NOVEL TECHNOLOGIES FOR ENHANCED PRODUCTION OF ETHANOL: IMPACT OF HIGH PRODUCTIVITY ON PROCESS ECONOMICS

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In these studies *Saccharomyces cerevisiae* NRRL Y-566 was used to produce ethanol from a concentrated glucose (250-300 g L^{-1}) solution. When fermentation media were supplemented with $CaCO_3$ and $CaCl_2$, ethanol concentrations, yield, and productivities were improved significantly. In control batch fermentation, the culture was able to produce 20.87 g L^{-1} ethanol with a productivity of 0.25 g L^{-1} h^{-1} when using 100 g L^{-1} sugar solution in feed. When supplemented with a solution of 0.40 g L^{-1} $CaCl_2$, ethanol concentration, yield, and productivity were improved to 90.0 g L^{-1}, 0.48, and 1.25 g L^{-1} h^{-1} (500 % increase), respectively. The effect of $CaCO_3$ supplementation was not as pronounced as that of $CaCl_2$. Using these parameters, the process economics for production of ethanol was performed and it was projected that supplementation with 0.40 gL^{-1} $CaCl_2$ would result in the production of ethanol for \$0.91 kg^{-1}. It was also projected that improving productivity to 37.5 g L^{-1} h^{-1} using cell recycle and supplementation with $CaCl_2$ would result in the production of ethanol for \$0.70 kg^{-1} employing *S. cerevisiae* NRRL Y-566. Using *Z. mobilis* in membrane cell recycle reactors and application of $CaCl_2$ can result in achieving high productivities (500-600 g L^{-1} h^{-1}) and reduction in ethanol production price to \$0.59 kg^{-1}.

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Introduction

Depletion of fossil fuel reserves, increased fuel demand due to world's population increase, and uncertainty in its availability have rekindled an interest in alternative biofuels that are renewable and sustainable in nature. Ethanol/or ethyl alcohol fermentation offers promising alternative as it can be produced from various renewable resources such as corn, molasses and agricultural residues and significant research on the production of ethanol from these resources has occurred over the last 3-4 decades.[1-6] However, the basic hurdle to the economical production of ethanol is its high cost of production. The major factors that affect the cost of ethanol production include:

- low reactor productivity,

- requirement of high energy for distillative recovery due to low product concentration in the broth, and often,

- low product yield.

All these factors can be addressed through the application of cutting edge science and technology as outlined below. Additionally, development of superior microbial cultures would be beneficial for this fermentation, however, this is beyond the scope of this article.

Improvement in the reactor productivity can be achieved by the application of suitable reactor designs such as high productivity biofilm reactors or cell recycle membrane reactors, effective nutrient management for efficient cell growth and fermentation, productivity or product enhancers, and process optimization .[3,4,6-12] It has also been shown that several compounds like unsaturated fatty acids and sterols, proteins, amino acids, vitamins, and metal ions can lead to improvements in alcohol fermentation productivity.[13-14] Reduction in energy requirement for distillative recovery can be reduced by increasing product concentration in the fermentation broth as this would reduce size of the distillation column and hence lower process and capital costs. Product yield can also be increased by reducing cell growth by recycling carbon to fermentation product. Some success has been achieved in the development of superior cultures that can improve both yield and product concentration in particular when using lignocellulosic sugars.[15]

Among various ethanol producing micro-organisms *Saccharomyces cerevisiae* has been used most commonly.[16] Objectives of the present studies were to improve and quantify reactor productivity, ethanol yield, and product concentration using *Saccharomyces cerevisiae* NRRL Y-566 on medium supplementation with product enhancers such as $CaCO_3$ and $CaCl_2$. Furthermore, process economics of the developed processes using *S. cerevisiae* NRRL Y-566 and *Zymomonas mobilis* were compared.

Materials and Methods

Organism and maintenance

Lyophilized *Saccharomyces cerevisiae* (NRRL Y-566) was obtained from the culture collection of National Center for Agricultural Utilization Research [formerly Northern Regional Research Laboratory (NRRL), United States Department of Agriculture], Agricultural Research Service, Peoria, IL. For rehydration, sterile water (0.3 mL) was added to the lyophilized yeast and entire content was transferred to a test tube containing 5 mL water and allowed to hydrate overnight. The culture was maintained on YEPD (yeast extract, peptone, and dextrose) medium consisting yeast extract 10 g L^{-1}; peptone, 20 g L^{-1}; glucose, 20 g L^{-1}; and agar, 15 g L^{-1}. The pH of the medium was adjusted to 5.0. Before inoculation, the medium was sterilized in an autoclave for 15 min at 121 ºC followed by cooling to 25 ºC.

