice | Waste cotton |
|---|---|---|---|
| $M$ ($10^3$ kg) | 5 | 5 | 5 |
| $D$ (day year$^{-1}$) | 300 | 300 | 300 |
| $d$ (day) | 1 | 1 | 3 |
| $X$ (g-carbohydrate g-raw-material$^{-1}$) | 0.3 | 0.75 | 0.9 |
| $Y$ (-) | 0.9 | 0.9 | 0.8 |

The CCSSF reactor is assumed to be 3 m in diameter and 6 m in height. This diameter is the maximum width that can be transported on load in Japan, which ensures mass production of the CCSSF system in a factory. Based on the given dimensions, the inner volume of the reactor will be about 40 $m^3$. The capacity of the reactor, *M*, was temporarily set at $5\times10^3$ kg considering that $5\times10^3$ kg of a solid medium is prepared by a drum-shaped mixer with similar size for production of enzymes by a fungus in a company (personal communication with Prof. Y. Katakura). The operation period, *D*, was assumed to be 300 days in a year considering the maintenance period. The fermentation time, *d*, for starchy materials is assumed to be 1 day because α-1,4 and α-1,6 linkages of starch are easy to be digested by amylases, whereas that for cellulosic material is assumed to be 3 days because β-1,4 linkage of cellulose with a rigid crystalline structure needs more time for digestion by cellulases. Since the moisture

content of food wastes is considered to be high and they contain protein and fat, the carbohydrate content, *X*, is assumed to be 0.3 g-carbohydrate g-raw-material$^{-1}$. The moisture content of rice is known to be about 15% and the carbohydrate content was reported to be 0.88 g-carbohydrate g-dry-weight$^{-1}$ (Kim and Dale, 2004). Thus, the carbohydrate content, *X*, is calculated to be 0.75 g-carbohydrate g-raw-material$^{-1}$. The carbohydrate content, *X*, of waste cotton was reported to be 0.9 g-carbohydrate g-dry-weight$^{-1}$ (Taherzadeh and Jeihanipour, 2009). The ethanol yield, *Y*, was assumed to be 0.9 and 0.8 for starchy and cellulosic materials considering that cellulosic substrates are more difficult to be digested by saccharifying enzymes compared with starchy ones.

*The costs for ethanol production*

In addition to capital and operating costs for ethanol production, other costs for utilities, maintenance, labor, waste treatment, and overhead are required. When the capital cost, operating cost and other costs are $C_{\mathrm{Capital}}$, $C_{\mathrm{Operating}}$ and $C_{\mathrm{Others}}$ (yen L$^{-1}$), respectively, the total production cost of ethanol, $C$ (yen L$^{-1}$) is given as

$$C = C_{\mathrm{Capital}} + C_{\mathrm{Operating}} + C_{\mathrm{Others}} \quad (4.2)$$

The capital cost is the sum of the construction cost for pretreatment, saccharification, fermentation, distillation and waste water treatment; being $C_p$, $C_s$, $C_f$, $C_d$, and $C_w$ (yen system$^{-1}$), respectively. When the year of depreciation for each facility is assumed to be $a$ (year) and the amount of ethanol produced by the system is $E$ (L year$^{-1}$), $C_{Capital}$ is given as

$$C_{Capital} = \frac{C_p + C_s + C_f + C_d + C_w}{aE} \quad (4.3)$$

The operating cost includes the costs for the raw materials, enzymes and yeast, being $C_B$, $C_{EN}$ and $C_Y$ (yen L$^{-1}$), respectively. Thus $C_{Operating}$ is given as

$$C_{Operating} = C_B + C_{EN} + C_Y \quad (4.4)$$

When the annual cost for others is $C_O$ (yen year$^{-1}$), the other cost per amount of ethanol is given as

$$C_{Others} = \frac{C_O}{E} \quad (4.5)$$

The value for each parameter is assumed as shown in Table 4.2. In this thesis, the pretreatment cost, $C_p$, was assumed to be zero because the target materials of the CCSSF system are starchy and cellulosic waste that do not require pretreatment. Since the CCSSF system consolidates saccharification, fermentation and distillation processes, the construction cost for these processes were combined. The construction cost of a CCSSF system with a capacity of $5\times10^3$ kg of raw material per batch was estimated to

be 200,000,000 yen system$^{-1}$ by Kansai Chemical Engineering Co. Ltd. The cost for waste water treatment, $C_w$, was assumed to be zero because the CCSSF system emits small amount of waste water. Thus, the total construction cost, $C_{s+f+d}$, was calculated to be 200,000,000 yen system$^{-1}$. When the year of depreciation, *a*, is assumed to be 10 years, the total construction cost was calculated to be 20,000,000 yen year$^{-1}$

The costs for saccharifying enzymes, $C_{EN}$, were estimated to be 5 and 43 yen L$^{-1}$ for starchy and cellulosic materials, respectively, based on the enzyme dosages recommended by manufacturers and their selling prices. For example, the recommended amount of Cellic CTEC2$^{TM}$ (Novozymes) is 0.05 g-enzyme-solution for 1 g cellulose, and its selling price is assumed to be 0.5 yen g-enzyme-solution$^{-1}$. Since one liter of ethanol (790 g) can be obtained from 1.74 kg cellulose assuming that the ethanol yield is 80%, the cost for the cellulase, $C_{EN}$, was calculated to be 43 yen L$^{-1}$.

