RSC Advances

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/advances

Two reactor continuous culture fermentation for the fuel ethanol production from the lignocellulosic acid hydrolysate using *Zymomonas mobilis* and *Scheffersomyces stipitis*

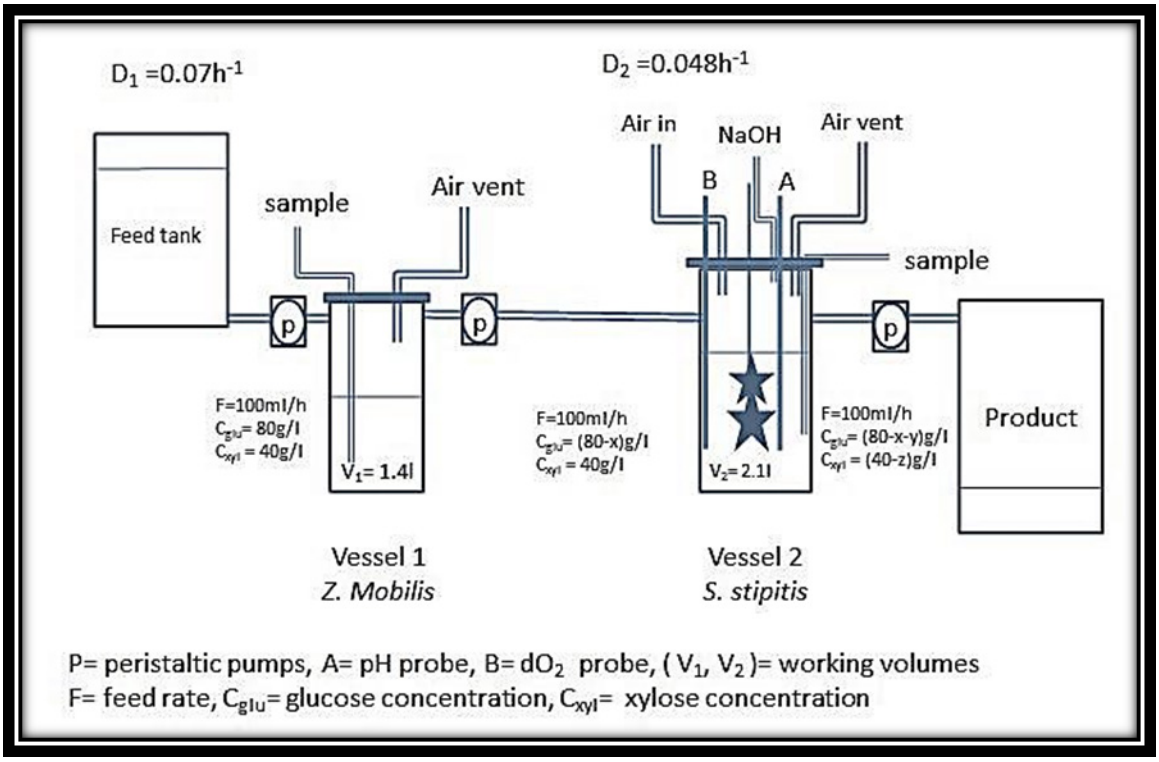
Gaurav Chaudhary (gauravgene@gmail.com),

Sanjoy Ghosh (ghsohfbs@iitr.ac.in)*

Biochemical Engineering Laboratory, Department of Biotechnology, Indian Institute of Technology Roorkee, Roorkee 247667 Uttarakhand, India

* Corresponding author. Tel.: +91 1332 285424(o)/285482(r) ; fax: +91 1332 285424/286151/273560 E-mail address: ghoshfbs@iitr.ac.in (S.Ghosh)

Graphical Abstract: Multistage ethanol production from lignocellulosic acid hydrolysate using two different microorganisms in two reactors enhances the utilization of all sugars (pentose and hexose) with higher ethanol productivity.



Abstract:

Conversion of mixed sugars into bioethanol was investigated in continuous two stage bioreactor connected in series. The first reactor was inoculated with *Z. mobilis* a hexose fermenting bacteria and the second reactor was inoculated with *S. stipitis,* a well-known pentose fermenting yeast. The reactors were switch on to the continuous mode after 24 h of batch run in the beginning. Different flow rates have been checked for complete utilization of hexose sugars and maximal xylose fermentation. Using this system at 100 ml/h flow rate an overall 1.37 g/l/h ethanol productivity (r_p) was obtained with un-detoxified acid hydrolysate as compared to 1.56 g/l/h with pure sugars. Using un-detoxified acid hydrolysate the maximum ethanol yield $Y_{p/s}$ with *Z. mobilis* was found to be 0.47 g/g (92 %) and with *S. stipitis* was found to be 0.45 g/g (88 %). Hexose sugars were completely utilized with approximately 40 % xylose utilization in un-detoxified acid hydrolysate. Whereas, during the fermentation of pure sugars more than 60 % xylose have been utilized with complete utilization of glucose.

Keywords: continuous culture, reactors-in-series, *Zymomonas mobilis, Scheffersomyces stipitis*, lignocellulosic ethanol, mixed sugar fermentation.

Introduction:

Lignocellulosics materials like herbaceous energy crops, agricultural waste. Short rotation hardwoods, etc., predominantly contains cellulose (31-50%), hemicellulose (9-22%) and lignin (18-29%)[1]. Cellulose on hydrolysis produces hexose sugar glucose which can be easily fermented into ethanol. On the other hand hemicellulose on hydrolysis produces the mixture of pentose (xylose, arabinose etc.) and hexose (glucose, galactose etc.) sugars. Thus conversion of all the possible sugars present in lignocellulosic biomass into ethanol is important for economical ethanol production from lignocellulosic materials.

Hexose sugars can be easily converted to ethanol by many microorganisms like yeast *Saccharomyces cerevisiae* and bacterium *Zymomonas mobilis* with a very fast rate but unlikely to other organisms they are unable to convert xylose. *Scheffersomyces stipitis* is yeast which can convert pentoses to ethanol with a relatively faster rate[2]. The oxygen supply during the fermentation largely affects the growth and ethanol production of *S. stipitis*[3]. Studies have shown that glucose or hexose sugars dominates the xylose or pentose sugars utilization by *S. stipitis*[4,5]. Thus, the mixed sugar fermentation is difficult to carry out than that of single sugar fermentation in a bioreactor, especially when the sugar (e.g. glucose and xylose) uptake or consumption rates are hugely different[6,7,8,9,10,11].

For effective and complete utilization of mixed sugars, the low glucose concentrations are thus important. Different methods have been employed to attain the xylose conversions. Complete utilization of sugars in batch culture is effective, but productivity is low[12]. In a continuous stirred tank reactor (CSTR) system with *S. stipitis* the conversion of xylose can achieved by keeping the

residence time relatively higher and lower glucose concentrations. This will also results in lower productivity. Co-culture of two microorganisms can also be employed for the purpose itself. Compatibility of two microorganisms is the major limitation along with the physico-chemical conditions requirements of two different types of microorganisms. A plug flow reactor can also be a beneficial for complete utilization of sugars. The glucose concentration decreases over the length of the reactor.