Preparation of inoculum and medium

The liquid inoculum development medium contained: yeast extract, 10.0 gL^{-1}; magnesium chloride, 1.00 gL^{-1}; ammonium sulfate, 1.00 gL^{-1}; potassium dihydrogen phosphate, 1.00 gL^{-1}; glucose, 50.00 gL^{-1}; $MnSO_4.7H_2O$, 0.01 gL^{-1}, and; $FeCl_3.2H_2O$, 0.01 gL^{-1}. The pH of the solution was adjusted with dilute sulphuric acid solution to pH 5.5 and then sterilized in an autoclave for 15 minutes at 121 ºC. After the medium (100 mL contained in 250 mL screw capped glass bottle) was cooled to room temperature, a colony of *S. cerevisiae* NRRL Y-566 was transferred to it. Then the culture was kept for growth in an incubator at 30 ºC at an agitation speed of 150 rpm.

Effect of glucose, $CaCO_3$, and $CaCl_2$ concentrations on the batch fermentation process

To evaluate the fermentation performance of the microbial culture and the effects of medium components, batch fermentations were performed in 250 mL Pyrex™ screw capped bottles. The bottles containing 100 mL fermentation medium (glucose 100-300 gL^{-1}) were autoclaved at 121 ºC followed by cooling to 30 ºC. Autoclaved and cooled fermentation media were supplemented with different concentrations of calcium carbonate ($CaCO_3$; 0.0, 0.5, 1.0, 2.0, and 3.0 g L^{-1}; in 250 g L^{-1} glucose solution) and calcium chloride ($CaCl_2$; 0.0, 0.2, 0.4, 0.8, and 1.2 g L^{-1}; in 250 g L^{-1} glucose solution) solutions separately ($CaCO_3$ was autoclaved separately and $CaCl_2$ solutions were sterilized by filtration through 0.22 µm filter) and then inoculated with 24 h old 6 % (v/v) pre-culture/inoculum developed above. All fermentations were conducted in triplicate at 30 ºC and the agitation was maintained at 150 rpm. Two mL samples were taken at regular intervals to measure glucose and ethanol concentrations.

Material balance, energy balance and economic analysis

To estimate ethanol prices, SuperPro Designer Software (version 9.0, built 8, special built 2012, Intelligen, Inc., Scotch Plains, NJ, USA) was used for material balance, energy balance and economic analysis. The equipment and chemical prices reported are for 2014 (SuperPro Designer). The ethanol prices reported are factory gate selling prices (FGSP) and do not include transportation costs. Various parameters that were used to estimate ethanol prices are presented in **Table 1**. The plant capacity was assumed to be 100,000 metric tons of ethanol production per year. In this fermentation there were two by-products (CO_2 and cell mass) and both were sold for credit. The plant was a grass rooted/green field plant with annual operation for 350 days per year. The capital would be borrowed at 7.0 % interest rate until it is paid off.

Analytical procedures

Ethanol concentration was measured using 7890A Agilent Technologies gas chromatograph (Agilent Technologies Inc., Palo Alto, CA, USA) equipped with a flame ionization detector (FID) and 30 m (length) x 320 µm (internal diameter) x 0.5 µm (HP-Innowax film) J x W 19091 N-213 capillary column. The operating conditions were: column temperature 150 ºC (isothermal); program run time: 5.5 min; ethanol retention time: 2.3 min; carrier gas: nitrogen; injector temperature: 175 ºC; detector temperature: 250 ºC; N_2 flow rate: 40 $mL \cdot min^{-1}$; H_2 flow rate: 60 $mL \cdot min^{-1}$; sample quantity: 1 µL with split ratio of 10:1. The supernatant was filtered through 0.22 µm cellulose acetate filter for GC analysis.

