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Recycling of yeast cells for simultaneous saccharification and fermentation of liquefied starch of rice flour

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ABSTRACT

Different concentrations (150, 200, 280 and 300 gL^{-1}) of rice flour were hydrolysed either by one-step (simultaneous) or two-steps liquefaction and saccharification with α-amylase (2.5 KNU g DS^{-1}) and glucoamylase (2.3 AGU g DS^{-1}) for 4h. Hydrolysis of rice flour suspension (10L) by two-steps process led to better starch hydrolysis than the single step process. The hydrolysate (DE 88.2) obtained by the hydrolysis of 280 gL^{-1} rice flour fermented by bakers yeast led 25.3 gL^{-1} ethanol at 48h. Addition of glucoamylase (0.53 AGU mL^{-1}) increased ethanol production to 64 gL^{-1} at 96h. When 280 gL^{-1} rice flour liquefied with 2.5 KNU g DS^{-1} was subjected to simultaneous saccharification with different amounts of glucoamylase at 48h, 95.3 gL^{-1} ethanol was obtained with 2.3 AGU g DS^{-1}. Replacing the commercial glucoamylase with that produced in the lab gave similar ethanol yield and efficiency. With yeast cell recycling, the efficiency and yield of ethanol production were decreased. Intermittent addition of nutrient medium improved the ethanol production by yeast recycling.

Key Words: Liquefaction, Saccharification, simultaneous saccharification and fermentation, cell-recycle, ethanol.

INTRODUCTION

Ethanol is a large volume low value product obtained by the fermentation of glucose/sucrose. For ethanol production much attention has been paid to make it economically viable. For this purpose two major factors such as cost of the substrate and mode of fermentation are taken into consideration. Most common organisms used for ethanol production are *Saccharomyces cerevisiae* and *Zymomonas mibilis.* As these organisms lack starch/cellulose hydrolyzing ability, modified carbon sources [1-4] and alternative organisms [1, 5-10] were tried. Naturally available amylolytic yeasts have shown low ethanol tolerances and limited dextrin conversions [10-13]. Genetically modified yeast possessing amylolytic properties was also tried for direct starch conversion to ethanol [6-7]. Since the use of alternative organisms to was not successful, simultaneous saccharification and fermentation of liquefied starch/cellulose was tried [14-22]. Since *Saccharomyces cerevisiae* is not viable at and above 50^{o}C, simultaneous liquefaction, saccharification and fermentation is not feasible. Hence thermotolerant yeasts were tried [23]. In another study raw cassava root starch was transformed into ethanol by one-step process of fermentation in which liquefaction, saccharification and fermentation were combined [24]. As these were not feasible in large-scale operations it is possible to combine saccharification and fermentation [11, 16-18]. Combining saccharification and fermentation reduces the use of more number of reactors and reactor volume[21]. Further the osmotic effect of glucose on the organisms and inhibitory effect of glucose on glucoamylase can be reduced.

We have reported the advantages of using one-step starch hydrolysis to glucose [25-28]. In this paper hydrolysis of starch in rice flour either by combining the liquefaction and saccharification (one-step) or by carrying out the above two processes in two-steps is reported. To compare the two processes and to select the best process, starch in rice

flour of different concentrations was hydrolysed by α-amylase and glucoamylase. To achieve high ethanol yield saccharification and fermentation were combined and the possibility of recycling yeast cells was studied.

MATERIALS AND METHODS

Materials

Rice was purchased from local market and pulverized in a domestic grinder. Thermostable α-amylase of *Bacillus licheniformis* (Termamyl 60L, Activity 67.5KNUg^{-1}) and glucoamylase from *Aspergillus niger* (Spiritamylase 150L, Activity 159.9AGUg^{-1}) were purchased from Novo Industries, Denmark. KNU – Kilo Novo Unit: AGU – Amylo Glucosidase Unit, DS - Dry Substance; One KNU is defined as the amount of enzyme which breaks down 5.26 g/h starch (Merk Amylum Soluble, Erg.B.6 Batch 9947275) at 37^{o}C and pH 5.6, whereas one AGU is defined as the amount of enzyme which hydrolyses 1μmol/min at pH 4.3 and 25^{o}C.

Microorganisms

Aspergillus niger CFTRI 1105 was from Central Food Technical Research Institute, Mysore, India and a bakers yeast, (Fermipan) was from Gist Borcades, The Netherlands.

Analytical method

Reducing sugar [29], total sugar [30], ethanol [31], viable yeast cell count [33] and glucoamylase activity [34] were determined by standard methods.