In the present study, continuous cultures with two bioreactors in series have been used. Glucose fermentation using *Z. mobilis* have been carried out in the first reactor, whereas the remaining glucose and xylose was fermented in the second reactor by *S. stipitis*. Different dilution rates were checked to maximize the utilization of both the sugars.

Results and discussion:

Reactors in series:

Z. mobilis and *S. stipitis*

The glucose concentration in the first reactor during a combination of *Z. mobilis* and *S. stipitis* in two reactors in series can be calculated from biomass balance of *Z. mobilis*

$$\frac{dC_x}{dt} = \mu_{max} \cdot \frac{C_s}{C_s + K_s} \cdot C_x - D.C_x \qquad (1)$$

In steady state it follows that

$$C_s = K_s \cdot \left(\frac{D}{\mu_{max} - D}\right) \qquad (2)$$

the glucose concentration at steady state can be calculated using equation 2 which shows that at dilution rates $0.5\mu_{max}$ and $0.67\mu_{max}$ the glucose concentration varies from 1 to 2 times of K_s Value. The Ks value of *Z. mobilis* was calculated during the anaerobic conditions was found to be 0.63 g/l. which shows that glucose concentration in inlet stream of second reactor will be low.

Equations for the *S. stipitis*

The specific substrate uptake rates for *S. stipitis* can be described by the equation given by Grootjen *et al*.[13]

$$q_{glu} = q_{glu,max} \cdot \frac{C_{glu}}{C_{glu} + K_{glu}} \quad (3)$$

$$q_{xyl} = q_{xyl,max} \cdot \frac{C_{xyl}}{C_{xyl} + K_{xyl} \cdot \left(1 + \frac{C_{glu}}{K_{i,xyl}}\right)} \quad (4)$$

These equations show the inhibition of xylose conversion by glucose. Substrate uptake rate is the rate limiting step in the conversion of sugars by *S. stipitis* under oxygen limited conditions. It has been shown that a low glucose concentration can also affect the xylose uptake rate.

Batch culture of *S. stipitis* with different ratios of xylose and glucose:

Figure 1 shows the fermentation profile of *S. stipitis* with different ratios of glucose / xylose (2, 0.2 and 0) and different kinetic parameters obtained are shown in table 1. The maximum ethanol concentration was reached to a level of approximately 3.6 % v/v using *S. stipitis* with 0.2 and 0 glucose/ xylose ratio respectively. Also ethanol yield was comparable i.e. 0.49 and 0.48 g/g respectively. The major difference was found in ethanol productivity i.e. 0.46 and 0.89 g/l/h respectively. This difference in the ethanol productivity might be due to time delay in switching over mechanism by glucose and xylose pathways in the first case. Formation of cell biomass

formation is more favored by C6 sugars (like glucose) than C5 sugars (like xylose). Hence, fluctuation of glucose concentration is directly reflected in cell biomass concentration. In a situation where available carbon goes more towards cell biomass formation, product (ethanol) formation suffers. More product comes in stationary phase (cell growth ceases) when almost all available carbon goes towards product formation. Fermentation with higher glucose concentrations leads to the higher cell biomass concentrations (15.48 g/l) and lower maximum ethanol concentration (1.87 % v/v). From these results it can be observed that the ethanol production using *S. stipitis* was favored by nil/low concentrations (concentration lesser than 10 g/L) of glucose.

***Z. mobilis* and *S. stipitis* in a two reactor system with continuous culture:**

The fermentation profiles of the two reactor system are shown in figure 2 and their kinetic parameters are presented in table 2. The different flow rates (50, 100, 200, 300 and 400 ml/h) were tried during continuous fermentation. The glucose consumption rates in the first reactor were found to be 3.45, 6.46, 5.66, 6.45 and 6.57 g/h respectively at different dilution rates and respective residual glucose concentrations were found to be 10.9, 15.35, 51.69, 58.48 and 63.51 g/l. Using the calculated kinetic parameters of *Z. mobilis* ($\mu_{max,}$ 0.33 h^{-1} and K_s, 0.63 g/l) the glucose concentration of 0.17 g/l was calculated in the first reactor at steady state at a flow rate of 100 ml/h. Also the measured concentration of glucose in the first reactor at steady state was found to be 15.35 g/l at 100 ml/h flow rate. The glucose concentrations in first reactor at steady with different dilutions rates shows that effective conversion of the glucose in the first reactor requires the lower dilution rates. The lower glucose consumption rate at 50 ml/h flow rate was due to lower concentrations of glucose. Furthermore, maximum ethanol productivity, 2.22 g/l/h was found at the flow rate of 100 ml/h with an ethanol yield ($Y_{p/s}$) of 0.49 g/g. From figure 2 c

and 3 d it is evident that at the flow rate higher than 100 ml/h, most of the glucose passes through the effluent stream of first reactor which completely or partially inhibits the xylose fermentation in the second reactor. The glucose was completely utilized in the second reactor at the flow rates of 50 and 100 ml/h which favors the xylose utilization in the second reactor by *S. stipitis*.

In the second reactor the maximum xylose consumption was found with 100 ml/h flow rate. The concentration of xylose in effluent stream was found to be 15.00 g/l. At the flow rates 50 ml/h and higher than 100 ml/h, 22.00 to 26.00 g/l xylose concentration was found in the effluent stream. Which shows that the optimal flow rate for xylose conversion is 100 ml/h (D= 0.048 with respect to second reactor). Although glucose concentration at a flow rate of 50 ml/h was found lowest (10.9 g/l) among all the flow rates observed in the inlet stream of second reactor, the utilization of xylose wasn't enough. What might happen is due to the lower cell biomass of *S. stipitis*. Using Coulter counter measurement negligible amount of *Z. mobilis* cell biomass was found in second reactor, this could be due to very less glucose concentration in the second reactor and the initial higher concentration of *S. stipitis* dominated over the growth of *Z. mobilis*.

Although, at higher dilution rates the ethanol productivities were found to be maximum, effective utilization of total sugars wasn't achieved, the lower dilution rate have been chosen for the further experiment with acid hydrolysate so that maximum sugars could be converted into ethanol. In contrast to the two reactor system used by Grootjen *et al*.[14] with *S. cerevisiae* and *S. stipitis,* the two reactor system in this present study with *Z. mobilis* and *S. stipitis* was more successful as in this system the significant amount of the ethanol was produced in the second reactor and hence making the process more efficient and economical. The overall ethanol production rate r_p of this system was found to be 1.56 g/l/h which is much higher as compared to

achieved by Grootjen *et al*.[14] i.e. 0.43 g/l/h in case of two reactor system using *S. cerevisiae* and *S. stipitis* and 0.51 g/l/h in case of three reactor system with *S. stipitis* alone.