Sugar concentrations in samples were analyzed using an Agilent 1100 HPLC system (Agilent Technologies Inc., Palo Alto, CA, USA) equipped with a refractive index detector at 45 ºC. Separation was achieved using a ICSep COREGEL-87H3 column (Transgenomic Inc., Omaha, NE, USA) maintained at 65 ºC with 4 mM H_2SO_4 as the eluent at a flow rate of 0.8 $mL \cdot min^{-1}$. Each fermentation sample was filtered through a 0.22 µm filter and diluted appropriately using deionized water.

Ethanol productivity was calculated as maximum ethanol concentration (g L^{-1}) during fermentation divided by fermentation time (h) and is expressed as g L^{-1} h^{-1}. Fermentation time was considered as time period between inoculation and time at which maximum ethanol concentration was produced. Ethanol yield was estimated as the amount of ethanol produced (g L^{-1}) divided by the amount of glucose utilized (g L^{-1}).

Results and Discussion

Effect of $CaCO_3$ and $CaCl_2$ on fermentation

Batch fermentation experiments were conducted with various initial glucose levels with an aim to obtain high ethanol concentration in the broth. The initial glucose concentrations in the batch experiments were 100, 150, 200, 250, and 300 gL^{-1}. The results of this experiment are presented in **Fig. 1A**. The figure indicates that the ethanol concentrations increased with increase in initial glucose concentrations and fermentation completed in 72 to 84 h. Maximum ethanol production of 34.3 gL^{-1} was obtained when glucose concentration was 250 gL^{-1}. However, when a higher concentration of glucose (300 gL^{-1}) was used, ethanol concentration decreased to 27.4 gL^{-1}.

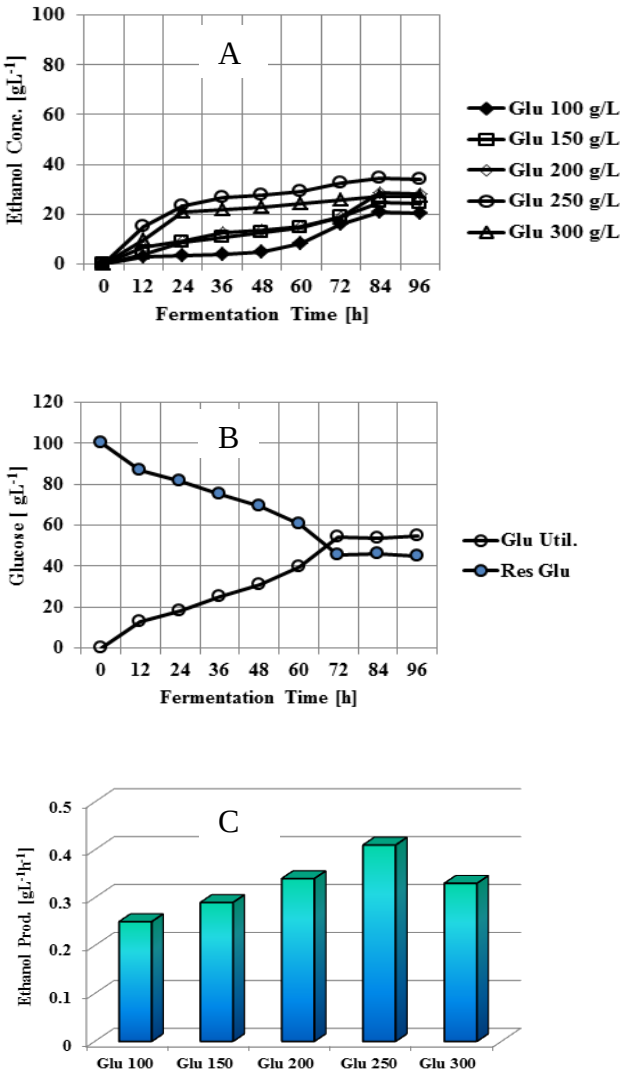

Figure 1. Production of ethanol from glucose using *S. cerevisiae* Y-566 in batch process. A: Ethanol concentration at various time periods and at different glucose concentrations; B: Glucose utilization and residual glucose at 100 gL^{-1} initial glucose; C: Ethanol productivity at different initial glucose levels.

In these fermentations ethanol yield of 0.39 ± 0.03 was obtained. Sugar utilization was also incomplete. In fermentation experiment with 100 gL^{-1} initial glucose, about 55.0 gL^{-1} glucose was utilized leaving behind 45.0 gL^{-1} as residual sugar. The concentrations of utilized and residual glucose are shown in **Fig. 1B**.