Hydrolysis of starch in rice flour

Simultaneous liquefaction and saccharification - one-step process

Rice flour suspended in tap water (160, 200, 280 and 300 L^{1}) was hydrolysed by α-amylase (2.5 KNU g DS^{-1}) and glucoamylase (2.3 AGU g DS^{-1}) at pH 5.0 and 70^{o}C. Total weight was adjusted to 10 kg with tap water and incubated at 70^{o}C for 4h while mixing. The hydrolysate was filtered through a Whatman Number one filter paper and the extract was analyzed for reducing [29] and total sugar [30].

Liquefaction and saccharification in two-steps – two-steps process

Rice flour suspended in tap water (160, 200, 280 and 300 gL^{-1}) at pH 7.0, hydrolysed with α-amylase (2.5 KNU g DS^{-1}) for 1h at 95^{o}C. For saccharification, to the cooled hydrolysate at pH 4.6, glucoamylase (2.3 AGU g DS^{-1}) was added and incubated at 50^{o}C for 3h while mixing.

Preparation of yeast inoculum

Dry yeast (5 gL^{-1}) was added to sterile sucrose solution (50 gL^{-1}) at pH 4.5, and incubated at 35^{o}C. The 18h old inoculum was inoculated (10%, v/v) to a sterile glucose solution (10 gL^{-1}) and incubated for 18h and used as inoculum.

Fermentation of rice flour hydrolysate obtained by two-steps process

Rice flour hydrolysate at pH 4.5 inoculated with (2.9x10^{8} cells mL^{-1}, 10%, v/v) yeast inoculum and incubated at 35^{o}C and 100 rpm. Ethanol produced [31] and residual total [30] and reducing sugar [29] were estimated.

Addition of glucoamylase to rice flour hydrolysate obtained by two-steps process

To sterile rice flour hydrolysate obtained by two-steps process, glucoamylase (0.53AGUgDS^{-1}), and yeast inoculum (2.9x10^{8} cell mL^{-1}, 10%, v/v) were added and incubated at 35^{o}C, (100 rpm).

Simultaneous saccharification with different concentrations of glucoamylase & fermentation

Rice flour suspended in tap water (280 gL^{-1}) at pH 7.0 was liquefied with α-amylase (2.5 KNU g DS^{-1}) at 95^{o}C and pH 7.0 for 1h. Liquefied starch solution at pH 4.5 was mixed with glucoamylase of different concentrations (0.53, 1.15 and 2.3 AGU g DS^{-1}) and yeast inoculum (10%, v/v).

Simultaneous saccharification by glucoamylase (prepared in the lab) & fermentation of liquefied starch

Production of glucoamylase by solid-state fermentation

Glucoamylase was produced by *Aspergillus niger* in solid medium [34]. Liquefied rice flour starch extract at pH 4.5 was subjected to simultaneous saccharification and fermentation (SSF) either with commercial or lab produced glucoamylase preparations (2.3 AGU g DS^{-1}) and yeast (10%, v/v).

Recycling of yeast cells for SSF by of liquefied starch

Liquefied rice flour starch extract (pH 4.5) mixed with lab produced glucoamylase (2.3 AGU g DS^{-1}) and yeast (10%, v/v). Ethanol [31] produced and the total [30] and reducing sugar [29] in the medium were monitored. At 48h

the cells were allowed to settle and the extract was decanted. Then to the cells liquefied rice flour starch and lab produced glucoamylase were added. This procedure was repeated till the ethanol production decreased.

Yeast cell recycling in SSF with intermittent addition of nutrient rich medium

During SSF with cell recycling, whenever ethanol yield started to decrease, to the residual cells fresh sterile nutrient medium (consisting of 4 gL^{-1} yeast extract, 1 gL^{-1} $(NH_4)_2SO_4$ and 10 gL^{-1} glucose) was added and incubated for 24. Then liquefied rice flour starch extract and glucoamylase produced in the lab (2.3 AGU g DS^{-1}) were added. Whenever ethanol production decreased, to the cells nutrient medium was added. This procedure was repeated till the ethanol production decreased.