Effect of dilution rate on different parameters

The dilution rate is the most important process parameter in continuous culture. During the ethanol fermentation using mixed sugars in two reactors system the dilution rates have been varied from 0.035 to 0.280 h^{-1} with respect to the first reactor and from 0.024 to 0.190 h^{-1} with respect to second reactor. The changes in overall productivities have been studied. With increasing the dilution rate the overall productivity of the system increased quadratically. Figure 3 a shows the graph between dilution rate v/s overall productivity. The substrate concentration in the outlet stream of both the reactors increased on increasing the dilution rate (figure 3 b).

The ethanol concentration in the outlet stream of first reactor was found to be decreasing from 4.50 % to 1.03 % v/v on increasing the dilution rate from 0.035 to 0.280 h^{-1} (figure 3 c). From the curve between ethanol concentration and dilution rate showed that ethanol concentration in the outlet stream was a quadratic function of dilution rate. The ethanol yield in the first reactor (by *Z. mobilis*) was also found to be quadratic function of dilution rate and initially decrease in ethanol yield was observed on increasing the dilution rate from 0.035 to 0.140 h^{-1} and then again increase in ethanol yield was observed when dilution rate was increased from 0.140 to 0.280 h^{-1} (figure 3 c). In contrast to the first reactor (*Z. mobilis*), the net ethanol concentration in second reactor (by *S. stipitis*) increased from 1.33 % to 2.13 % v/v as the dilution rate increased from 0.024 to 0.190 h^{-1}. The relations between ethanol concentration and ethanol yield with dilution rate in second reactor have been shown in figure 3 d. This increase in ethanol concentration at higher dilution rate might be due to the presence of higher glucose concentration in the inlet

stream (figure 3 b). However, ethanol yield was found to be lower at higher dilution rate with *S. stipitis* in second reactor. Further it has been found that increase in ethanol concentration was linear with respect to increase in dilution rate, whereas ethanol yield was a quadratic function of dilution rate (figure 3 d).

Fermentation of acid hydrolysate using two reactors in series:

The ethanol production using two reactors in series have been studied with un-detoxified acid hydrolysate. The fermentation profile of un-detoxified acid hydrolysate in two reactor system has been shown in figure 4 and different kinetic parameters are given in table 2. The maximum ethanol concentration at the steady state in first reactor was found to be 3.98 % v/v, whereas the maximum ethanol concentration in second reactor reached to a level of 5.39 % v/v. In comparison to fermentation of pure sugars, using un-detoxified acid hydrolysate, the ethanol productivity r_p of *Z. mobilis* in the first reactor wasn't significantly affected, while ethanol yield $Y_{p/s}$ of *Z. mobilis* decreased by 0.02 g/g.

In the second reactor the glucose and xylose concentrations varies in the range of 1 - 2 and 23 – 28 g/l respectively at steady state. This indicates the partial inhibition of *S. stipitis* by toxic compounds formed during acid hydrolysis. Comparing with results of pure sugars, the ethanol yield $Y_{p/s}$ decreased by small extent but ethanol productivity r_p was affected and decreased to 0.54 g/l/h from 0.90 g/l/h. The overall ethanol productivity of the system was found to be 1.37 g/l/h.

Conclusion:

From the results of batch cultures it can be concluded that the two microorganisms used in this study, *Z. mobilis* and *S. stipitis* are not compatible for the co-culture due to of their different

aeration requirement. They can perform much better separately than together. This can either be achieved by sequential culture of *Z. mobilis* and *S. stipitis* or by using two reactors in series. *Z. mobilis* must be used in the first reactor in order to ensure glucose is maximally used by it and very less glucose range is left in the outlet stream of first reactor and glucose level in the second reactor must be very low so that *S. stipitis* can utilize xylose in the second reactor with much better efficiency. Dilution rate of the first reactor must be kept lower than the second reactor. Further overall ethanol productivity was observed less due to of lower dilution rates which might be increased by use of high cell density culture of *S. stipitis* in the second reactor.

Experimental:

Microorganisms:

Zymomonas mobilis MTCC 91 was obtained from the IMTECH Chandigarh, India and was maintained on glucose agar plates containing (g/l) glucose 20, yeast extract 10, KH_2PO_4 2 and agar 15 and stored at 4 ^oC. Yeast strain *Scheffersomyces stipitis* CBS 6054 was obtained as generous gift from Prof. Thomas Jefferies FPL, U.S. Department of Agriculture and was maintained on YPD agar plates containing (g/l) yeast extract 10, peptone 20, dextrose 20 and agar 20 and was stored at 4 ^oC.

Synthetic media:

Inoculum medium contains (g/l) yeast extract 10, $MgCl_2$ 1, $(NH_4)_2SO_4$ 1, KH_2PO_4 1, with glucose 30 for *Z. mobilis* and xylose 30 for *P. stipitis*. Unless otherwise stated, the same media was used as production (fermentation) media, except the carbon sources, 40g/l glucose and 20g/l xylose as mixed sugars. Media were sterilized at 121^oC for 20 min and sugars were separately autoclaved from yeast extract and inorganic salt solution.

Inoculum preparation:

Inoculum was prepared by inoculating the single colonies from agar plates of *Z. mobilis* and *S. stipitis* in their respective media containing glucose or xylose in 250 ml Erlenmeyer flask with 100 ml inoculum medium. *Z. mobilis* was grown at 30^{o}C in static condition for 24 h whereas *S. stipitis* was grown at 30^{o}C with 150 rpm shaking for 24 h.

Batch culture of *Z. mobilis* and *S. stipitis* in a bioreactor:

Experiments have been carried out to study the effect of aeration on the ethanol production by *Z. mobilis* in a 7 l New Brunswick Bioflow 110 bioreactor system with 3 l working volume. The composition of fermentation media was the same as described above with 60 g/l glucose. The effect different xylose/glucose ratios (1/2, 5/1 and pure xylose) were also investigated on ethanol production by *S. stipitis* in the same bioreactor with 3 l working volume. The silicone (0.1 % v/v) was used as antifoam agent to foam in the reactor.

Continuous culture using reactors in series of *Z. mobilis* (in first reactor) and *S. stipitis* (second reactor):

The continuous culture experiments have been carried out using two reactors in series, the overall scheme has been shown in figure 5. In this case, the working volume of first and second reactor was 1.4 l and 2.1 l respectively. That means the residence time in first reactor was 2/3rd of the second reactor. This difference in residence time is required as the glucose conversion rate by *Z. mobilis* in first reactor is much higher than that of *S. stipitis*. The fermentation in first reactor was carried out without aeration and agitation, whereas aeration rate of 0.2 vvm was maintained in the second reactor at an agitator speed of 150 rpm. The first reactor was inoculated later (after 6 h) with *Z. mobilis* in contrast to second reactor which was inoculated with *S. stipitis*. After 24 h

of batch mode operation, the reactors were set on to continuous mode. It has been observed from the literature that *Z. mobilis* and *S. stipitis* works very well at 30 ^{o}C and hence this temperature was used all over the study whereas in case of continuous mode of fermentation the inoculum concentration does not have any serious effect and hence the inoculum concentration was not optimized.