In these five experiments run at various sugar concentrations (100-250 gL^{-1} glucose), ethanol productivities ranged from 0.25 to 0.41 $gL^{-1}h^{-1}$ (**Fig. 1C**) which showed an upward ethanol production trend in relation to initial glucose concentration. Since, 250 gL^{-1} glucose was found to produce more ethanol with improved productivity, it was used for further studies. The concentration of ethanol in 100 gL^{-1} glucose fermentation should have been more than that in 250 gL^{-1} fermentation due to the fact that high sugar concentrations are inhibitory to the cell. It is plausible that the initial osmotic pressure exerted on *S. cerevisiae* NRRL Y-566 at high sugar concentration may have induced ethanol production.

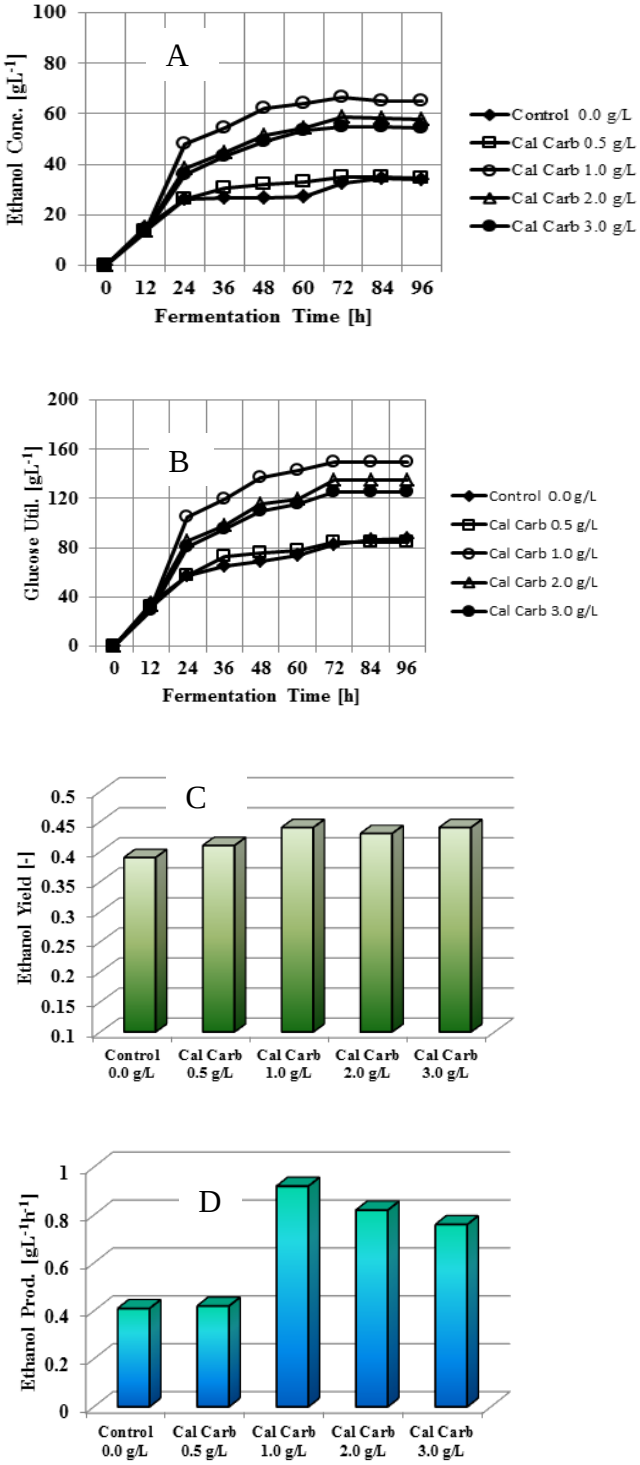

Figure 2. Effect of $CaCO_3$ on ethanol production and productivity from glucose (250 g L^{-1}) in batch process. A: Ethanol at various levels of $CaCO_3$; B: Glucose utilization; C: Ethanol Yield; and D: Ethanol productivity.

