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ARTICLE

Effect of salt on growth and ethanol production of ethanologenic yeasts – A preliminary investigation

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ABSTRACT

Seawater can reduce the freshwater footprint in bioethanol industries by replacing it in several bioprocessing steps in bioethanol production. However, the yeasts must be salt tolerant and be able to produce ethanol in seawater. In the present study, the salt tolerance capacity of two ethanologenic yeasts was checked in YPD and YPX media in the presence and absence of salt by comparing their growth and fermentation ability to produce ethanol. *P. stiptis* NCIM 3498 performed better regarding ethanol production when compared to *S. cerevisiae* NCIM 3570. The presence of salt did not impact the glucose and xylose fermentation by the yeasts.

1. Introduction

The depletion of freshwater around the globe is gaining attention and warranted the third world war. Several countries have revised their water policy and suggested to save the freshwater as much as possible by reducing their utilization, reusing after proper treatment and replacing them with a suitable alternative. Seawater can be a suitable alternative to the freshwater in industrial applications due to its 97% freshwater and 3% mineral composition (Indira et al., 2018). Due to the depletion of petroleum resources, several nations have started to utilize biofuels produced from biomass. Bioethanol is one of the highly used biofuels for transportation. Bioethanol is produced mainly from corn and molasses; however, recently several nations have started to produce it from bamboo, and rice straw. Lignocellulosic biomass is the most abundant and low-cost carbon substrate on earth that renders it an attractive feedstock for biofuel production. It consists of cellulose, hemicellulose and lignin (Nigam, 2001). Cellulose is a linear chain mucopolysaccharide with D-glucose units joined through β-1,4- linkage. Hemicellulose is a heteropolysaccharide comprising of xylose, arabinose (pentoses), mannose, glucose and galactose (hexoses) and sugar acids (Zhang and Lynd, 2004). Hemicellulose upon hydrolysis liberates xylose as major sugar (Sun and Cheng, 2002). So feasible biomass to ethanol conversion process requires an optimized use of all sugars in cellulosic and hemicellulosic fractions and an organism able to ferment both pentose and hexose (Lynel et al., 1999). It has been calculated by several researchers that almost 6 to 7 litres of fresh water are utilized to produce one litre of bioethanol in ethanol industries. Though it is feasible to utilize

Nomenclature and Abbreviation	
HPLC	High performance liquid chromatography
YPD	Yeast extract peptone dextrose
YPX	Yeast protein extraction kit
OD	Optical density

a higher quantity of fresh water to produce bioethanol, this has to be seriously addressed due to the threat of a shortage of fresh water for public use in future. Hence, there must be a suitable alternative to freshwater to be used in bioethanol industries to save the freshwater for future use. Seawater, being 97% freshwater with only 3% of minerals, emerging as a suitable alternative to replace the freshwater in industries. It has been proved that seawater can be utilized in various stages of bioethanol production. However, it requires halotolerant cellulolytic enzymes and ethanologenic yeasts to work in seawater to convert the cellulose to ethanol (Zaky et al., 2018). Hence the present study is undertaken to check the ability of native yeasts to grow and produce ethanol in the presence of salt. *Saccharomyces cerevisiae*, the most widely used yeast cannot utilize xylose. Some common ethanologens such as *Pichia stiptis, Candida sheathe*, and *Pachysolen tannophilus* can ferment both glucose and xylose into ethanol. Among these, *Pichia stiptis* is the most favourable industrial strain because of its high ethanol yield efficiency from xylose and also ability to ferment a wide range of polymers including cellobiose (Agbogbo et al., 2006). Yeast like other organisms adapts to survive in adverse growth conditions and protect cells from detrimental effects. In response to salinity stress, yeasts such as *Saccharomyces cerevisiae* reduce the stress effects by accumulating osmolytes particularly glycerol and activating cation transport systems H+-ATPase and Na+/H+ antiporter on plasma membrane for removing excess Na+ ions (Kogej et al., 2007). The objective of the present work is to study the effect of 3% NaCl (0.5 M) on growth and ethanol production of two native yeasts *Pichia stiptis* and *Saccharomyces cerevisiae*.