Preparation of acid hydrolysate and fermentation using two reactors in series

The acid hydrolysate was prepared as described earlier by Chaudhary *et al*.[15]. 30% aqueous ammonia was used to pretreat the Kans grass biomass for 40 days at 30 ^{o}C temperature. The pretreated biomass was filtered and washed with deionized water until the pH of the filtrate drops to the neutral range. Further 60 % sulfuric acid was used to solubilize the carbohydrate content of pretreated biomass at 30 ^{o}C for 4 h. The slurry thus obtained was diluted to 10 % sulfuric acid concentration by adding deionized water and subjected to the final hydrolysis at 100 ^{o}C in an autoclave for 1 h. The acid hydrolysate thus obtained filtered, neutralized and concentrated to 100 g/l. Further pure glucose and xylose were added to maintain their concentration 80 and 40 g/l respectively. The pure sugars from the synthetic media were replaced by the acid hydrolysate. The fermentation of acid hydrolysate was carried out using the same scheme of two reactors in series as described in the previous section. The flow rate was maintained at 100 ml/h.

Analytical methods:

The total reducing sugars in the fermentation broth was estimated by DNS method[16] and the pentoses were measured by using phloroglucinol method[17]. Ethanol concentrations were

estimated using Gas Chromatography technique (Dani, GC) as earlier described by Chaudhary *et al*.[15]. Dry cell biomass was estimated turbidometrically.

Calculations:

Ethanol yield $Y_{E/S}$ = Ethanol produced (E) / Substrate consumed (S) 5

Percentage ethanol yield (conversion efficiency) = (actual ethanol yield / theoretical ethanol yield) * 100 6

Ethanol productivity (r_p) = (Ethanol Produced / substrate consumed) / time 7

Abbreviations:

C: concentration (g/l); *D:* dilution rate (per h); *K:* Monod constant (g/l); t: time (h); q: specific production or consumption rate (g/g/h); μ: growth rate (per h); F: feed rate (ml/h); **Subscripts:** x: biomass; s: substrate; i: inhibition; glu: glucose; xyl: xylose; max: maximum

Acknowledgement: Authors are thankful to University Grant Commission, New Delhi, India for providing financial assistance as Senior Research Fellowship (SRF) to Mr. Gaurav Chaudhary.

References:

1. N. Mosier, R. Hendrickson, N. Ho, M. Sedlak and M. R. Ladisch, *Bioresour. Technol.,* 2005, **96**, 1986-1993.
2. P. Bruinenberg, P. M. Bot, J. Dijken and W. A. Scheffers, *Appl. Microbiol. Biotechnol.,* 1984, **19,** 256-260.
3. D. R. J. Grootjen, R. G. J. M. van der Lans and K. C. A. M. Luyben, *Enzyme Microb. Technol.,* 1990, **12**, 20-23.
4. S. G. Kilian and N. Van Uden, *Appl Microbiol Biotechnol,* 1988, 27, 545-548.

5. J. C. Preez, M. Bosch and B. A. Prior, *Appl. Microbiol. Biotechnol.,* 1986, **23,** 228-233.
6. R. J. Bothast, N. N. Nichols and B. S. Dien, *Biotechnol. Prog.,* 1999, **15**, 867-875.
7. N. N. Nichols, B. S. Dien and R. J. Bothast, *Appl. Microbiol. Biotechnol.,* 2001, **56**, 120-125.
8. M. Moniruzzaman, B. S. Dien, C. D. Skory, Z. D. Chen, R. B. Hespell, N. W. Y. Ho, B. E. Dale and R. J. Bothast, *World J Microbiol Biotechnol,* 1997, 13, 341-346.
9. J. C. Saez-Miranda, L. Saliceti-Piazza and J. D. McMillan, *Biotechnol. Prog.,* 2006, **22**, 359-368.
10. B. C. Saha, L. B. Iten, M. A. Cotta and Y. V. Wu, *Biotechnol. Prog.,* 2008, **21**, 816-822.
11. S. Govindaswamy and L. M. Vane, *Bioresour. Technol.,* 2010, **101**, 1277-1284.
12. D. P. Bayrock and W. Michael Ingledew, *J. Ind. Microbiol. Biotechnoy,* 2001, **27**, 87-93.
13. D. R. J. Grootjen, R. G. J. M. van der Lans and K. C. A. M. Luyben, *Enzyme Microb. Technol.,* 1991, **13**, 648-654.
14. D. R. J. Grootjen, M. L. Jansen, R. G. J. M. van der Lans and K. C. A. M. Luyben, *Enzyme Microb. Technol.,* 1991, **13**, 828-833.
15. G. Chaudhary, L. K. Singh and S. Ghosh, *Bioresour. Technol.,* 2012, **124**, 111-118.
16. G. L. Miller, *Anal chem.*, 1959, 31, 426-428.
17. T. J. Eberts, R. H. B. Sample, M. R. Glick, and G. H. Ellis, *Clin Chem.,* 1979, 25, 1440–1443

Tables:

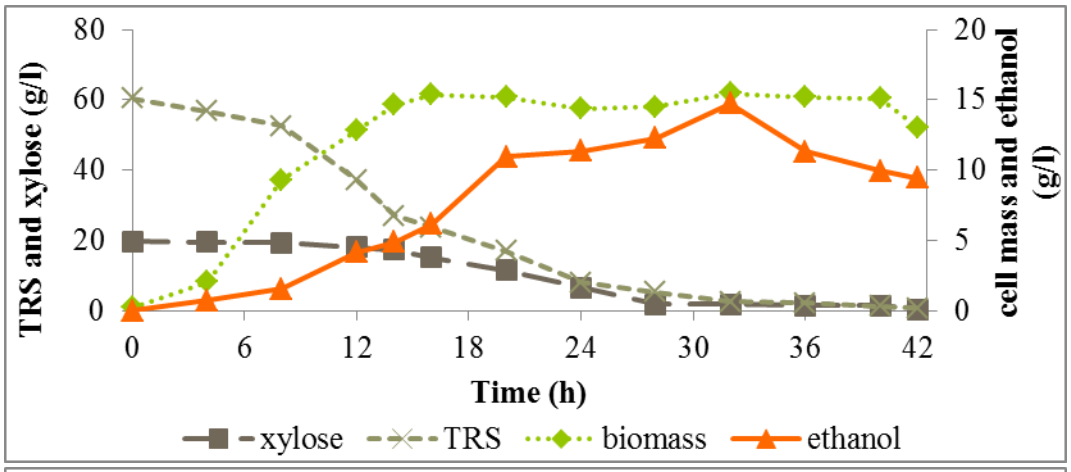
Table 1: different fermentation parameters for bioethanol production using *S. stipitis* with different proportion of glucose and xylose