The cost for yeast, $C_Y$, was estimated to be 4 yen L$^{-1}$ based on the following assumptions and calculations. The average specific ethanol production rate during CCSSF was assumed to be 0.4 g-ethanol g-cell$^{-1}$ h$^{-1}$ based on the actual data that the maximum specific ethanol production rate of *S. cerevisiae* TJ14 was 1.2 g-ethanol g-cell$^{-1}$ h$^{-1}$ (see chapter 2). When yeast cells are assumed to be used for 100 h maintaining the average specific production rate, one gram of yeast produces 40 g

ethanol. Thus, 20 g yeast is required to produce 1 L (790 g) of ethanol. Since the cheapest carbon source for production of yeast is cane molasses with a price of 50 yen $kg^{-1}$ and the typical yield of baker's yeast is known to be 0.4 g-cell g-$sugar^{-1}$, the cost is calculated to be 0.125 yen g-$cell^{-1}$. Thus, the production cost of yeast was estimated to be 0.2 yen g-$cell^{-1}$ after taking into acount the equipment and other operating costs. For production of one liter ethanol, a production cost for yeast, $C_Y$, amounting to 4 yen (=0.2×790/40) is required.

The cost for others, $C_O$, was estimated to be 10,000,000 yen $year^{-1}$ assuming that one operator is employed for one system.

**Table 4.2** Summary of parameter values for cost estimation

| Main parameters | Subparameters | | | |
|---|---|---|---|---|
| (Yen $L^{-1}$) | (Yen $L^{-1}$) | (Yen $year^{-1}$) | (Yen $system^{-1}$) | Value |
| $C_{Capital}$ | | | $C_p$ | 0 |
| | | | $C_s$ | |
| | | | $C_f$ } $C_{s+f+d}$ | 200,000,000 |
| | | | $C_d$ | |
| | | | $C_w$ | 0 |
| $C_{Operating}$ | $C_B$ | | | 0 |
| | $C_{EN}$ | | | 5 or 43 |
| | $C_Y$ | | | 4 |
| $C_{Others}$ | | $C_O$ | | 10,000,000 |

*Sensitivity analysis of production cost*

When one assumes the selling price of ethanol, $S$ (yen $L^{-1}$), one can calculate the balance of payment, $BP$ (yen $year^{-1}$), as

$$BP = E \times \left( S - C_{\text{capital}} - C_{\text{Operating}} - C_{\text{Others}} \right) \tag{4.7}$$

In this thesis, $S$ is assumed to be 100 yen $L^{-1}$ which is the target price determined by the Ministry of Agriculture, Forestry and Fisheries of Japan (MAFF)

. As a result, $BP$ is expressed as the following equation consisting of ten parameters.

$$BP = E \times S - \frac{C_{\text{p}} + C_{\text{s+f+d}} + C_{\text{w}}}{a} - E \times C_{\text{B}} - E \times C_{\text{EN}} - E \times C_{\text{Y}} - C_{\text{O}} \tag{4.8}$$

where

$$E = 0.719 \times M \times \frac{D}{d} \times X \times Y \tag{4.9}$$

and $C_{\text{s+f+d}}$ is the construction cost of the CCSSF system.

Since each parameter was estimated based on assumptions and may vary with location of the facility, type of raw material, social situation and so on, sensitivity analysis was used to assess these parameters and to identify which parameter has the greatest influence on the production cost of ethanol. The seven parameters, namely fermentation time, $d$; the capacity of the CCSSF system, $M$; yield, $Y$; constuction cost,

$C_{s+f+d}$ ; year of depreciation, $a$; enzyme cost, $C_{EN}$; and other costs, $C_O$, were Varied from 50 to 200%, while ethanol yield ranged from 70 to 100%. However, the carbohydrate content, $X$; operation period, $D$ and cost for yeast, $C_Y$, were fixed. Table 4.3 shows the standard value of each parameter for the cases involving the use of starchy food waste, off-spec rice and cellulosic waste cotton as the representative biomass.

**Table 4.3** Standard condition of variable assumption in CCSSF

| | Variable | Starchy | | Cellulosic |
|---|---|---|---|---|
| | | Food waste | Off-spec rice | Waste cotton |
| $\boldsymbol{M}$ | Biomass ($10^3$ kg batch$^{-1}$) | 5 | 5 | 5 |
| $D$ | Operation period (day year$^{-1}$) | 300 | 300 | 300 |
| $\boldsymbol{d}$ | Fermentation time (day batch$^{-1}$) | 1 | 1 | 3 |
| $X$ | Carbohydrate content (g g$^{-1}$) | 0.3 | 0.75 | 0.9 |
| $\boldsymbol{Y}$ | Yield/theoretical yield (–) | 0.9 | 0.9 | 0.8 |
| $S$ | Ethanol price (yen L$^{-1}$) | 100 | 100 | 100 |
| $C_p$ | Construction for pretreatment ($10^8$ yen year$^{-1}$) | 0 | 0 | 0 |
| $\boldsymbol{C_{s+f+d}}$ | Cost for CCSSF system ($10^8$ yen year$^{-1}$) | 2 | 2 | 2 |

| | | | | |
|---|---|---|---|---|
| $C_w$ | Cost for waste water treatment ($10^8$ yen year$^{-1}$) | 0 | 0 | 0 |
| ***a*** | Year of depreciation (year) | 10 | 10 | 10 |
| $C_B$ | Biomass cost (yen L$^{-1}$) | 0 | 0 | 0 |
| $\boldsymbol{C_{EN}}$ | Enzyme cost (yen L$^{-1}$) | 5 | 5 | 43 |
| $C_Y$ | Yeast cost (yen L$^{-1}$) | 4 | 4 | 4 |
| $\boldsymbol{C_O}$ | Other costs ($10^6$ yen year$^{-1}$) | 10 | 10 | 10 |

Parameters written in bold characters were examined in the sensitivity analysis.

## 4.3 Results

*Estimation of ethanol production cost in CCSSF*

To demonstrate the advantages of CCSSF system, the bioethanol production cost needs to be estimated. As shown in Table 4. 4, the amount of ethanol produced by a unit of CCSSF in a year, *E*, from the food wastes, the off-spec rice and the waste cotton were calculated to be 292, 730 and 260 kL year$^{-1}$, respectively, based on Eq. 4.9. Using these values for *E*, the capital costs for production of ethanol, $C_{Capital}$, for the three biomasses were calculated to be 68, 27 and 77 yen L$^{-1}$, respectively, based on Eq. 4.3. Furthermore, the operating costs, $C_{Operating}$, for the food waste, the off-spec rice and waste cotton were calculated using Eq. 4.4 and give the values 9, 9 and 47 yen L$^{-1}$,

respectively. Lastly, the other costs, $C_{Others}$, for the food waste, the off-spec rice and waste cotton were calculated to be 34, 14 and 38 yen $L^{-1}$, respectively, based on Eq. 4.5. From the computed values, the total production cost of ethanol, *C*, from the food wastes, the off-spec rice and the waste cotton were found to be 111, 50 and 162 yen $L^{-1}$.