Subsequently, experiments were performed where 0.0 to 3.0 gL^{-1} $CaCO_3$ (**Fig. 2A**) was supplemented to the medium. Fermentation medium containing 0.0 g L^{-1} calcium carbonate was considered as the control. The results confirmed that supplementation with calcium carbonate improved ethanol production. At a $CaCO_3$ concentration of 1.0 g L^{-1}, 66.63 g L^{-1} ethanol was produced. This is an increase of 94.1 %.

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Further increase in $CaCO_3$ concentration did not improve ethanol production. At $CaCO_3$ concentrations of 2.0 and 3.0 gL^{-1}, ethanol concentrations of 59.0 and 55.0 gL^{-1} were obtained, respectively. Utilization of glucose was the highest (150.0 gL^{-1}) at 1.0 gL^{-1} $CaCO_3$ concentration (**Fig. 2B**). As compared to the control experiment, ethanol yield improved slightly (**Fig. 2C**) to 0.44. It should be noted that productivity improved due to increased production of ethanol and faster fermentation. At a $CaCO_3$ concentration of 1.0 gL^{-1} fermentation was complete in approximately 72 h as opposed to 84 h for the control fermentation. In this run a productivity of 0.92 $gL^{-1}h^{-1}$ was obtained (**Fig. 2D**) which is 224% of that achieved in the control run.

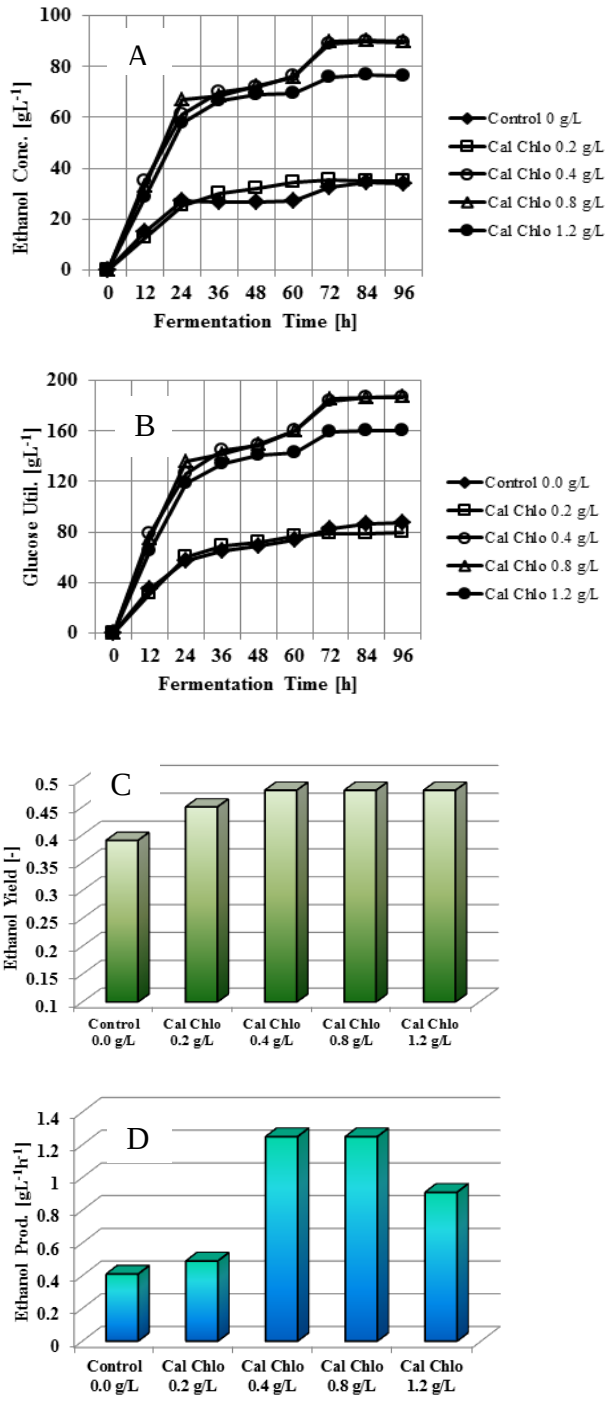

Figure 3. Effect of $CaCl_2$ on ethanol production and productivity from glucose (250 gL^{-1}) in batch process. A: Ethanol; B: Glucose utilization; C: Yield, and; D. Productivity.

