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Fermentation of High Gravity Glucose by Free and Immobilized *saccharomyces cerevisiae* S1 by Cell Recycling

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Abstract: The potential economic benefits in ethanol production could be realized by conducting fermentation at 40^{o}C and above. In this study, thermotolerant yeast isolated and developed in our laboratory was used in the cell recycle operations to find the possibility to improve the high gravity fermentation. Fermentation of glucose (300 gL^{-1})−PYN medium by free and agar immobilized *Saccharomyces cerevisiae* S1 cells took 36 h to utilize glucose completely and produced 100 and 143 gL^{-1} ethanol, respectively. When the free and agar immobilized *Saccharmyces cerevisiae* S1 cells were subjected to repeated batch operation in glucose (300 gL^{-1})−PYN medium, it was possible to continue the process with 2 and 37 cycles respectively. When the free cells were used in the repeated batch fermentation with glucose (300 gL^{-1})−2PYN medium supplemented with 26.8 gL^{-1} soy flour, it was possible to continue the process for 7 repeated cycles. However, stuck fermentation was observed with advancing cycles with increasing residual sugar with free cells. Thus, cell recycle batch process was possible with additional nutrients in the medium while with immobilized cells such additional nutrients were not necessary.

Keywords: : Stuck fermentation, high gravity fermentation, *Saccharomyces cerevisiae*, cell recycling, free cells

1. Introduction

Traditional fermentation system use freely suspended yeast cells in batch bioreactors (Verbelen *et al.*, 2006). Recycling of yeast cells (Matano *et al.*, 2013) and continuous alcohol fermentation (Deshphande, 2002; Vasconcelos *et. al.*, 2004) are useful to reduce the fermentation time and to increase the ethanol productivity. Immobilization of microbial cells showed certain technical and economical advantages over free cell fermentation (Verbelen *et al.*, 2006). Several theories have been put forward to explain the enhanced fermentation capacity of immobilized microorganisms (Ivanova *et al.*, 1996; Lin and Tanaka, 2006).

The potential economic benefits in ethanol production could be realized by conducting fermentations at 40^{o}C and above. This has

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generated considerable interest in the selection of thermotolerant yeast strains (D'Amore, *et al.*, 1988). It has been estimated that the cooling cost during fermentation was reduced by 10 % when the fermentation was carried out at 37°C instead of 32 °C (Anderson *et al.*, 1985). Further the ethanol recovery cost can also reduced considerably when operating at high temperature. Certain difficulties such as high ambient temperatures, coupled with an exothermic fermentation reaction lead to inhibition of the fermentation ability of yeast (Anderson *et al.*, 1985). This further emphasized the need for thermotolerant strains. In the present study the efficiency of a locally isolated thermotolerant yeast strain (*Saccharomyces cerevisiae* S1, Balakumar *et al*., 2001) for the fermentation of high concentration of glucose at 40 °C was investigated, by comparing the recycling of the cells in the free and immobilized forms to improve the high gravity fermentation at 40°C to reduce the distillation cost.

2. Materials and Methods

Materials: Soybean from local market was powdered and dried at 80 °C. Culture media & Technical agar were purchased from Oxoid Limited, UK, and all the other chemicals were purchased from Sigma-Aldrich, USA.

Saccharomyces cerevisiae S1: *Saccharomyces cerevisiae* S1 is a locally improved thermo tolerant strain (Balakumar *et al.*, 2001), maintained in glucose (50 gL^{-1}) -peptone, yeast extract and nutrient (PYN)–agar slants in refrigerator at 4 °C.

Analytical methods: Glucose (Miller, 1959), ethanol (Varley *et al.*, 1980) and viable cell count (Sami *et al.*, 1994) were determined by standard methods.

Peptone, Yeast extract and Nutrient (PYN) medium: The PYN medium contained (gL^{-1}) peptone, 3.5, yeast extract, 3.0, $MgSO_4.7H_2O$, 1.0, KH_2PO_4, 2.0; and $(NH_4)_2SO_4$, 1.0 at pH 5.0 (Balakumar and Arasaratnam, 2009). Based on the needs, different amounts of glucose were added to the medium and represented as glucose (amount in gL^{-1}) – PYN medium (Balakumar and Arasaratnam, 2009). When double the amount of the nutrients of PYN medium was used, the medium is presented as glucose (amount in gL^{-1}) – 2PYN medium'.