2. Materials and methods

2.1 Microorganism and media

Saccharomyces cerevisiae NCIM 3570 and *Pichia stiptis* NCIM 3498 were obtained from National Centre for Industrial Microorganisms, National Chemical Laboratory, Pune, India and maintained on 50% glycerol stock at -20°C. Cryopreserved yeasts were streaked on YPD agar media containing 1% yeast extract, 2% peptone, 2% dextrose and 2% agar and grown for 48 h at 30°C. Grown colonies were then inoculated in 20 ml of sterilized YPD broth in 50 ml centrifuge tubes and incubated at 30°C, 150 rpm for 48 h. After 48 h, the culture (20 ml) was transferred to 200 ml YPD broth and incubated for 48 h at 30°C and 150 rpm. The culture broth was harvested after 48 h using a centrifuge at 5000 rpm for five min. The yeast pellets were then washed 2-4 times in sterile deionised water until a clear supernatant was obtained. To the washed pellets 5-10 ml of sterile water was resuspended to form a concentrated liquid with OD600 of 50 (served as inoculum). Experiments were performed in Erlenmeyer flasks containing 50 ml of YPD broth and YPX broth (the same composition as YPD but dextrose was replaced with 2% xylose) with and without NaCl (3% or 0.5 M). Each fermentation flask was inoculated with an inoculum to reach an initial cell concentration of 0.5 OD.

2.2 Effect of salt on growth

A growth study was performed by measuring the OD at 600 nm after 0, 5, 12, 24, 48, 72, 96 and 120 h of growth of *S. cerevisiae* in YPD broth and *P. stipitis* in YPD and YPX broth.

2.2 Effect of salt on ethanol production

Fermentation was also monitored for five days for the time intervals mentioned in section 2.2 taking 1 ml samples to measure the concentration of glucose, xylose and ethanol using an HPLC (Shimadzu, Japan) equipped with refractive index detector and Agilent HiPlex H+ column. Samples were filtered through 0.2 µm filter and injected into HPLC. H_2SO_4 (5 mM) was utilized as mobile phase at the flow rate of 0.4 ml/minute. The concentration of glucose, xylose and ethanol present in the samples after fermentation intervals were calculated based on the area values of the respective standards. Glucose and xylose consumed during the fermentation intervals were calculated based on the following equation:

Glucose or xylose (%) consumed by yeast cells between sampling time (fermentation time) = Concentration of glucose measured at To - Concentration of glucose or xylose measured at Tt

Where; To is a time of initiation fermentation, and Tt is the time of completion of fermentation for respective sampling intervals.

Ethanol yield (g/g) was calculated by the following equation:

$$Ethanol\ yield\left(\frac{g}{g}\right) = \frac{Measured\ ethanol\ in\ sample\ (g)}{Theoretical\ ethanol\ (g)}$$

The theoretical yield of ethanol is 0.51 g of ethanol per 1.0 g of glucose, i.e. 2 moles of ethanol per mole of glucose

and 0.51 g of ethanol per 1.0 g of xylose, i.e. 1.67 moles of ethanol per mole of xylose. Theoretical ethanol was calculated based on the concentration of glucose/xylose consumed during the sampling interval time. In the present study, instead of taking the measured ethanol in the sample, ethanol produced between the sampling time intervals was considered. Ethanol produced between the sampling time interval was calculated by the following equation:

Ethanol (%) produced between sampling intervals = Concentration of ethanol measured at Tt - Concentration of ethanol measured at To

Where; To is a time of initiation fermentation, and Tt is the time of completion of fermentation for respective sampling intervals.