RESULTS AND DISCUSSION

Hydrolysis of starch rice flour

Starch in rice flour can be hydrolyzed either by combining the liquefaction and saccharification (one-step) or by carrying out the above two process in two-steps [26]. To compare the two processes and to select the best process, starch in rice flour of different concentrations was hydrolysed with α-amylase (2.5 KNU g DS^{-1}) and glucoamylase (2.3 AGU g DS^{-1}). At all rice flour concentrations studied the recovery of reducing sugars and the DE were higher when the hydrolysis was performed in two-steps than in one-step process. With increase in rice flour concentration from 160 to 300 gL^{-1} the recovery in one-step process decreased than that in the two-steps process (Table 1). When the process was carried out in 10mL level, it was observed that the one-step process was superior to the two-steps process [26] but scaling up of the process has shown that one-step process is inferior to the two-steps process. The reduction in recovery and DE in one-step process could be because the conditions are not optimum to both α-amylase and glucoamylase [26]. For further studies the hydrolysate obtained from 280 gL^{-1} rice flour suspension was selected because mixing of the 300 gL^{-1} rice flour suspension in the initial stages is difficult.

Table 1: Recovery of reducing sugars and DE values obtained by the hydrolysis of different concentrations of rice flour either by one –step (simultaneous liquefaction and saccharification) or two-steps (liquefaction and saccharification in separate steps) processes using α-amylase (2.5 KNU g^{-1} DS) and glucoamylase (2.3 AGU g^{-1} DS) for 4h

Rice flour (gL^{-1})	Recovery (%)		DE (%)	
	One-Step	Two-Steps	One-Step	Two-Steps
160	85.4	99.4	76.6	95.0
200	70.8	93.8	74.2	91.4
280	56.8	92.1	70.0	88.2
300	55.0	90.9	69.3	83.8

In all the processes the total volumes of the reaction mixture were 10 litres.

Fermentation of rice flour hydrolysate

Fermentation of rice flour hydrolysate (280 gL^{-1} rice flour by the two-steps process, DE 88.2%, 220.6 gL^{-1} total sugar) by yeast produced 25.3 gL^{-1} of ethanol at 48h, and extended incubation time up to 96h has not increased the ethanol production (Control, Table 2). The efficiency of ethanol production was 83.9% while the yield was 22.9%. Of the total sugar available in the medium, 72.7% of the sugars were not utilized. The incomplete utilization of sugar could be due to several reasons. The DE of the hydrolysate used was 88.2 and hence some sugars were present as oligosaccharides, having more than two sugar units, which cannot be utilized by yeast because the organism can use only mono and disaccharides [7]. Another reason would be that high amount of reducing sugar would have exerted osmotic pressure and inhibited the ability of the cells to multiply and ferment [2]. Inoculum was prepared in glucose solution and it is devoid of other nutrients. The rice flour hydrolysate extract contained 0.4 gL^{-1} soluble proteins and 0.2 gL^{-1} total amino acids. Thus even though the organism was activated in glucose solution, for fermentation studies, proteins and amino acids containing rice flour hydrolysate was used as the medium. Therefore insufficiency of nutrients cannot be considered as a serious factor to affect the ability of the organisms to produce ethanol. The main factor that could be considered is the incomplete hydrolysis of starch. Therefore an experiment was performed by adding glucoamylase to the hydrolysate (DE 88.2) and to perform simultaneous saccharification of the residual oligosaccharides and fermentation.

Addition of glucoamylase to rice flour hydrolysate obtained by two-steps process during fermentation

After the addition of glucoamylase, the sugar in the hydrolysate was better utilized and ethanol production efficiency was 78.4%, while the yield was 46.9% at 48h (Test, Table 2). At 72h, the efficiency of ethanol production and ethanol yield were 83.2 and 53.5% respectively. With further increase in incubation time, the ethanol yield has not increased significantly. These experiments were carried out in 10 litre volumes. The sugar utilization was better in the test than in the control. In the test and the control 72.7 and 34.1% of the total sugar respectively was left at 48-

96h. Thus the addition of glucoamylase has improved the saccharification and the sugar was utilization for feermenttion. However this has not continued beyond 72h. This could be due to insufficient amount of glucoamylase added or the yeast cells have reached the lag phase and have not fermented the sugars produced by saccharification process. But it is evident from the results that there was no increase in the reducing sugar concentration after 48h (Table 2). Thus from the results, it can be concluded that the glucoamylase added was not sufficient for the hydrolysis of the residual tri-, tetra- and oligosaccharides in the medium. As the results obtained with the addition of glucoamylase with simultaneous fermentation gave better ethanol yield and ethanol production efficiency an experiment was carried out to find the suitable concentration of glucoamylase for SSF.