**Table 4.4** Estimation of ethanol production cost for starchy and cellulosic materials

| | | Starchy | | Cellulosic |
|---|---|---|---|---|
| | | Food waste | Off-spec rice | Waste cotton |
| *E* | Amount of ethanol (kL $year^{-1}$ $system^{-1}$) | 292 | 730 | 260 |
| $C_{Capital}$ | Capital cost (yen $L^{-1}$) | 68 | 27 | 77 |
| $C_{Operating}$ | Operating cost (yen $L^{-1}$) | 9 | 9 | 47 |
| $C_{Others}$ | Other cost (yen $L^{-1}$) | 34 | 14 | 38 |
| *C* | Total cost (yen $L^{-1}$) | 111 | 50 | 162 |

*Sensitivity analysis of production cost*

In Fig. 4.1, the balance of payment of starchy food waste was calculated following Eq. 4.7. The vertical axis corresponds to the balance of payment and the

horizontal axis is the parameter normalized by dividing with the standard value. The point where all the line intersect represents the balance of payment under the standard conditions. Each line shows the change in the balance of payment when each parameter varies from 0.5 to 2.0. The slope of each line (or curve) indicates the criticalness of the parameter.

In Fig. 4.1a, the balance of payment, *BP*, is calculated to be –3.5 million yen under the standard conditions, which indicates that the expenditure is more than the revenue. Furthermore, it was found that the contributions of enzyme cost, $C_{EN}$ and other costs, $C_O$, to the balance of payment are relatively small. The slope of the curve for the year of depreciation, *a*, becomes steep during the early year of plant operation. This is because the year of depreciation is the denominator of Eq. 4.8. The slope of ethanol yield, *Y*, is relatively high, although the effect on the balance of payment is small. The influence of the construction cost, $C_{s+f+d}$; the fermentation time, *d* and the capacity of CCSSF system, *M*, to the balance of payment are relatively large, especially the balance is drastically improved when the fermentation time, *d*, is shorten or the construction cost, $C_{s+f+d}$,is reduced.

Since the construction cost, $C_{s+f+d}$, is relatively large compared to the total cost, *C*, and sensitive in case of the food waste, the case when half of the construction cost is

supported by the government ($C_{s+f+d}$=100,000,000 yen system$^{-1}$) was studied. As shown in Fig. 4.1b, the balance of payment became +6.5 million yen under the standard conditions.

When food wastes are used as materials for the CCSSF, one would obtain a treatment fee, in other words “inverse onerous contact”, from food companies in cases that they outsource the treatment of their food wastes to other companies. In such cases, the material cost would have a negative value. When the treatment fee is higher than 12 yen L$^{-1}$ ($C_B$> -12 yen L$^{-1}$), the balance for payment of ethanol production by the CCSSF system becomes the revenue under standard condition (Fig. 4.1c).