Inoculum: The yeast cells were grown in sterile (100 in gL^{-1}) – PYN medium, for 18h at 40 °C. Total cell count of the inoculum was taken before sedimentation and allowed to sediment at 4 °C for 6h. Sedimented cells were used as free cell inoculum or taken for the preparation of immobilized cells.

Immobilization of yeast: Sterile aqueous agar (40 gL^{-1}, 75 mL) was cooled to 50 °C and the sedimented cells, re-suspended in sterile normal saline (25 mL) were vigorously mixed by shaking at 50 °C in a water bath for 10 min. The liquid agar / yeast cell mixture was trickled via a syringe (0.7 mm) into sterile paraffin oil (300 mL) at 4 °C. After 2 h, the beads were washed with distilled water.

Comparison of the performance of free and agar immobilized *Saccharomyces cerevisiae* S1 in glucose (300 gL^{-1})-PYN medium . Agar immobilized *Saccharomyces cerevisiae* S1 cells were inoculated to 100 mL glucose

(300 gL^{-1})−PYN medium and incubated at 40 ^{o}C with shaking (100 rpm). Residual sugar and ethanol were monitored. Free sediment cells from 100 mL of 18 h old inoculum was treated instead of immobilized cells.

Cell Recycle Batch Fermentation

Repeated batch operation of free and agar immobilized Saccharomyces cerevisiae S1 in glucose (300 gL^{-1}) − PYN medium

The first batch operation was started as said above. At the end of the first batch, after complete utilization of glucose, the agar immobilized *Saccharomyces cerevisiae* S1 was recovered from the spent medium by allowing the beads to settle and decanting the spent medium. Then 100 mL of glucose (300 gL^{-1})−PYN medium was added to the settled beads and incubated. This process was repeated till the immobilized cells ceased to produce ethanol. In addition to the cells and ethanol the cells leaked into the medium was monitored.

Sedimented free cells from 100 mL of 18 h old inoculum was treated as of immobilized cells. Upon reaching the complete utilization of glucose, cells were allowed to sediment and the supernatant was decanted. Fresh medium (100 mL) was added to the sedimented cells and this cycle was repeated till the free cells ceased ethanol-producing ability.

Recycling of free Saccharomyces cerevisiae S1 in (300gL^{-1})−2PYN medium supplemented with soy flour

The glucose (300 gL^{-1})−2PYN medium supplemented with soy flour (26.8 gL^{-1}) was prepared. After sterilization, the medium was allowed to settle and the clear medium was used (to prevent the accumulation of soy flour particles in the subsequent cycles). The medium was inoculated with 10 % (v/v) *Saccharomyces cerevisiae* S1 inoculum and, aerated for 12 h and then incubated at 40 ^{o}C with shaking (100 rpm). Residual sugar, ethanol and biomass were measured with time.

4. Results and Discussions

4.1 Performance of free and agar immobilized Saccharomyces cerevisiae S1 in glucose (300 gL^{-1})−PYN medium

Sediment cells from 100mL inoculum or agar immobilized yeast was used as inoculum for the fermentation of glucose (300 gL^{-1})-PYN medium at 40 ^{o}C. Ethanol produced was100 and 143 gL^{-1} with free and agar immobilized yeast, respectively (Table 1) at 36 h. Decreased production of ethanol by free cells could be attributed to increased nutritional demands (Alvers *et al*., 2009). In one of the previous study, fermentation of glucose (300 gL^{-1})−PYN required the supplementation of the medium with double the concentration of the nutrients of PYN or soy flour (26.8 gL^{-1}) to produce 140 gL^{-1} ethanol at 36 h (Balakumar and Arasaratnam, 2014). Residual sugar in the spent medium where agar immobilized yeast cells was used, complete utilization of glucose as observed (Table 1). Ethanol production rate was 2.8 and 3.9 $gL^{-1}h^{-1}$, respectively with suspended free cells and agar-immobilized yeast, respectively. Ethanol production rate was increased by 39 % by immobilizing the cells. This study seems to confirm the early findings (Holcberg and Margalith,

Table 1: Ethanol production in glucose (300 gL^{-1}) – PYN medium by free and immobilized yeast cells at pH 5.0 and 40 °C. The ethanol and residual glucose were measured at 36 h.