Table 2: Effect of glucoamylase (0.0-control and 0.53-test AGU g L^{-1}) on the fermentation of rice flour hydrolysate obtained by the hydrolysis of 280 gL^{-1} rice flour in two-steps process (DE 88.2)

Time (h)	Reducing sugar (gL^{-1})		Total sugar (gL^{-1})		Ethanol (gL^{-1})	
	Test	Control	Test	Control	Test	Control
0	194.6	194.6	220.6	220.6	-	-
24	118.6	149.7	140.5	175.7	35.6	20.7
48	68.6	132.3	88.7	160.3	51.7	25.3
72	59.6	132.3	78.7	160.3	59.0	25.3
96	58.0	132.3	75.2	160.3	64.0	25.3

Simultaneous saccharification by different concentrations of glucoamylase & fermentation

In this experiment when the liquefied starch was used for ethanol production (no glucoamylase was added) highest amount of ethanol was produced at 48h and the ethanol yield and ethanol production efficiency were 15.7 and 43.4% respectively (Table 3). When glucoamylase of 0.53, 1.15 and 2.3 AUG g DS^{-1} activity were added, highest ethanol was produced at 72, 72 and 48h. At these respective glucoamylase concentration and time the ethanol yields and efficiencies were 47.8 & 91.3%; 72.5 & 94.0% and 90.7 & 93.6%. With increase in added glucoamylase concentration, ethanol yield was increased. Ethanol production efficiency was increased and was almost same with 1.15 and 2.3 AGU g DS^{-1} glucoamylase concentrations. Since the highest ethanol production and ethanol yield were achieved at 48h with 2.3 AGU g DS^{-1} glucoamylase, it was decided to use this glucoamylase concentration to obtain highest ethanol production yield and efficiency in short time. Thus here the same glucoamylase concentration used with two-steps process was able to provide better ethanol yield and ethanol production efficiency. Similar high substrate concentration was fermented in acceptable time when high enzyme levels were used for saccharification [20]. The results indicated that the reducing sugars produced by the saccharification process was better utilized by yeast and the ethanol production efficiency was improved as the initial reducing sugar concentration was less, which usually exert osmotic effect on yeast and reduces the cell metabolism [2 & 20]. Simultaneous saccharification and fermentation was able to improve ethanol production yield and efficiency. One advantage with this enzyme was that the optimum pH for the activity of glucoamylase was 4.6 [26] and the fermentation was also carried out at pH 4.5. Thus the enzyme activity was 100% at this pH. Further glucoamylase is also inhibited by high concentrations of glucose [14]. Glucoamylase also has the reversing effect of saccharification and forming polymers of glucose, if it is incubated with the glucose for long time [35]. Since the highest glucoamylase concentration was selected, it was decided to replace the commercial enzyme with lab-produced glucoamylase [34].

SSF of liquefied starch in rice flour with lab prepared or commercial glucoamylases and yeast

Highest ethanol produced in the medium containing commercial and home made glucoamylase were 95.3 and 96.8gL^{-1} at 48h, i.e. there was no significant difference in the ethanol production efficiency (86.4 & 87.8) and yield (94.2 & 95.3%). Hence the commercial glucoamylase can be replaced with the home made glucoamylase.

Yeast cell recycling and SSF

To avoid the repeated inoculum preparation cell recycling was tried. When liquefied rice flour extract of DE of 45.3 having 210.6 gL^{-1} total sugars was used as medium (without additional protein & other nutrients) in the first cycle at 48h, 96.8 gL^{-1} ethanol was obtained. With increase in the cell recycle process from I to VII, ethanol produced has decreased from 96.8 to 11.9 gL^{-1}. Ethanol production efficiency and yield were also decreased. Decrease in ethanol production could be due to the removal of young cells with the decanted spent medium and accumulation of dead cells in the residue obtained when the fermentation medium was allowed to settle. This was supported with the viable cell number in the residue (Table 5).

Table 3: Simultaneous saccharification and fermentation of sugars in liquefied rice flour hydrolysate (DE 45.3) by different amounts of glucoamylase and yeast. Control indicates the activity of glucoamylase on liquefied starch at 35°C and pH 4.5 without the inoculation of yeast.