Kinetic parameters	Xylose/Glucose: 1/2	Xylose/Glucose: 5/1	Xylose only
Initial total reducing sugars (g/l)	60.00	60.00	60.00
Maximum ethanol concentration (% v/v)	1.87	3.64	3.59
Maximum cell concentration (g/l)	15.48	13.39	13.26
Maximum time (h)	32.00	62.00	32.00
Sugar consumption (%)	95.92	98.33	98.93
Ethanol yield coefficient $Y_{p/s}$ (g/g)	0.26	0.49	0.48
Cell biomass yield $Y_{x/s}$ (g/g)	0.27	0.23	0.22
Ethanol productivity r_p (g/l/h)	0.46	0.46	0.89
Growth rate r_x (g/l/h)	0.48	0.22	0.41
Remaining sugars (g/l)	2.45	1.00	0.64
Sugar consumption rate (g/l/h)	1.80	0.95	1.86

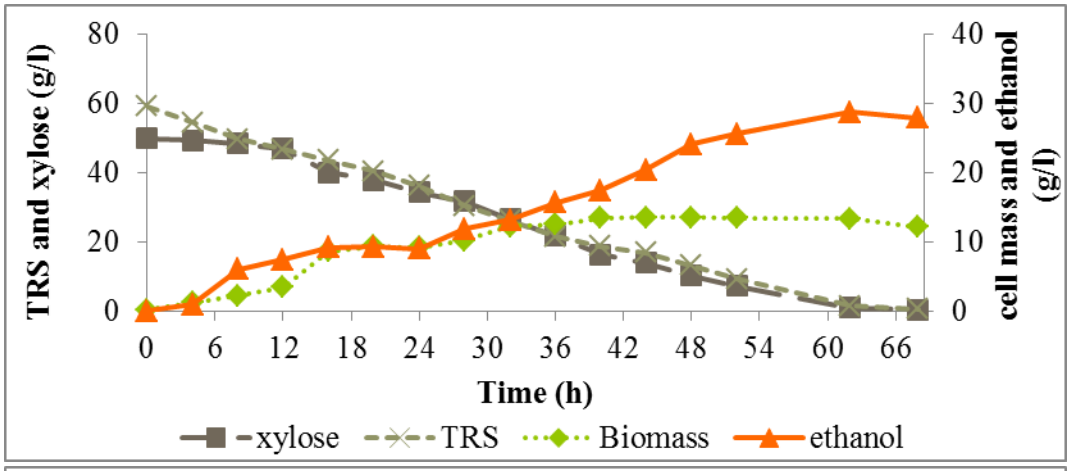
Table 2: Comparison of different fermentation parameters for bioethanol production at different flow rates during continuous culture using pure sugars and with un-detoxified acid hydrolysate

	Pure sugars										Un-detoxified acid hydrolysate	
Kinetic parameters	**reactor 1 (1.4 l)**					**reactor 2 (2.1 l)**					**Reactor 1**	**Reactor 2**
Feed rate, F (ml/h) →	**50**	**100**	**200**	**300**	**400**	**50**	**100**	**200**	**300**	**400**	**100**	**100**
Glucose concentration in inlet stream, G_i (g/l)	80.00	80.00	80.00	80.00	80.00	10.90	15.35	51.69	58.48	63.51	80.00	12.72
Glucose concentration in outlet stream , (g/l)	10.90	15.35	51.69	58.48	63.51	0.00	0.00	8.28	7.48	5.15	12.72	1.24
Xylose concentration in inlet stream (g/l)	40.00	40.00	40.00	40.00	40.00	40.00	40.00	40.00	40.00	40.00	40.00	40.00
Xylose concentration in outlet stream (g/l)	40.00	40.00	40.00	40.00	40.00	22.07	15.00	22.10	25.23	25.56	40.00	26.57
Ethanol concentration in inlet stream (% v/v)	0.00	0.00	0.00	0.00	0.00	4.50	4.25	1.47	1.12	1.03	0.00	3.98
Ethanol concentration at steady state (% v/v)	4.50	4.25	1.47	1.12	1.03	5.83	6.38	3.33	3.92	4.08	3.98	5.39
Cell concentration at steady state, X (g/l)	6.66	11.96	5.18	4.81	4.44	1.38	2.73	2.27	2.09	2.78	10.94	3.65
Ethanol yield , $Y_{p/s}$ (g/g)	0.51	0.49	0.41	0.41	0.49	0.36	0.46	0.24	0.33	0.33	0.47	0.45
Biomass yield, $Y_{x/s}$ (g/g)	0.10	0.19	0.18	0.22	0.27	0.05	0.07	0.037	0.03	0.04	0.16	0.15
Ethanol productivity r_p (g/l/h)	1.24	2.22	1.63	1.86	2.28	0.25	0.90	1.39	3.09	4.58	2.20	0.54

a)



b)



c)

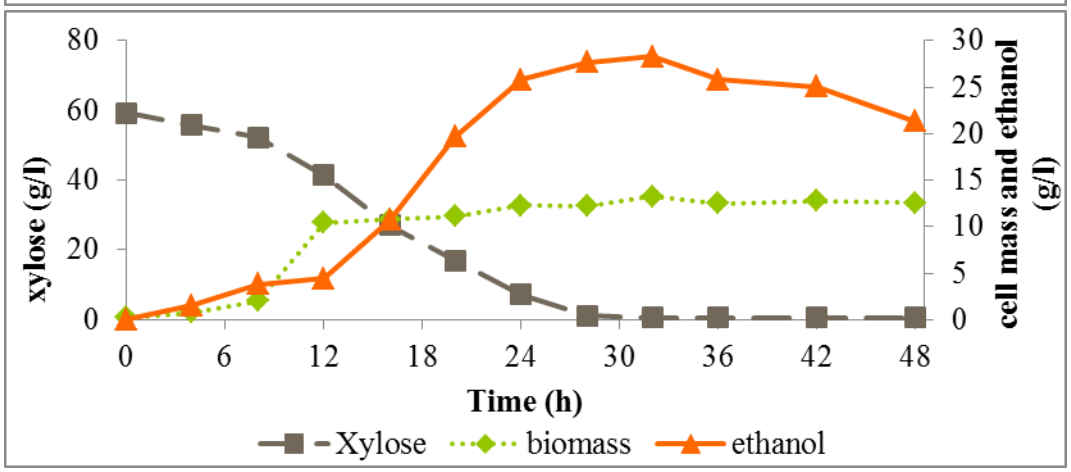
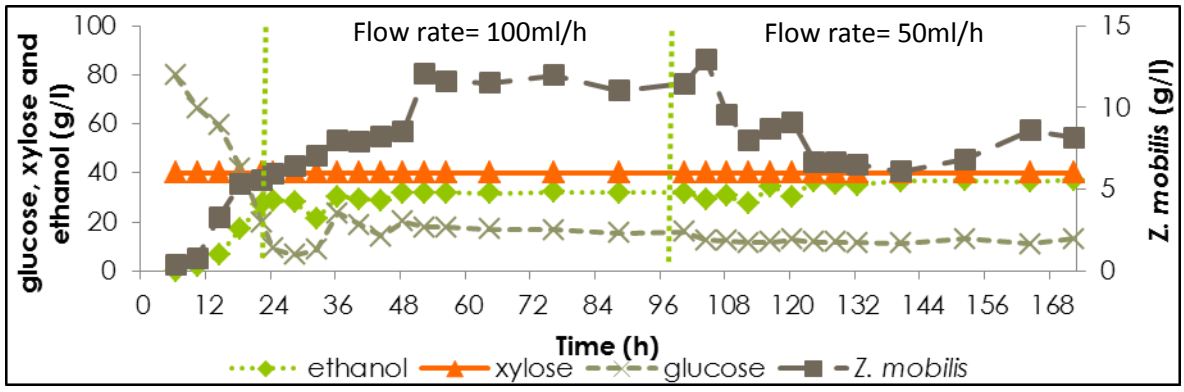