Parameter	Ethanol (gL^{-1})	
	Free cells	**Immobilized cells**
Ethanol (gL^{-1})	100	143
Residual sugar (gL^{-1})	80	0.0
Glucose utilization (%)	73.3	100
Ethanol production rate ($gL^{-1}h^{-1}$)	2.8	3.9
Ethanol production efficiency (%)	65.2	65.2

Efficiency was calculated as the amount of alcohol produced / Theoretical amount of ethanol that could be produced from the added amount of glucose x 100; Theoretically 1g glucose gives 0.511g alcohol.

1981; Verbelen *et al.*, 2006; Lin and Tanaka, 2006). The membrane properties would have been altered by the immobilization process, affecting the permeability of the cells towards higher penetration of the substrate as well as faster removal of the end products from the cells (Verbelen *et al.*, 2006). Further glucose in the solution diffuses through the gel matrix, reaches the cells and disappears when fermented into ethanol and CO_2. This creates a gradient of glucose, which permits its flow from the solution into beads.

As long as the rate of diffusion is lower than the rate of fermentation, the local concentration of the sugar would be non inhibitory to the reaction. Further, continuous ethanol removal from the fermentation site is important to establish a non-inhibitory level of ethanol (Holcberg and Margalith, 1981). The free and immobilized cells were used for the cell recycle batch fermentation.

4.2 Cell Recycle Batch Fermentation

Repeated batch operation of free and agar immobilized *Saccharomyces cerevisiae* S1 in glucose (300 gL^{-1}) − PYN medium

The free and immobilized cells recovered from the first batch operation were used for subsequent repeated cycle (Figure 1). At 36 h fermentation in the first batch the free count was taken in the medium. Initial free cell number in the free suspended medium was 2 x 108 cells mL^{-1} and at 36 h fermentation it was 2.8 x 108 cells mL^{-1}. In the first and 2nd batch operations with the immobilized cells, the cell leakage was insignificant. When the free cells were used the process was active only for two repeated batches. Whereas when the immobilized cells were used they performed in a prolific manner up to 30 cycles with constant production of ethanol (139 ± 3.0 gL^{-1}). Carragenan entrapped yeast cells completely fermented 300 gL^{-1} glucose, while 138 gL^{-1} residual glucose was obtained with free cells (Barros *et al.*, 1987). The difference in fermentation behavior may be due to the higher cell viability in the immobilized cell system than their free cell

counterpart (Holcberg and Margalith, 1981). No cell leakage was observed till 10th batch and from batch 11 to 16th batches, very few free cells appeared in the medium. At 17th batch the cell leaking was prominent and 1 x 105 cells appeared in the medium and steady increase in the free cells was observed with the subsequent recycles.

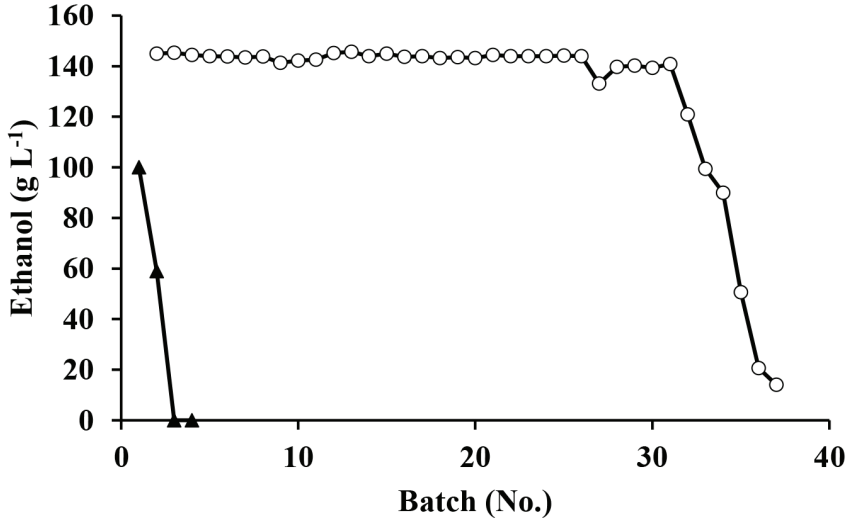

Figure 1: Repeated cell recycle ethanol production in glucose (300 gL^{-1}) PYN medium by(o) free and (▲) agar immobilized *Sacharomyces cerevisae* S1 at 40 °C.