Next, experiments with calcium chloride ($CaCl_2$) supplementation were performed where $CaCl_2$ ranging from 0.0 to 1.20 gL^{-1} was added to the medium. At $CaCl_2$ concentrations of 0.40 to 1.20 gL^{-1} fermentation improved dramatically. At a $CaCl_2$ concentration of 0.40 gL^{-1} maximum ethanol production (89.8 gL^{-1}) was achieved (**Fig. 3A**). This ethanol concentration is 262% of that achieved in the control experiment. Although, there was marked increase in ethanol production, the fermentation broth still contained residual sugars. Glucose consumption increased from 86.4 gL^{-1} to 186.5 gL^{-1} when 0.40 gL^{-1} calcium chloride was added to the medium. The utilization of sugars is shown in **Fig. 3B**. In these fermentations, ethanol yield also increased with a maximum of 0.48 at $CaCl_2$ concentrations ranging from 0.40-1.20 gL^{-1} (**Fig. 3C**). The highest ethanol productivity of 1.25 $gL^{-1}h^{-1}$ was achieved at a $CaCl_2$ concentration of 0.40 gL^{-1} (**Fig. 3D**).

An early study on effect of $CaCl_2$ on ethanol fermentation was performed by Bajpai and Margaritis using a bacterial strain of *Zymomonas mobilis*.[17] In these studies they indicated that there was no appreciable change in rates of cell mass production and ethanol formation in the medium containing up to 2.0 gL^{-1} $CaCl_2$. Further increases in $CaCl_2$ concentrations, resulted in decreased cell growth and ethanol production rates. These studies were followed by Sreekumar and Basappa [18] who demonstrated that supplementation of $CaCl_2$ and $CaCO_3$ to the fermentation medium enhanced ethanol production. The only difference between studies performed by these two groups was that Bajpai and Margaritis[17] used 100 gL^{-1} glucose solution while Sreekumar and Basappa[18] used 200-400 gL^{-1} glucose in their medium. Hence, it was concluded that calcium salts enhance ethanol concentration and yield in presence of high sugar concentration. It should be noted that there was no mention of increase in ethanol productivity and the microorganism used was a bacterium and not yeast.

Similar studies were performed by Nabais et al. for the production of ethanol using yeasts.[19] The cultures that were used included *S. bayanus* IST 154, *S. cerevisiae* IGC 3507 III and *Kluyveromyces marxianus*. It was observed that supplementation of fermentation medium with $CaCl_2$ resulted in the rapid production of higher concentrations of ethanol from high glucose concentration (320 gL^{-1}). It was also reported that calcium in optimal concentrations somehow protects the culture from toxic effects of ethanol, [19-20] and hence, results in the accumulation of higher concentration of ethanol in the broth which is economically beneficial for the product recovery. Similar studies were also performed for a commercial substrate (corn semolina) with similar observations.[21] In these studies it was reported that mineral salts take part in yeast metabolism as the activators of enzymes or are part of the enzyme in their active center. However, none of these authors investigated the effect of calcium carbonate on *S. cerevisiae* fermentation and on ethanol productivity.

Although supplementation of concentrated glucose medium with calcium salts results in enhanced production of ethanol, from process engineering point of view, use of concentrated sugar solution is preferred [4, 8, 22, 23] as it would reduce capital and process operational costs thus benefitting the economics of the process. Additionally, application of concentrated sugar solution would result in more concentrated product[9] in the broth which would further

reduce energy requirement for product separation by distillation. In these studies we were able to use 250 gL^{-1} sugar solution and accumulated approximately 90.0 gL^{-1} ethanol. In our process, use of calcium salts in combination with concentrated sugar solution resulted not only in enhanced ethanol concentration, they also enhanced yield, and productivity. The productivity was improved by 500% which would dramatically impact the economics of the process that is presented below.

Process Economics

Based on the data generated above, process economics of ethanol production was evaluated. For this purpose a plant with annual capacity of 100,000 tons of ethanol per year was considered with 350 working days per year. The process economic details of the plant are presented in **Table 1** while a process flow diagram is shown in **Fig. 4**.

Table 1. Parameters that were used to evaluate the process economics of ethanol production.