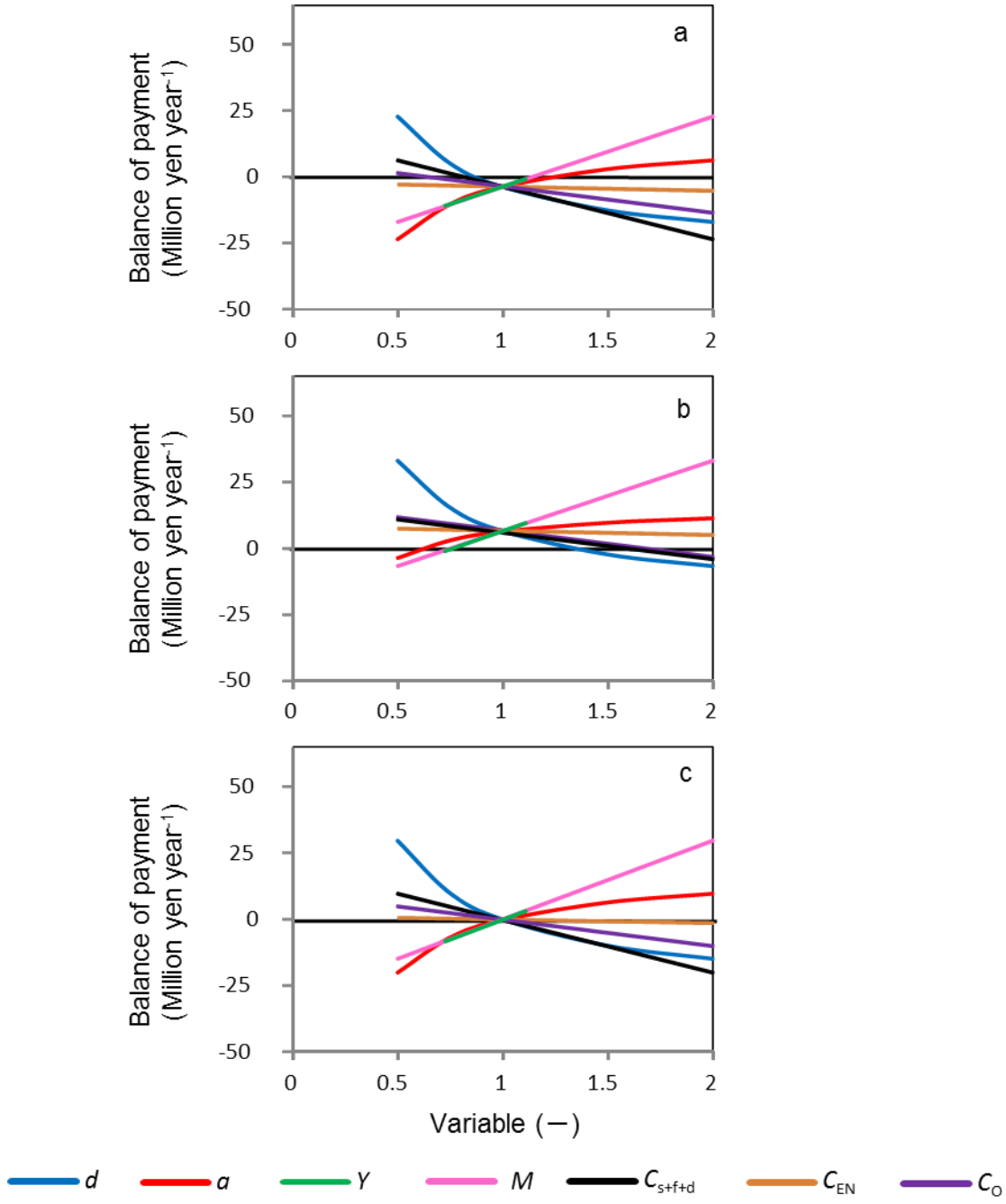


**Fig. 4.1** Sensitivity analysis of the balance of payment, *BP*, for the production of ethanol from starchy food waste based on Eq. 4.7. (a) under the standard conditions, (b) half of the construction cost is supported by the government ($C_{s+f+d}$=100,000,000 yen system$^{-1}$), (c) the treatment fee is 12 yen L$^{-1}$ for starchy food waste material.

In case of the waste cotton, a cellulosic material (Fig. 4.2a), the balance of payment is calculated to be −16 million yen under the standard conditions. It was found that the effects of the enzyme cost, $C_{EN}$, and the other costs, $C_O$, to the balance are relatively small. The changes in the slope of the curve for the year of depreciation, *a*, and ethanol yield, *Y*, are almost similar to that of the food waste. The influences of the capacity of CCSSF system, *M*; the fermentation time, *d*, and the construction cost, $C_{s+f+d}$, on the balance of payment were found to be relatively large; however, the values for slope were lower compared to those of the food waste. An increase in the capacity of CCSSF system, $C_{s+f+d}$, or a reduction of the fermentation time, *d*, does not have very significant effect on the reduction of the production cost as compared to the case for food waste.

When half of the construction cost, $C_{s+f+d}$, is supported by the government, the balance of payment becomes −6 million yen under the standard condition (Fig. 4.2b), which is still unprofitable. The treatment of waste cotton is expected to generate a treatment fee. When the treatment fee is higher than 24 yen $L^{-1}$, the balance of payment consequently becomes the revenue at standard conditions (Fig. 4.2c).