Time (h)	Glucoamylase AGU mL^{-1}																			
	0.0					0.53					1.15					2.3				
	Control		Test			Control		Test			Control		Test			Control		Test		
	Sugar (gL^{-1})		Sugar (gL^{-1})		Ethanol (gL^{-1})	Sugar (gL^{-1})		Sugar (gL^{-1})		Ethanol (gL^{-1})	Sugar (gL^{-1})		Sugar (gL^{-1})		Ethanol (gL^{-1})	Sugar (gL^{-1})		Sugar (gL^{-1})		Ethanol (gL^{-1})
	Reducing	Total	Reducing	Total		Reducing	Total	Reducing	Total		Reducing	Total	Reducing	Total		Reducing	Total	Reducing	Total	
0	95.3	210.6	95.3	210.6	0.0	95.3	210.6	95.3	210.6	0.0	95.3	210.6	95.3	210.6	0.0	95.3	210.6	95.3	210.6	0.0
24	100.2	210.0	77.9	193.2	5.2	150.3	211.3	68.4	158.2	23.3	185.3	210.1	120.3	130.6	38.6	198.6	210.0	96.2	104.3	48.6
48	102.8	211.0	23.3	134.6	16.5	175.9	211.8	5.8	100.4	45.2	208.6	212.5	52.1	59.4	74.6	209.3	211.2	7.8	8.3	95.3
72	103.6	210.8	23.1	132.3	16.5	190.2	211.4	5.6	100.4	50.9	209.3	210.4	43.2	48.3	76.3	210.0	212.6	6.6	7.2	95.2

Table 4: Simultaneous saccharification and fermentation of liquefied starch in rice flour (DE 45.3) at pH 4.5 and 35°C by commercial glucoamylase (Spiritamylase 2.3 AGU g DS^{-1}) or locally produced glucoamylase (2.3 AGU g DS^{-1}) and yeast (Fermipan, 10^8 Cells mL^{-1})

Time (h)	Glucoamylase (2.3 AGU g DS^{-1})					
	Commercial			Locally Prepared		
	Sugar (gL^{-1})		Ethanol (gL^{-1})	Sugar (gL^{-1})		Ethanol (gL^{-1})
	Reducing	Total		Reducing	Total	
0	95.3	210.6	-	95.3	210.6	-
24	96.2	104.3	48.6	98.4	100.5	48.3
48	7.8	8.3	95.3	6.3	7.4	96.8
72	6.6	7.2	95.2	6.5	7.3	96.5

Table 5: Simultaneous saccharification and fermentation of liquefied starch in rice flour (DE 45.3) at pH 4.5 and 35°C by locally prepared glucoamylase (2.3 AGU g DS $^{-1}$) and recycling of yeast cells

Cycle Number	Time (h)	Viable Cell No.	Sugar (gL^{-1})		Ethanol		
			Reducing	Total	Amount (gL^{-1})	Efficiency (%)	Yield (%)
I	0		95.3	210.6	0.0		
	24		98.4	100.5	48.3		
	48	8.0×10^8	6.3	7.4	96.8	95.8	91.9
II	0		95.3	210.6	0.0		
	24		125.5	128.5	32.1		
	48	7.9×10^7	15.4	16.0	76.1	78.2	72.2
III	0		95.3	210.6	0.0		
	24		136.2	138.2	21.8		
	48	4.5×10^6	19.3	20.4	57.3	60.2	54.3
IV	0		95.3	210.6	0.0		
	24		116.2	119.2	20.0		
	48	6.2×10^5	30.6	32.5	42.5	47.7	40.3
V	0		95.3	210.6	0.0		
	24		129.8	134.8	14.9		
	48	3.1×10^4	40.3	44.8	32.6	39.3	30.9
VI	0		95.3	210.6	0.0		
	24		140.4	143.4	9.85		
	48	1.6×10^4	58.4	60.8	22.0	29.3	20.8
VII	0		95.3	210.6	0.0		
	24		146.5	149.6	6.8		
	48	7.8×10^3	100.1	103.2	11.9	22.1	11.2

Thus alternative measure has to be taken to maintain the viable cells in the medium. One alternative could be the addition of nutrient rich synthetic medium to activate the cells in between the cycles. The usual nutrient medium used for the activation of yeast cells consist of 4.0 gL^{-1} yeast extract, 1.0 gL^{-1} $(NH_4)_2SO_4$ and 10 gL^{-1} glucose [36]. The rice flour hydrolysate contained 0.4 gL^{-1} soluble proteins and 0.2 gL^{-1} total amino acids. For single batch of experiment with yeast, the nutrients available in rice flour hydrolysate was acceptable but for repeated cell use, the yeast cells seems to require nutrient rich medium. Therefore an experiment was carried out by adding nutrient rich medium at the end of first batch/cycle fermentation. Then the nutrient rich medium was added after III cycle of the process. This was repeated till the ethanol production reduced.