After 20th batches of operation clear micro-colonies were found on the immobilized yeast-agar beads. From 30th cycle the immobilized cells showed decreasing performance up to 36th batch of the operation and afterwards the immobilized cells were inactive. Cell recycling of *Saccharomyces cerevisiae* CAIM13 was possible for four repeated cycle (Al-Judaibi, 2011)

The use of agar immobilized yeast cells was economical in terms of less nutritional demands and the free cell operation requires fresh inoculum for every third batch operation. For example to carry out 30 repeated cycles free cell inoculum should be prepared for 15 cycles. Hence, the use of immobilized cells for the fermentation of glucose (300 gL^{-1})– PYN medium is more economical than free cell fermentation. In cell recycle operations yeast cells are centrifuged and re-inoculated into other fermentors (Ingledew, 1993). In this experiment, cells were recovered by keeping the fermented medium at 4 °C for 3 h. The unproductive down time could be reduced by the use of centrifuges. In this experiment by approximately 10 fold increase in cell density (the inoculum was obtained by sedimentation of 100 mL inoculum), increased ethanol production (from 82 to 100 gL^{-1}), with reduction in fermentation time (from 48 to 36 h) were observed. Therefore, by increasing the inoculum potential, ethanol production efficiency could be increased.

However, in the second cell recycle batch ethanol produced was 71.2 gL^{-1} and the third batch of cell recycle was inactive. The decrease in the performance of free cells could be attributed to the cell death with the repeated cycles. The viabilities in the second and third cycles were 58.7 and 7.1 %, respectively. Further with the removal of the spent medium, the young cells which would not have sedemented also been removed. This is not possible with the alginate-entrapped cells. Bakers' yeast (Fermipan) on simultane -ous saccharification and fermentation was reported with intermittent addition of nutrient medium where liquefied rice flour starch was used (Arasaratnam *et al.,* 2012). In the report only liquefied rice flour starch was used and the ethanol yield was 91.9 % in the first cycle and it decreased to 22.3 % in the 9th cycle (Arasaratnam *et al.*, 2012). However, intermittent addition of fresh nutrient medium has helped to continue the recycling of free cells for 9 cycles (Arasaratnam *et al.*, 2012). There was no well-defined method available for the determination of viability of the agar immobilized yeast cells. However, the appearance and growth of micro-colonies within the agar beads from 20th batch operation reveals that the cells are still active. Alginate trapped *Saccharomyces cerevisiae* was able to perform in the repeated continuous process for 6 cycles from 95 to 85 gL^{-1} (Ivanova *et al.*, 2011).

Recycling of free *Saccharomyces cerevisiae* S1 in glucose (300 gL^{-1})−2PYN medium supplemented with soy flour

It has been observed that in the previous experiment, the nutrients of the glucose (300gL^{-1})−PYN medium were insufficient to continue the recycle process with free cells. Hence, the free cell recycling was carried out with the medium having double the concentration of PYN medium supplemented with soy flour (26.8 gL^{-1}), where the nitrogen content of the soy flour was equal to the nitrogen content of PYN medium. The first batch fermentation took 36h to produce 139.09 gL^{-1} ethanol with complete glucose utilization (Figure 2). The biomass has increased from 0.75 to 8.0 gL^{-1} at the end of the first batch operation. When the sediment from the first batch was mixed with fresh medium, the residual glucose reached 5.0 gL^{-1} at 20 h and 142 gL^{-1} ethanol was produced. In five batches of subsequent cycles 118, 98, 90, 79 and 51 gL^{-1} ethanol was produced with 50.0, 88.0, 111.0, 134.0 and 190 gL^{-1} residual glucose. Stuck fermentation was observed with advancing cycles with increasing residual sugar. No fermentation was observed after 7th batch of cell recycle operation. In the first batch 10.67 fold increase in the biomass was observed and in the second batch the increase was from 6.6 to 9.3 gL^{-1}. Even though the increase in biomass in the second batch was less than the first batch, the alcohol produced was higher in the second cycle (142 gL^{-1}). This could be due to the higher inoculum size (6.6 gL^{-1}) obtained at the second batch. With the advancing cycles, the increase in the biomass and the ethanol produced were reduced simultaneously. Figure 3 shows the sugar utilization, residual sugar, ethanol production rate and ethanol production efficiency with cell recycle operation. Sugar utilization was 97 % for the first two cycles. It has gradually