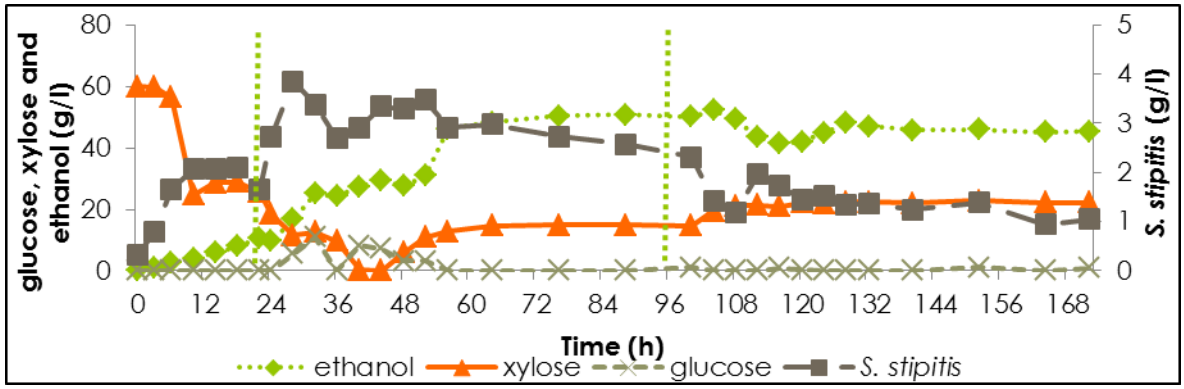
Figure 1: Effect of different proportion of glucose and xylose concentration on ethanol production using *S. stipitis.*

Reactor 1



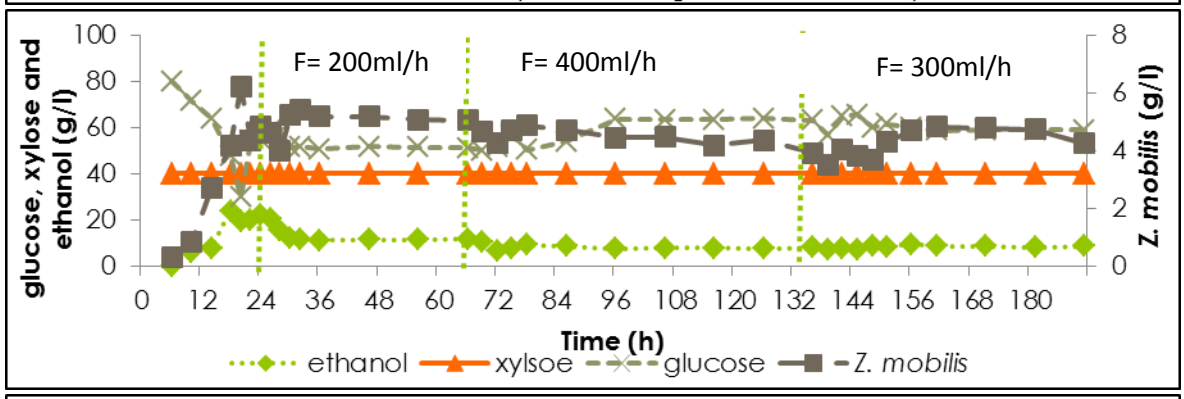
a)

Reactor 2



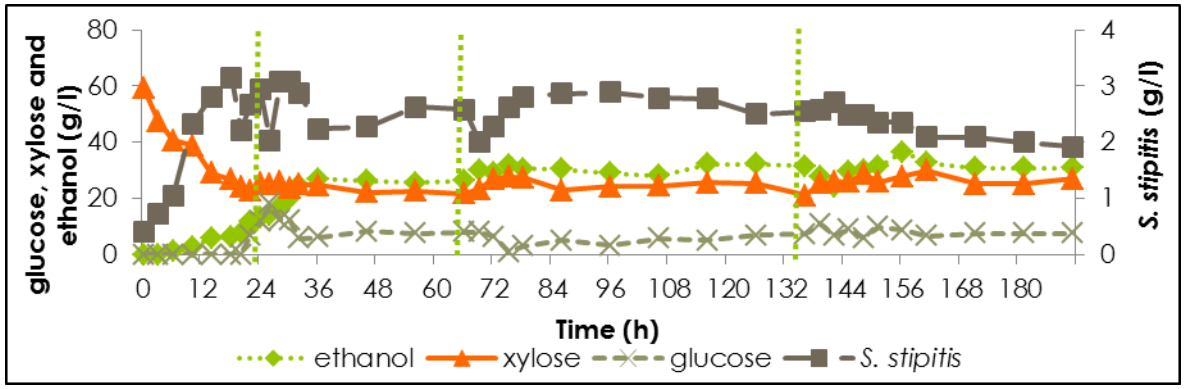
b)

Reactor 1



c)