Yeast cell recycling in SSF with intermittent addition of nutrient medium

When the nutrient medium was added performance of the cells was improved. In the first two cycles after the addition nutrient medium (i.e. in II and III cycles, Table 6) the ethanol yield and efficiency were the same as in I cycle. In the IV cycle (Table 6) this was decreased as in the II cycle of the previous experiment (Table 5). Thus intermittent addition of nutrient medium improved the performance of the cells as with the freshly prepared inoculum.

Since the ethanol production has decreased after the IV cycle (Table 6) again fresh nutrient medium was introduced and the cells were incubated for 24h. Then the cells were allowed to sediment and liquefied rice flour extract with glucoamylase was added. Ethanol production was improved in the V cycle but was less than that in I, II and III cycles and it was better than the IV cycle. However the performance in the VI cycle was reduced and not comparable III cycle. Further decrease in ethanol production was seen in the VII cycle. Addition of fresh nutrient medium has also not helped in keeping the performance of the cells in VIII and IX cycles. In a continuous ethanol production using *Saccharomyces cerevisiae*, immobilized on gelatine coated glass beads and crosslinked with glutaraldehyde nutrients rich medium was added intermittently to recharge the cells in immobilized system [36]. Here when the cells are decanted loss of young viable cells cannot be avoided unless a membrane filter is used. Addition of nutrient medium did not help to maintain the viable cells in the residue. The results indicated that the use of immobilized cells is superior to this process [36]. However here we were able to produce ethanol in the range of 69.8 to 96.8 gL^{-1}; by cell recycling using simultaneous saccharification and fermentation process in 6 cycles, with intermittent addition of nutrient medium.

The advantage with this procedure is the achievement of high ethanol concentration by removing the inhibitory effect of sugars at high concentration. Simultaneous saccharification and fermentation offers the advantage of removing the osmotic stress on the cells and reducing the reactor volume [2 & 17] in addition to the energy expenditure [22]. Further Simultaneous saccharification and fermentation also can reduce the total time of the process [20]. Thus the Simultaneous saccharification and fermentation is turned out to be an economical process [21].

Table 6: Simultaneous saccharification and fermentation of liquefied starch in rice flour (DE 45.3) at pH 4.5 and 35°C by locally prepared glucoamylase (2.3 AGU g DS^{-1}) and recycling of yeast cells, while intermittently adding nutrient medium.

Cycle Number	Time (h)	Viable Cell (No.)	Sugar (gL^{-1})		Ethanol		
			Reducing	Total	Amount (gL^{-1})	Efficiency (%)	Yield (%)
	0		95.3	210.6	0.0		
I	24		98.4	98.4	48.3		
	48	8.0×10^8	6.3	7.4	96.8	95.8	91.9
Addition of fresh sterile nutrient medium							
	0		95.3	210.6	0.0		
II	24		97.4	99.4	48.9		
	48	6.5×10^8	5.8	6.4	97.3	95.3	90.5
	0		95.3	210.6	0.0		
III	24		98.3	100.2	38.1		
	48	9.3×10^7	6.1	7.2	85.8	84.4	81.5
	0		95.3	210.6	0.0		
IV	24		124.3	129.6	31.9		
	48	4.3×10^7	17.3	18.4	74.3	77.3	70.6
Addition of fresh sterile nutrient medium							
	0		95.3	210.6	0.0		
V	24		113.2	114.3	38.6		
	48	$6.4\ 10^7$	38.3	36.3	85.3	97.9	81.0
	0		95.3	210.6	0.0		
VI	24		128.4	134.3	28.3		
	48	9.8×10^6	53.2	55.2	69.8	89.8	66.3
	0		95.3	210.6	0.0		
VII	24		138.6	142.7	25.4		
	48	4.3×10^6	69.4	73.8	58.4	85.4	55.5
Addition of fresh sterile nutrient medium							
	0		95.3	210.6	0.0		
VIII	24		133.2	138.6	28.2		
	48	9.4×10^5	87.2	89.3	48.3	79.6	45.9
	0		95.3	210.6	0.0		
IX	24		165.4	168.2	17.3		
	48	2.7×10^4	110.3	112.4	23.5	47.9	22.3

CONCLUSION

Time reduction in the industrial processes makes the process economical. When raw starch has to be used as carbon source combining liquefaction and saccharification or SSF can reduce the process time. From this study it can be concluded that the SSF is better than combining liquefaction and saccharification. Further combining cell recycling with SSF has shown that intermittent addition of nutrient medium is useful to improve the cell viability in reactors to enhance ethanol production.

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