decreased to 36.72 % at the 7th batch. Ethanol production rate was 3.86 and 7.1 $gL^{-1}h^{-1}$ for 1st and 2nd cycles respectively and dropped to 1.6 at the 7th cycle. Ethanol production efficiency (% of theoretical maximum) was 96.5 and 97 % for the first two cycles and decreased to 33.26 % at the 7th cycle. The best performance of the cells was observed in the 2nd cell recycle batch of fermentation.

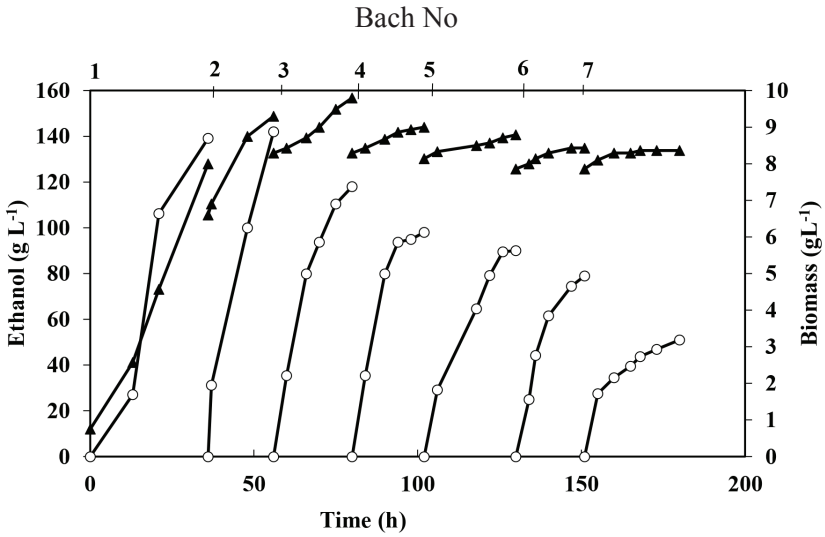

Figure 2: The time course on (O) ethanol and (▲) biomass production of the repeated batch cell recycle operation with Saccharomyces cerevisiae S1 at 40°C in glucose (300 gL^{-1}) – PYN medium supplemented with soy flour (26.8 gL^{-1}).

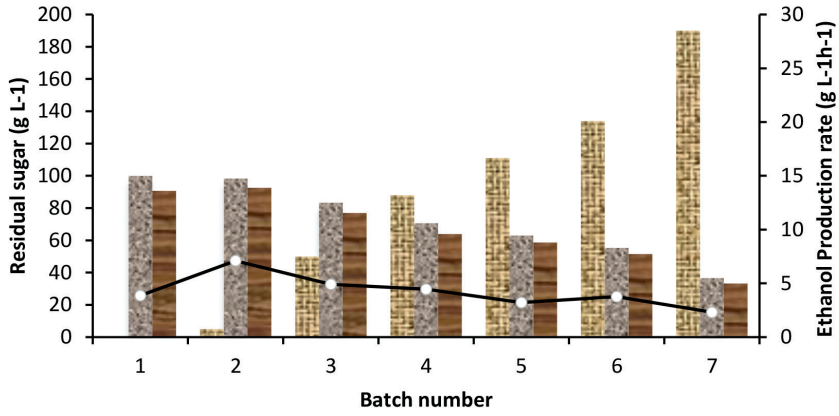

Figure 3: Cell recycle operation in glucose (300gL^{-1})-2PYN medium supplemented with soy flour (26.8 gL^{-1}) at 40°C. (▩) Residual sugar; (O) ethanol production rate; (▩) glucose utilization and (▩) ethanol production efficiency were obtained at the end of each batch operation.

4. Conclusions

The strain is capable of producing alcohol at 40°C, and thereby eliminates the problem of cooling the fermenter, particularly in large-scale fermentations. *Saccharomyces cerevisiae* S1 was able to perform better in the immobilized form (37 cycles) than in the free form (2 cycles). The free cells required additional nutrients for cell recycling process.

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