3. Results and discussion

S. cerevisiae was grown in YPD media containing glucose in the presence and absence of NaCl (3% or 0.5 M). To compare the growth in presence and absence of salt, the growth observed between the sampling intervals was considered. The results revealed that NaCl (3% or 0.5 M) is not completely arresting the growth of the yeast and they can grow. However, the growth rate was reduced in salt water when compared to fresh water. Presence of NaCl reduced the growth of yeast to the maximum of 15 fold between 24 to 48 h. There was very less reduction (1.3 to 4.7 fold) observed in the growth rate between other time intervals (0 to 5, 12 to 24, 48 to 72, 72 to 96 and 96 to 120 h). *S. cerevisiae* is a salt-sensitive yeast while other yeasts such as *Zygosaccharomyces rouxii* have been shown to be more tolerant of the presence of salt (Dakal et al., 2014). It was interesting to note that between 5 to 12 h period, the growth rate in the presence of salt is 1.5-fold increase when compared to fresh water (Figure. 1). *P. stipitis* was grown in YPD (glucose amended) and YPX (xylose amended) media in the presence and absence of NaCl (3% or 0.5 M). Results show that the growth of *P. stipitis* was affected by the salt during the initial period of growth until 24 and 48 h in YPD and YPX, respectively, then the growth increased when compared to freshwater. Maximum growth reduction by salt was observed in xylose amended media between 12 and 24 h of growth. Glucose amended media in the presence of salt has shown only seven-fold decrease of growth compared to freshwater (Figure. 2 and 3). Among the two yeasts studied, *P. stipitis* has shown the highest growth in the presence of salt when they are grown in xylose amended media. In the presence of xylose, *P. stipitis* shown higher resistance to salt (3% or 0.5 M) than in the presence of glucose. Compared to *P. stipitis*, *S. cerevisiae* showed poor growth in the presence of salt. Casey et al. (2010) have reported that osmotic stress induced by the presence of salts is an important factor that affects the performance of yeasts during fermentation.

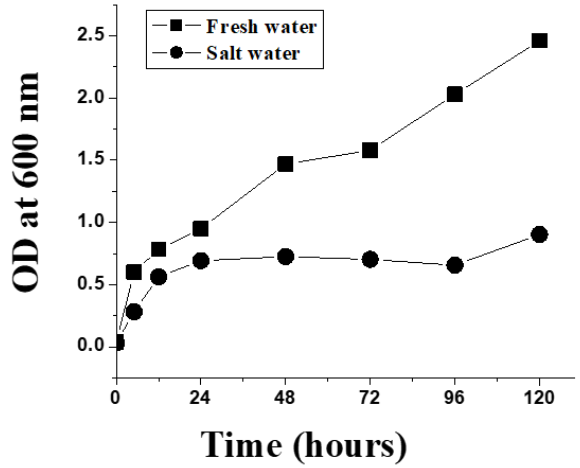

Figure. 1. Effect of NaCl (3%) on the growth of *S. cerevisiae* NCIM 3570 in glucose amended media

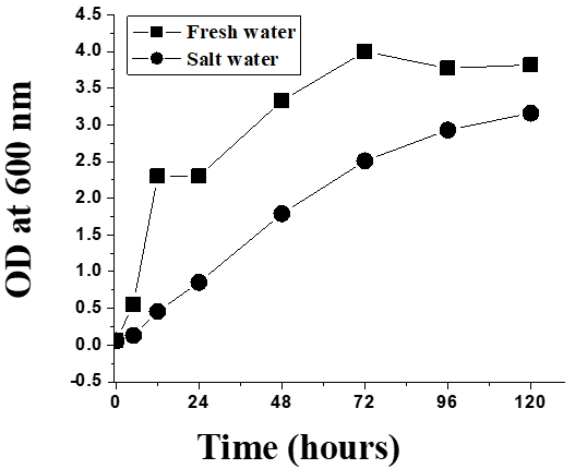

Figure. 2. Effect of NaCl (3%) the growth of *P. stipitis* NCIM 3498 in media with glucose