Plant Capacity:	100,000 metric tons ethanol $year^{-1}$
Ethanol yield:	0.48
Glucose price:	\$0.20 kg^{-}
Plant operation:	350 $days \cdot year^{-1}$; continuous process
Plant life:	15 years
Glucose conc. in feed:	186 $g \cdot L^{-1}$
Ethanol conc in effluent:	89-90 $g \cdot L^{-1}$
Plant:	Grass rooted or green field
Plant & capital details:	Depreciation 10 % straight line, depreciation period 10 years, tax 40 % on profit
Product recovery:	Distillation
Year of analysis:	2014, construction period 30 months, construction start 2014 & start up period 4 months

The total direct fixed capital (TDFC), working capital (WC), and start costs (SC) were projected to be \$192.5 x 10^6, \$4.7 x 10^6, and \$9.6 x 10^6, respectively (**Table 2**). Ethanol, cell mass, and carbon dioxide were considered as revenue streams with cell mass and CO_2 selling prices of \$0.05 kg^{-1} each. Using these parameters ethanol production cost was projected to be \$0.91 kg^{-1} (\$2.83.US gal^{-1}). The operating cost of the plant was projected to be \$91.32 x 10^6. $year^{-1}$. For these calculations ethanol productivity of 1.25 $gL^{-1}h^{-1}$ was considered as obtained in the above studies using $CaCl_2$ as productivity enhancer.

In numerous publications, it has been presented that the productivity of ethanol, and acetone-butanol-ethanol (ABE) can be increased by a factor of 30-45 by application of cell recycle technology.[7, 24-26] The reason behind this productivity increase is the high cell concentration that can be achieved in the cell recycle bioreactor.

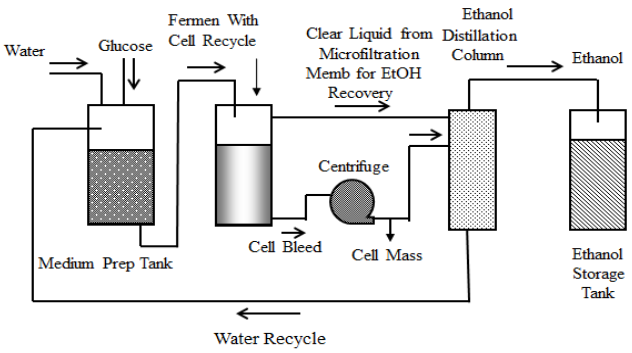

Figure 4. A schematic diagram of ethanol production from glucose by *S. cerevisiae* Y-566 or *Z. mobilis* in cell recycle continuous process employing $CaCl_2$ to enhance ethanol productivity.

In these cell recycle bioreactors cell concentration in excess of 80-100 $g \cdot L^{-1}$ can be achieved as compared to cell concentration in free cell batch reactors which is usually of the order of 3-5 g L^{-1}. Considering this increase in productivity, we assumed that the productivity can be increased by a factor of at least 30. Use of 0.40 g L^{-1} $CaCl_2$ resulted in an increase in productivity by a factor of 5. A combination of application of $CaCl_2$ and cell recycle technology is expected to result in a productivity of 37.5 g L^{-1} h^{-1} (1.25 x 30). Using this productivity we calculated price of ethanol production to be \$0.70$\cdot kg^{-1}$ (\$2.18$\cdot$US gal^{-1}). The various economic details for this process are presented in **Table 2**.

The productivity of ethanol production by *Z. mobilis* in batch reactors is reported to be 4-6 $g \cdot L^{-1} h^{-1}$ [25, 27] which was improved to 120 $g \cdot L^{-1}$ h^{-1}. Further improvement in this productivity by application of $CaCl_2$ is possible. If use of $CaCl_2$ can increase this productivity by a factor of 5, the cell recycle experiment would result in a productivity of 600 $g \cdot L^{-1} h^{-1}$. We considered a productivity of 500 $g \cdot L^{-1} h^{-1}$ and performed a cost estimation. For this plant total capital investment was estimated to be \$20.0 x 10^6 and it was projected that by using this technology ethanol can be produced for \$0.59 kg^{-1}.