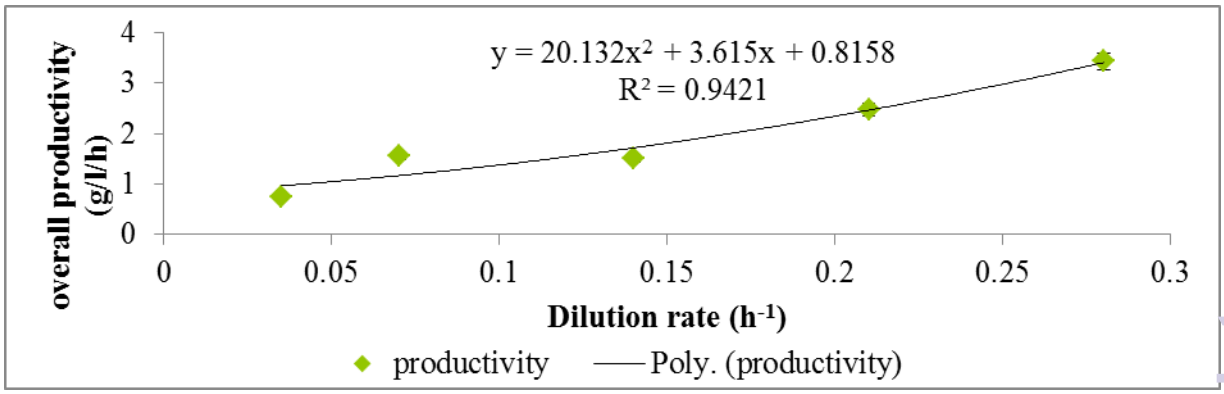
Reactor 2

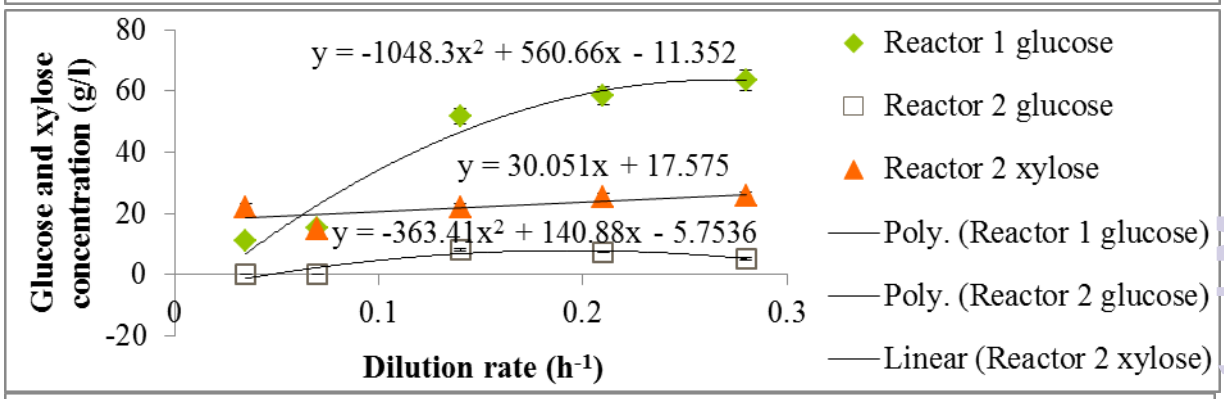


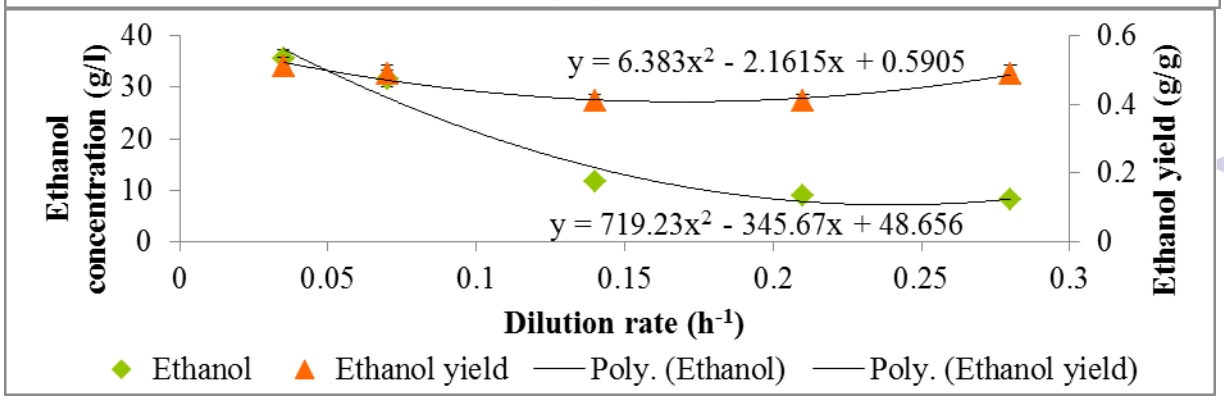
d)

Figure 2: fermentation profile of two vessel system with pure sugars: vessel 1 containing *Z. mobilis*, vessel 2 containing *S. stipitis* (a) and (b) at flow rate 50 and 100 ml/h (c) and (d) at flow rate 200, 300 and 400 ml/h.

a)







b)

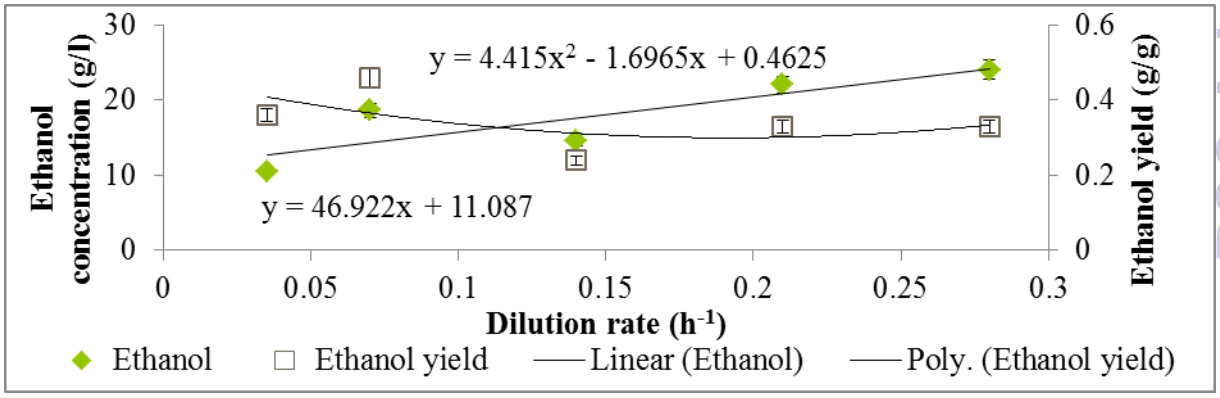
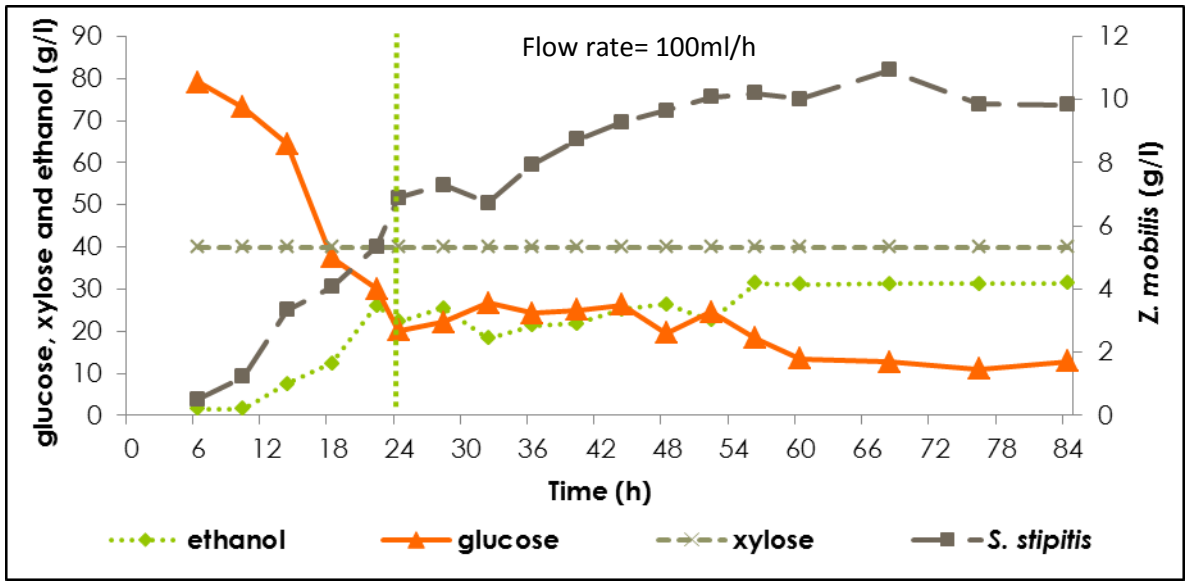