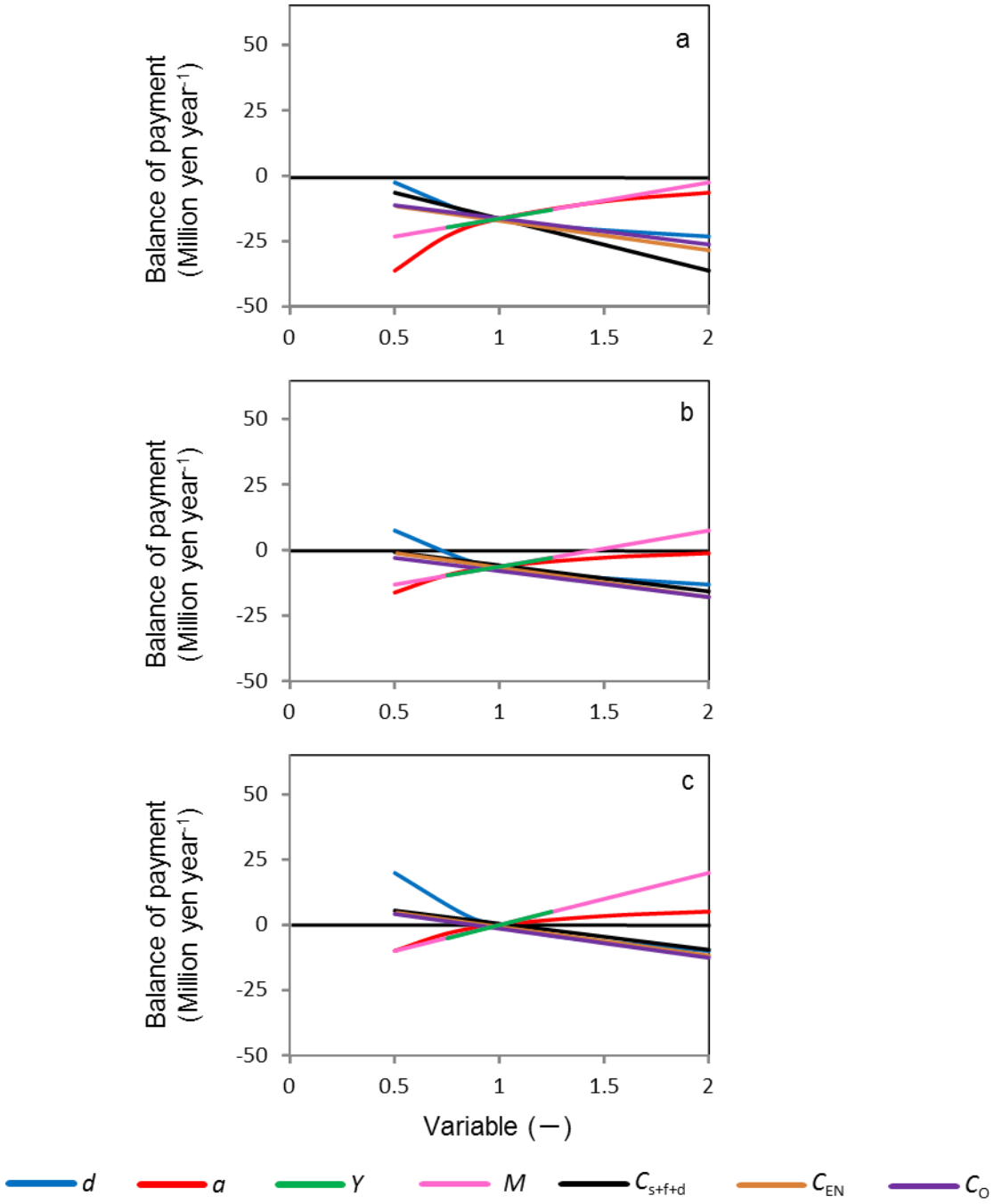


**Fig. 4.2** Sensitivity analysis of the balance of payment, *BP*, for the production of ethanol from cellulosic waste cotton based on Eq. 4.7. (a) under the standard conditions, (b) half of the construction cost is supported by the government ($C_{s+f+d}$=100,000,000

yen system$^{-1}$), (c) half of the construction cost, $C_{s+f+d}$, is supported by the government and the treatment fee is 24 yen L$^{-1}$ for the cellulosic material.

The balance of payment for the off-spec rice, which is a representative starchy material with high carbohydrate content, was estimated as shown in Fig. 4.3. Under the standard conditions, 730 kL of ethanol can be produced from off-spec rice in a year using the CCSSF system, and can generate a balance of payment amounting to +36 million yen. It was found that the effect of fermentation time, *d*, and the capacity of CCSSF, *M*, to the balance are relatively large. The change in the slope of the curve for the year of depreciation, *a*, and ethanol yield, *Y*, are almost similar to that of the food waste. The contributions of the construction cost, $C_{s+f+d}$; the enzyme cost, $C_{EN}$, and the other costs, $C_O$, are relatively small.

Although the cost for raw materials, $C_B$, was assumed to be zero in this chapter, one must pay a procurement cost for off-spec rice because it is a valuable material. As shown in Table 4.5, however, it is possible to gain profit until a procurement cost of 24 yen kg-rice$^{-1}$. When half of the construction cost, $C_{s+f+d}$, is supported by the government, one can pay a maximum of 31 yen kg-rice$^{-1}$. For this estimation, the off-spec rice is considered to be one of the most benefitial materials for bioethanol production in Japan.

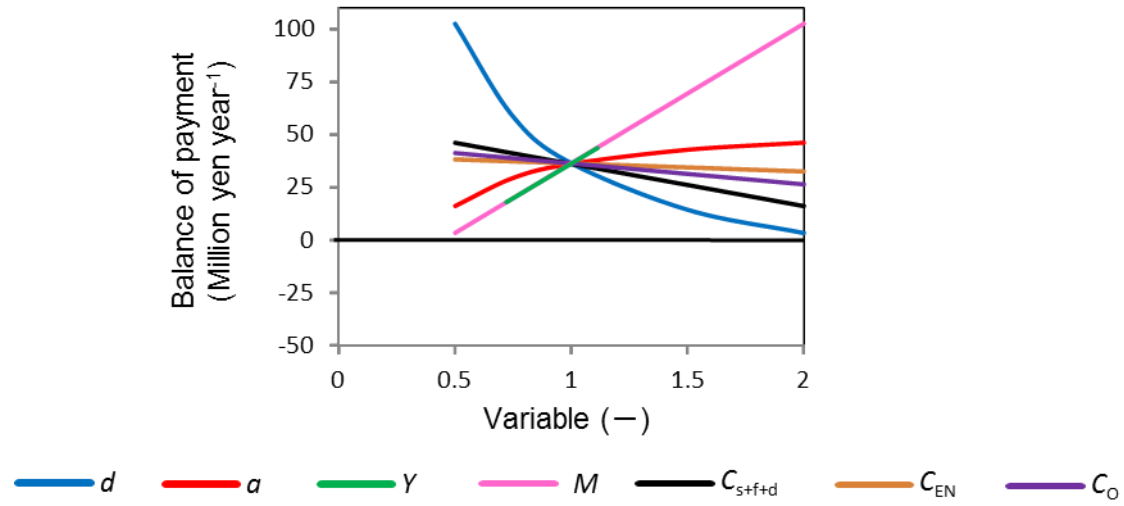


**Fig. 4.3** Sensitivity analysis of the balance of payment for the production of ethanol from the off-spec rice based on Eq. 4.7 under the standard conditions.

**Table 4.5** Influences of price for off-spec rice on the balance of payment.

| $C_{s+f+d}$ (yen system$^{-1}$) | $C_B$ (yen L$^{-1}$) | $C_B$ (yen kg-rice$^{-1}$)* | *BP* (million yen) |
|---|---|---|---|
| $2\times10^8$ | 0 | 0 | 36 |
| $2\times10^8$ | 50 | 24 | 0 |
| $1\times10^8$ | 64 | 31 | 0 |

* The carbohydrate content and the ethanol yield were assumed to be 0.75 and 0.90, respectively.

## 4.4 Discussions

*Comparison of production cost by CCSSF system with those by conventional systems*

The purpose of this thesis is to develop a new geometrically-distributed production system that produces ethanol at a reasonable cost with low energy consumption even in small scale. In this chapter, the production cost was classified into the capital, operating and other costs and these costs were estimated for three representative raw materials (Table 4.4).

Firstly, the capital costs for the starchy food waste, off-spec rice and waste cotton were estimated to be 68, 28 and 77 yen $L^{-1}$, respectively. The capital costs for off-spec rice was found to be comparable with the target price (100 yen $L^{-1}$) determined by the MAFF, whereas those for the starchy food waste and waste cotton were relatively higher. These capital costs were calculated from the CCSSF system which the capacity of ethanol production is $10^2$–$10^3$ kL $year^{-1}$, while that by conventional commercial plants is $10^4$–$10^6$ kL $year^{-1}$. Thus, it can be concluded that the CCSSF system has a good cost performance even in small scale.