In conclusion it has been shown that *S. cerevisiae* NRRL Y-566 was able to grow and produce ethanol in concentrated sugar solutions (250-300 g L^{-1}). With the use of $CaCO_3$ and $CaCl_2$ both ethanol concentrations and productivities were improved significantly. In a control batch fermentation the culture produced less than 20.87 g L^{-1} ethanol when using 100 g L^{-1} sugar solution with a productivity of 0.25 g $L^{-1} h^{-1}$. When using 0.40 g L^{-1} $CaCl_2$ solution, both ethanol concentration and productivity were improved to 90.0 g L^{-1} and 1.25 g L^{-1} h^{-1}, respectively. Also ethanol yield was improved to 0.48 which is 94 % of theoretical value and is close to commercial yield. Using these parameters, ethanol's process economics was performed and it was projected that supplementation with 0.40 g L^{-1} $CaCl_2$ would result in the production of ethanol for \$0.91 kg^{-1}. It was also projected that improving productivity to 37.5 g $L^{-1} h^{-1}$ would result in the production of ethanol for \$0.70 kg^{-1}. It is possible to achieve this productivity with the combination of $CaCl_2$ supplementation and the use of cell recycle technology when employing *S. cerevisiae* NRRL Y-566.

Table 2. Process economics of ethanol production from corn derived glucose using *Saccharomyces cerevisiae* Y-566 and *Zymomobilis mobilis*.

Parameters	*S. cerevisiae* Y-566		*Z. mobilis*
	Prod. 1.25 g L^{-1} h^{-1}	**Prod. 37.5 g L^{-1} h^{-1}**	**Prod. 500 g L^{-1} h^{-1}**
A Direct fixed capital [$]	192,525,000	79,547,000	20,010,000
B Working capital [$]	4,674,000	4,674,000	4,674,000
C Startup cost [$]	9,626,000	3,977,000	1,000,000
D Total investment (A+B+C) [$]	206,825,000	88,198,000	25,684,000
E Investment charged to project [$]	206,825,000	88,198,000	25,684,000
F **Production Rates**			
CO_2 [kg·$year^{-1}$]	104,168,400	104,168,400	104,168,400
Cell mass [kg·$year^{-1}$]	49,749,840	49,749,840	49,749,840
Ethanol [kg·$year^{-1}$]	100,000,000	100,000,000	100,000,000
G **Revenue Price**			
CO_2 [$·$kg^{-1}$]	0.05	0.05	0.05
Cell mass [$·$kg^{-1}$]	0.05	0.05	0.05
H **Revenues/savings**			
CO_2 [$·$year^{-1}$]	5,208,420	5,208,420	5,208,420
Cell mass [$·$year^{-1}$]	2,487,492	2,487,492	2,487,492
Ethanol [$·$year^{-1}$]	91,000,000	70,000,000	59,000,000
Total revenues [$·$year^{-1}$]	**98,695,912**	**77,695,912**	66,695,912
I **Annual Operating Cost (AOC)**			
Actual AOC [$.$year^{-1}$]	91,318,000	70,021,000	58,799,000
J **Unit Production Cost/Revenue [$.$kg^{-1}$]**	**0.91**	**0.70**	**0.59**
K Gross Profit (H-I) [$.$year^{-1}$]	7,377,912	7,385,000	18,608,000
L Taxes (40%) [$.$year^{-1}$]	2,951,165	2,954,000	7,443,000
M Depreciation [$.$year^{-1}$]	18,289,000	7,557,000	1,901,000
N Net profit (K-L+M) [$.$year^{-1}$]	22,715,747	11,988,000	24,231,000

Prod. - Productivity

Table 3. A summary of production of ethanol from glucose using *S. cerevisiae* NRRL Y-566 supplemented with $CaCO_3$ and $CaCl_2$.

Process	Initial sugar [g L^{-1}]	Max. ethanol concn. [g L^{-1}]	Yield [-]	Productivity [g L^{-1} h^{-1}]
Control	100	20.83	0.39	0.25
$CaCO_3$ (1.0 g L^{-1})	250	65.63	0.44	0.92
$CaCl_2$ (0.40 g L^{-1})	250	90.00	0.48	1.25

Using *Z. mobilis* in membrane cell recycle reactors and application of $CaCl_2$ could result in achieving high productivity (500-600 g L^{-1} h^{-1}) and reduction of ethanol production price to \$0.59 kg^{-1}. The results obtained in these studies have been summarized in **Table 3**.

In brief the objectives mentioned in the introduction section of this article have been achieved. Three most important factors (ethanol concentration, yield, and productivity) for ethanol production from corn or corn derived glucose have been improved.

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