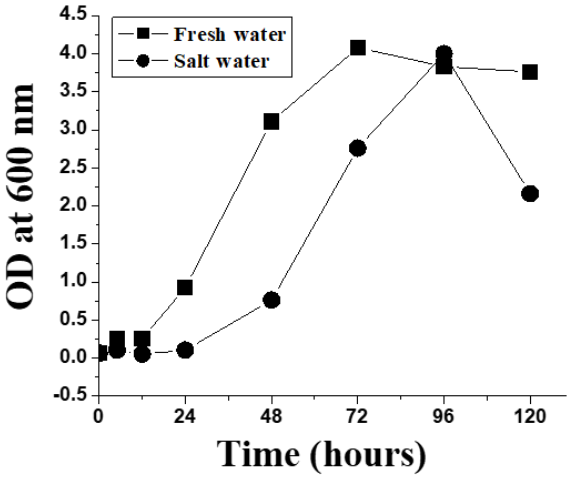

Figure. 3. Effect of NaCl (3%) the growth of *P. stipitis* NCIM 3498 in media with xylose

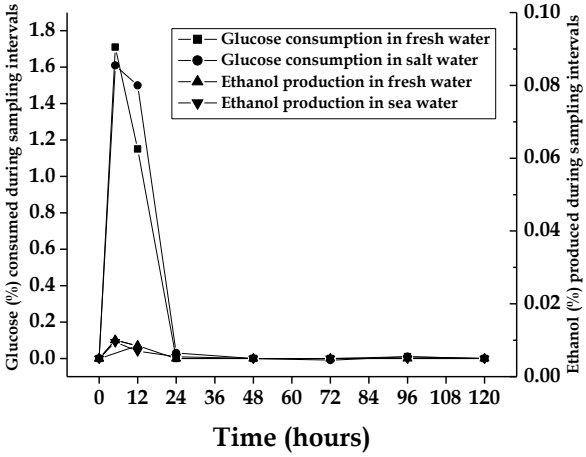

Figure. 4. Effect of NaCl (3%) on glucose consumption and ethanol production by *S. cerevisiae* NCIM 3570

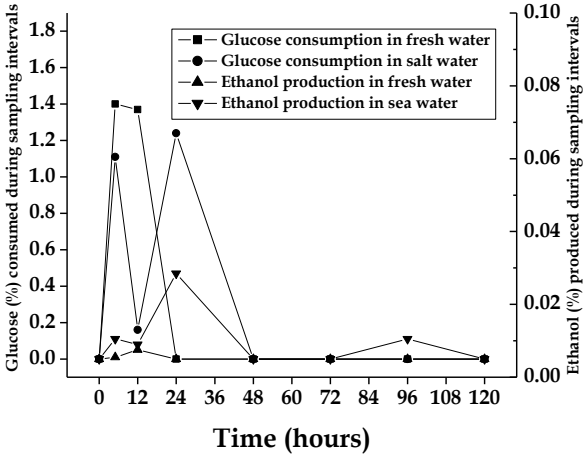

Figure. 5. Effect of NaCl (3%) on glucose consumption and ethanol production by *P. stipitis* NCIM 3498 in media with glucose

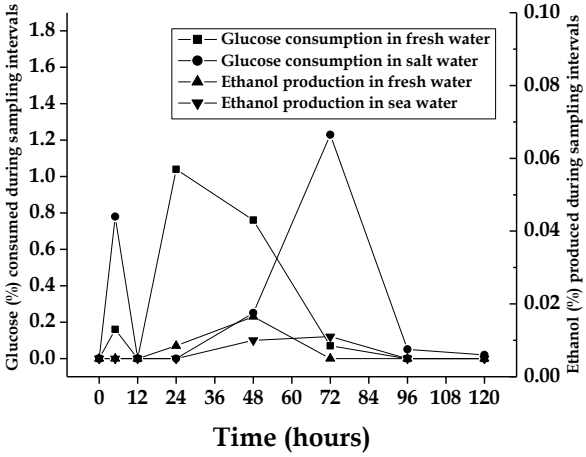

Figure. 6. Effect of NaCl (3%) on glucose consumption and ethanol production by *P. stipitis* NCIM 3498 in media with xylose