Secondly, the operating and other costs are combined and compared with those of ethanol produced from various materials by conventional systems (Table 4.6) The sums of the operating and the other costs of the CCSSF for the food waste, the off-spec

rice and the waste cotton were estimated to be 43, 23 and 85 yen $L^{-1}$, respectively. The operating and the other costs of off-spec rice (23 yen $L^{-1}$) are comparable with those for sugarcane and sugar beets in the US (20 and 17 yen $L^{-1}$, respectively). In addition, the operating and the other costs of the CCSSF for the food waste (43 yen $L^{-1}$) is also comparable to that for sugar beets in Europe (41 yen $L^{-1}$).

**Table 4.6** Summary of estimated ethanol production costs

(Source:http://www.usda.gov/oce/reports/energy/EthanolSugarFeasibilityReport3.pdf)

| Region | Raw material[2] | Cost [1] | | |
|---|---|---|---|---|
| | | Material | Processing | Total |
| U.S. | Corn wet milling | 0.40 ( 9) | 0.63 (14) | 1.03 (22) |
| U.S. | Corn dry milling | 0.53 (11) | 0.52 (11) | 1.05 (23) |
| U.S. | Sugarcane | 1.48 (32) | 0.92 (20) | 2.4 (52) |
| U.S. | Sugar beets | 1.58 (34) | 0.77 (17) | 2.35 (51) |
| U.S. | Molasses[3] | 0.91 (20) | 0.36 ( 8) | 1.27 (27) |
| U.S. | Raw sugar[3] | 3.12 (67) | 0.36 ( 8) | 3.48 (75) |
| U.S. | Refined sugar[3] | 3.61 (78) | 0.36 ( 8) | 3.97 (86) |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Brazil | Sugarcane[4] | 0.30 | ( 7) | 0.51 | (11) | 0.81 | (17) |
| E.U. | Sugar beets[4] | 0.97 | (21) | 1.92 | (41) | 2.89 | (62) |

[1] Dollars per gallon. Values in parenthesis are in yen per liter assuming that 1 Dollar is 82 yen. Excludes capital cost.

[2]Raw material cost for U.S. corn (wet and dry milling) are net raw material costs; raw material costs for U.S. sugar cane and sugar beets are gross raw material costs

[3]Excludes transportation costs

[4]Average of published estimate

*Critical parameters for cost reduction*

The total cost of the CCSSF, *C*, for the food waste, the off-spec rice and the waste cotton were estimated to be 111, 50 and 162 yen $L^{-1}$, respectively. Since the total cost for the off-spec rice is estimated to be half of the target price (100 yen $L^{-1}$), a significant amount of profit will be expected. For the food waste, it would be possible to achieve the target price by a cost reduction, whereas it would not be easy for the waste cotton.

Based on Eq. 4.8, the equation to compute for *BP* could be rearranged as Eq 4.10.

$$BP = E\{S - (C_{\mathrm{B}} + C_{\mathrm{EN}} + C_{\mathrm{Y}})\} - \left\{ \frac{C_{\mathrm{p}} + C_{\mathrm{s+f+d}} + C_{\mathrm{w}}}{a} + C_{\mathrm{O}} \right\} \quad (4.10)$$

To balance the payment, the first term on the right side of Eq. 4.10 must be a positive value. After this, therefore, it is presupposed that the selling price is higher than the operating cost. To improve the balance of income, two options can be considered; one is to increase *E* in the first term and another is to reduce the second term in Eq. 4.10.

The amount of ethanol produced in a unit of CCSSF system in a year, *E*, is a function of the capacity of CCSSF system, *M*; the operation period, *D*; the fermentation time, *d*; the carbohydrate content, *X*, and the ethanol yield, *Y*, as shown in Eq. 4.9. However, *D*, *X* and *Y* are practically difficult to increase. Thus, it is necessary to increase *M* and/or decrease *d* to improve the balance of payment.

The capacity of CCSSF is temporarily assumed to be $5\times10^3$ kg batch$^{-1}$ based on the actual case as described earlier. If the capacity is increased to double, the amount of ethanol produced in a unit of the CCSSF system in a year becomes double, resulting in a decrease in the production cost into half. In this case, however, it is important to ensure the homogenous mixing of the contents of the reactor. If the mixture forms lumps (clotting), the ethanol content inside of the lumps would be high and the temperature

would increase due to fermentation, whereas the ethanol content on the surface of lumps would be low and the temperature would be low due to latent heat. These distributions of the ethanol content and temperature would reduce the fermentation ability of yeast that can lead to an increase in the cost for yeast and would reduce the concentration of the recovered ethanol that will result in an increase in the cost for dehydration of the derived ethanol solution. Therefore, it is important to design a reactor that ensures a homogenous mixing of the mixture.

To reduce the fermentation time, $d$, it is required to increase the amount of saccharifying enzymes because the rate limiting step of CCSSF is saccharification. In the case of the starchy food waste, when the fermentation time, $d$, is shorten to be 12 h (half of the standard condition) by adding double amount of the enzyme to the fermentation mixture, the amount of ethanol produced in a year, $E$, increases from 292 to 584 kL. Subsequently, the capital cost, $C_{\mathrm{Capital}}$, is reduced from 68 to 34 yen $L^{-1}$, and the other costs, $C_{\mathrm{Others}}$, are reduced from 34 to 17 yen $L^{-1}$, whereas the operating cost, $C_{\mathrm{Operating}}$, increases from 9 to 14 yen $L^{-1}$ because the enzyme cost, $C_{\mathrm{EN}}$, increases from 5 to 10 yen $L^{-1}$. As a result, the total cost of ethanol production, $C$, is reduced from 111 to 65 yen $L^{-1}$ (Table 4.7).