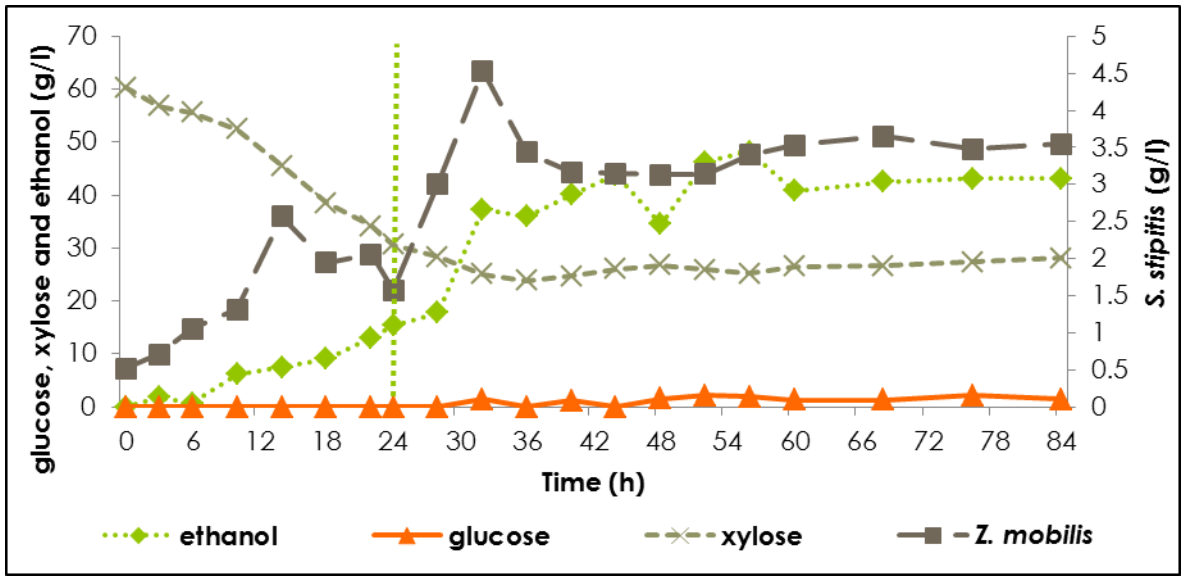
Figure 3: Effect of dilution rate on (a) overall ethanol productivity of two reactor system (b) different substrate concentartions at steady state outlet streams (c) ethanol concentration and ethnaol yield at steady state in reactor one with *Z. mobilis.* (d) ethanol concentration and ethanol yield at steady state in second reactor with *S. stipitis.*

Reactor 1



a)

Reactor 2



b)

Figure 4: Fermentation profile of two vessel system with acid hydrolysate (a) vessel 1 containing *Z. mobilis* (b) vessel 2 containing *S. stipitis* at flow rate 100 ml/h.

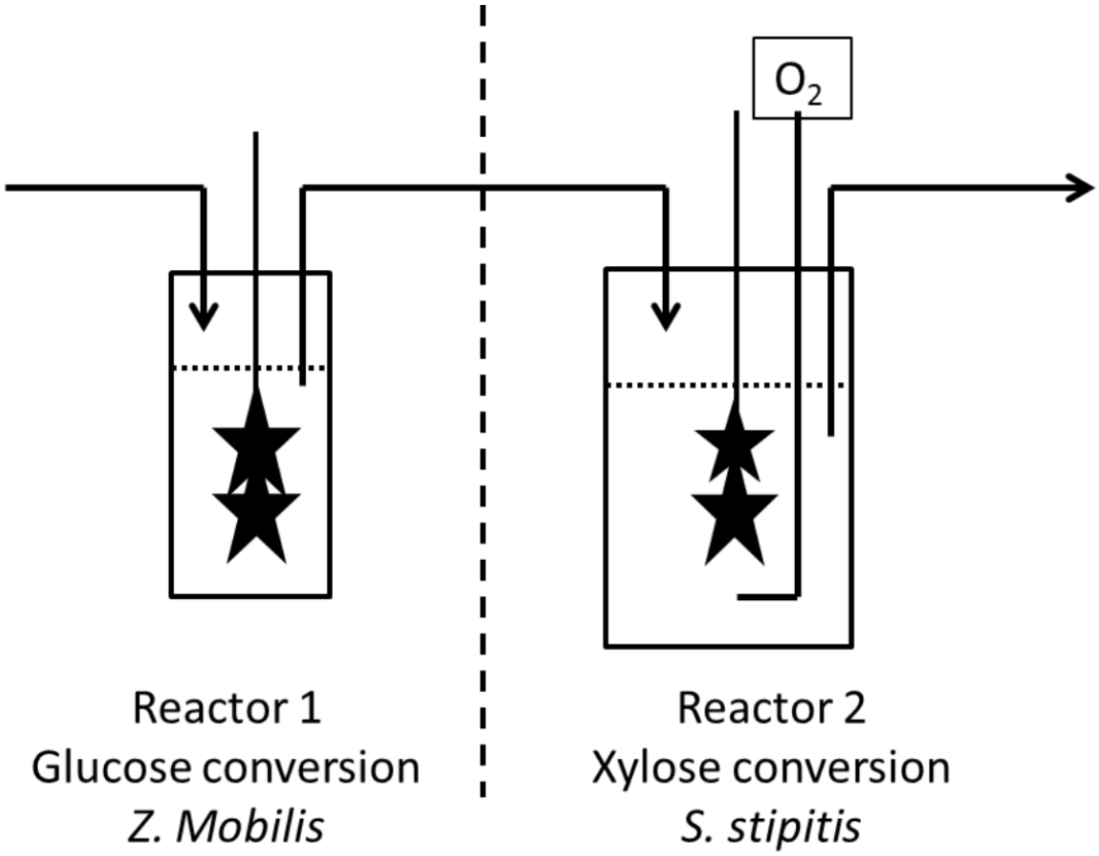


Figure 5: schematic diagram of two reactor system for continuous bioethanol production using mixed sugars.