In the case of cellulosic biomass, however, an increase in the saccharification rate would not be expected even when the amount of saccharifying enzymes is increased. It is known that the rate limiting step for saccharification of cellulose is the digestion of its crystalline region by cellobiohydrolase and that the turnover of the enzyme is quite lower than those of amylases. It is also known that the production rate of cellobiose does not increase even when the amount of cellobiohydrolase is increased, and the surface of cellulose is saturated with the enzyme molecules. To shorten the fermentation time for cellulosic materials, therefore, a new cellobiohydrolase with a high turnover for digestion of the crystalline region of cellulose needs to be developed. Alternatively, a new technology that increases the effective substrate concentration of cellulose needs to be developed.

**Table 4.7** Effects of doubling the amount of saccharifying enzyme on the costs of ethanol production from the starchy food waste

| Condition | Standard | Double amount of enzyme |
|---|---|---|
| Fermentation time (h) | 24 | 12 |
| Ethanol production (kL $year^{-1}$) | 292 | 584 |
| Capital cost (yen $L^{-1}$) | 68 | 34 |
| Operating cost (yen $L^{-1}$) | 9 | 14 |
| Other costs (yen $L^{-1}$) | 34 | 17 |
| Production cost (yen $L^{-1}$) | 111 | 65 |

## 4.5 Conclusions

In this chapter, the production costs of three biodegradable municipal solid wastes (BMSW), the starchy food waste, the off-spec rice and the cellulosic waste cotton, by the CCSSF system were estimated to be 111, 50 and 162 yen $L^{-1}$, respectively. In case of starchy material, the production cost was comparable to the target price, 100 yen $L^{-1}$, that was determined by the MAFF. However, the production cost needs to be further reduced in order to earn sufficient profit in the case of cellulosic material. The

capital cost of CCSSF system was small even in a small scale since the CCSSF is a simple and compact system as compared with conventional systems that perform saccharification, fermentation and recovery of ethanol independently.

The possible approaches in order to reduce the production cost of the CCSSF system is the reduction of fermentation time and/or the increase of the capacity of the CCSSF system since these two parameters are found to be the critical parameters in the sensitivity analysis. The fermentation time could be reduced by increasing the amount of saccharifying enzymes and/ or increasing the performance of the saccharifying enzymes. The capacity of the CCSSF system could be increased if a good design of a reactor that enables a homogenous mixing of the mixture is developed.

Nomenclature

| | |
|---|---|
| $a$ | year of depreciation of facility, year |
| $BP$ | balance of payment, yen year$^{-1}$ |
| $C$ | total ethanol production cost, yen L$^{-1}$ |
| $C_B$ | cost for raw materials, yen L$^{-1}$ |
| $C_{capital}$ | capital cost, yen L$^{-1}$ |
| $C_d$ | construction cost for distillation, yen system$^{-1}$ |

| | |
|---|---|
| $C_{EN}$ | cost for enzymes, yen $L^{-1}$ |
| $C_f$ | construction cost for fermentation, yen $system^{-1}$ |
| $C_O$ | annual cost for others, yen $L^{-1}$ |
| $C_{Operating}$ | operating cost, yen $L^{-1}$ |
| $C_{Otherrs}$ | other cost, yen $L^{-1}$ |
| $C_p$ | construction cost for pretreatment, yen $system^{-1}$ |
| $C_s$ | construction cost for saccharification, yen $system^{-1}$ |
| $C_{s+f+d}$ | construction cost of the CCSSF system, yen $system^{-1}$ |
| $C_w$ | construction cost for waste water treatment, yen $system^{-1}$ |
| $C_Y$ | cost for yeast, yen $L^{-1}$ |
| $D$ | operation period, day $year^{-1}$ |
| $d$ | fermentation time, day $batch^{-1}$ |
| $E$ | amount of ethanol obtained in a unit of CCSSF system, kL $year^{-1}$ |
| $M$ | capacity of CCSSF system, $10^3$ kg $batch^{-1}$ |
| $Mw_e$ | molecular weight of ethanol, g-ethanol mol-1 |
| $Mw_g$ | molecular weight of glucose unit in carbohydrate, g-carbohydrate $mol^{-1}$ |
| $S$ | ethanol selling price, yen $L^{-1}$ |

| | |
|---|---|
| $X$ | carbohydrate content of material, g-carbohydrate g-raw-material$^{-1}$ |
| $Y$ | ethanol yield against theoretical yield, – |

Greek letters

| | |
|---|---|
| $\rho_e$ | specific gravity of ethanol, kg L$^{-1}$ |

## Chapter 5 General conclusion and future perspective

### 5.1 General conclusion

Bioethanol has experienced unseen levels of attention due to its value as a renewable and sustainable energy source to save the earth. Currently, bioethanol, as an alternative to gasoline, is produced worldwide mainly from the first generation biomasses such as corn and sugarcane, and the amount of the production reaches to 85.9 billion litter in 2010. However, use of these eatable biomasses for production of ethanol has resulted in the raise in food prices and the shortage of food in developing countries. From this viewpoint, at the 2008 Hokkaido-Toyako Summit, the G8 leaders came to a general agreement "accelerate on the second generation biofuels, which do not require food crop as raw material, in order to bring them into practical production" (2008 Hokkaido-Toyako G8 Summit Interim Compliance Report). Since lignocellulosic biomass is one of the main biomasses in the second generation, an efficient production system of ethanol from this biomass is necessary.

In Japan, from the beginning, the first generation biomasses has not been available for production of bioethanol because Japan's food self-sufficiency ratio is only 40%. Although the second generation biomasses, such as rice straw and waste woods,

are available, the amount of these is quite insufficient for the demand of ethanol as an alternative fuel. Thus, in addition to these main biomasses, food wastes, waste paper and cotton need to be used as the second generation biomass. Since the biomasses in Japan are bulky and scattered in low density, the collection and transportation of them to conventional large scale production facilities are costly and energy-consuming. With the aim to reduce the total production cost and save energy-input for production of ethanol in Japan, alternative new systems that produce ethanol from local biomasses has been required. As one of the most efficient alternative systems, this thesis proposes the CCSSF system that realizes an energy-saving production of ethanol at reasonable costs even in a small scale that ensures flexible handling of various biomasses.

The CCSSF system consists of two parts, ethanol conversion and product recovery, as described in chapter 2. Since the CCSSF is the solid-state fermentation that minimize water, the content of ethanol increases rapidly during the fermentation. To avoid the ethanol inhibition for fermentation ability of yeast, therefore, ethanol converted from biomass is removed as vapor and recovered to the condenser. The concentration of the recovered ethanol becomes higher when the ethanol content in the fermentation mixture is maintained at higher level. As the results, one can save energy for not only recovery but also dehydration of ethanol because the amount of water

evaporated together with ethanol decreases. However, when the ethanol content was maintained at 75–85 g kg-mixture$^{-1}$, it was impossible to perform the repetitive fermentation because the fermentative activity of yeast decreased markedly, whereas ethanol solution at 509±64 g L$^{-1}$ was recovered. If the fermentative activity cannot be maintained, one must supply yeast cells for every batch of fermentation, resulting in an increase in energy and cost for preparation of yeast cells. In the present study, the ethanol content in repetitive fermentation was controlled at 30-50 g kg-mixture$^{-1}$ at 37°C, whereas the average concentration of the recovered ethanol was 233 g L$^{-1}$.

The CCSSF system discharges little waste water that requires energy and cost for treatment. In addition, it would be possible to recycle the residual wastes as fertilizer for agricultural lands where the biomass is harvested because the water content is low (it saves transportation cost).

Generally, bacterial contamination is one of the most serious problems in the bioethanol production process. Lactic acid bacteria are known as the major contaminants in ethanol fermentation. Although addition of antibiotics and antiseptics can prevent contamination, they are costly and not environmental friendly since the wastes of bioethanol production aim to be recycled as fertilizer. Therefore, alternative methods for preventing contamination with low environmental burden and cost are

required. In chapter 3, a simple and practical method was proposed to prevent bacterial contamination in CCSSF. When exogenous ethanol was premixed with the fermentation mixture and ethanol content was maintained, ethanol yield increased with an increase in ethanol content and reached 0.50 g $g^{-1}$ at an initial ethanol content of 60 g $kg^{-1}$, which is almost the same as the theoretical yield. In practical productions of ethanol, by recycling a portion of produced ethanol to the next batch of CCSSF, it is possible to repress contamination without any additional cost. The combination of CCSSF and the present method will realize one of the most ideal bioethanol production processes that is cost-saving and environmentally friendly.

With the aim to evaluate the usefulness of CCSSF for saving the cost for production of ethanol and find parameters that are critical for the cost, the sensitivity analyses of the balance of payment were performed in chapter 4. When a drum shape reactor with a capacity of 5 tons of biomass (3 m in outer diameter and 6 m in length) was supposed to be used, the production costs of ethanol from the starchy food waste, the off-spec rice and the cellulosic waste cotton were estimated to be 111, 50 and 162 yen $L^{-1}$, respectively. Then, the effects of the change in the level of each parameter on the total cost were analyzed. The fermentation time and the capacity of CCSSF system were found to be the most critical among the parameters required for calculation of the

total cost. For the starchy food waste, for example, when the fermentation time is shorten to be half (12 h) by duplicating the amount of enzyme, the production cost was calculated to be reduced from 111 to 65 yen $L^{-1}$ (Table 4.7).

## 5.2 Future perspective

To popularize bioethanol as a sustainable fuel, it is necessary to develop further efficient production systems for saving the cost and energy-input. The main motivation of this study is to realize a cost and energy saving production of ethanol in Japan where a limited amount of biomass is distributed at a low density. Even in a small scale that ensures geometrically-distributed production of ethanol from various biomasses, it was found that the CCSSF system enables to produce ethanol at a reasonable cost from the off-spec rice. For the food waste and the waste cotton, it would be possible to reduce the production cost by the further improvement of the system.

An automated system that can control the ethanol content in the reactor should be developed for easy operation. This will lead to reduction of the cost for operation including cost for labor because one labor can operate several of the CCSSF systems. High concentrations of ethanol can be recovered, when the ethanol content in the reactor is maintained at high levels. At high ethanol content, however, it is difficult to repetitive

the fermentation because of a decrease in the fermentative activity of yeast. Development of yeast strains that are tolerant to high concentrations of ethanol would save the costs for yeast and the dehydration.

Since reduction of the fermentation time can reduce the production cost dramatically as mentioned above, it is necessary to develop an efficient saccharification process, especially for lignocellulosic materials. It is known that the rate limiting step of the fermentation process is hydrolysis of crystalline region of cellulose by cellobiohydrolase consisting of catalytic domain and cellulose binding domain (CBD). It is also known that the velocity of an enzyme reaction becomes non-proportional to the amount of the enzyme when the surface of its substrate is saturated with the enzyme molecules. Igarashi et al. (1997) reported that most of cellobiohydrolase molecules on the surface of cellulose is in "non-productive adsorption" state, resulting in a decrease in apparent activity of cellobuihydrolase (Xu and Ding, 2007; Bommarius et al., 2008). It is reported that removal of CBD reduces non-productive adsorption (Kristensen 2009) but it also reduces the association rate of substrate and enzyme (Nidetzky et al., 1993). However, since the CCSSF is performed under low moisture contents where substrate and enzyme molecules are close together, the association rate would be maintained.

Thus, removal of CBD from cellobiohydrolase would accelerate the saccharification process.

In Japan, one major problem with bioethanol production is the availability of raw materials for the production. The availability of biomasses for bioethanol can vary considerably from season to season and depend on geographic locations. The price of the raw materials is also highly unstable, which can highly affect the production costs of the bioethanol. With the aim to reduce the production cost and produce bioethanol throughout a year, the bioethanol produced from various carbohydrate-content-wastes on CCSSF system have to be investigated.

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