Two percentage of glucose was taken for the study. It has been estimated that more than 2% of glucose has been consumed by yeast cells. The extra glucose would have come from other sources like yeast extract upon hydrolysis in water. It has been observed that almost 99.9% of the initial glucose taken was consumed within 12 h of fermentation (Table 1). However, some residual glucose was measured after 12 h which may be due to the release of glucose from the dead cells. Ethanol production was also similar to the glucose consumption with ethanol yield of 0.11 after 5 and between 6 to 12 h in the absence of sodium chloride. In the presence of sodium chloride, yeast cells are consuming almost 99% of the glucose within 24 h of fermentation. This was evident with the concomitant production of ethanol with ethanol yield of 0.11 (after five h), 0.05 (6 to 12 h), 0.62 (13 to 24 h). From the data, it is very clear that the presence of salt is not hindering the fermentation and ethanol production ability of the *S. cerevisiae* cells. It is interesting to note that the ethanol yield was higher (0.62) in the presence of salt during 13 to 24 hours when compared to fresh water (Table 1). This may be due to the slower adaptation of the *S. cerevisiae* cells to the salt during the fermentation period.

It has been observed that 99.9% of the initial glucose taken was consumed by *P. stipitis* within 12 h and 24 h of fermentation both in fresh water and salt water, respectively. Glucose consumption was evident in the production of ethanol for both conditions. *P. stipitis* produced very less amount of ethanol from glucose with ethanol yield of 0.02 and 0.07 between 0 to 5 and 6 to 12 h of fermentation in freshwater, respectively. However, the ethanol production was continued until 24 h in salt water with ethanol yield of 0.19 (0 to 5 h), 0.97 (6 to 12), and 0.74 (13 to 24 h) (Table 2). It is interesting to note that ethanol yield is higher in salt water than freshwater. *P. stipitis* took a long time to consume the xylose. The xylose took initially was also consumed *P. stipitis* consumed almost 99.9% of the glucose initially taken within 12 and 24 h of fermentation in absence and presence of salt, respectively. However, they took a long time to consume the xylose. Xylose consumption of seen even up to 72 h in fresh water and throughout the fermentation time in salt water. There was no xylose consumption observed between 6 to 24 h in fresh water and 6 to 24 h in salt water. Ethanol production was also similar to the xylose consumption by *P. stipitis*. In the absence of salt, *P. stipitis* was able to produce ethanol with ethanol yield of 0.13 between 13 to 24 h and 0.60 between 25 to 48 h of fermentation, whereas in the presence of salt they were able to produce ethanol only between 25 to 48 h and 49 to 72 h with ethanol yield of 0.83 and 0.20, respectively (Table 3). The ethanol production from xylose by *P. stipitis* is higher in salt water which is similar to that of glucose. Compared to *S. cerevisiae, P. stipitis* performed better in the presence of salt. The maximum ethanol yield recorded in the present study is 0.97 which was observed in salt water by fermentation of glucose by *P. stipitis* during 6 to 12 h of fermentation, followed by 0.83. The maximum ethanol yield recorded in the present study was in salt water fermentation by *P. stipitis* from glucose (0.97) between 6 to 12 h, followed

by the xylose fermentation in salt water by *P. stipitis* (0.83) between 25 to 48 h.

Casey et al. (2013) have reported that salts reduce the consumption of glucose and hence the growth of cell and production of ethanol. They have also elaborated that at low salt concentrations (up to 0.2 M), sodium chloride was the only salt found to have an inhibitory effect on glucose consumption, and however, the effect was statistically insignificant. In the present study, 0.5 M sodium chloride was used, and poor growth of yeasts was observed for both *S. cerevisiae* and *P. stipitis* in the presence of sodium chloride. In contrary to their statement, the consumption of glucose and xylose by the yeasts in our study were not greatly affected in the presence of sodium chloride. Yeasts have several different mechanisms to encounter the osmotic stress produced by the sodium chloride in fermentation media. All of these mechanisms require energy or carbon which results in the need for additional energy and carbon. Casey et al. (2013) concluded that the presence of 0.5 M sodium chloride had reduced the xylose consumption up to 60% compared to the control. However, in the present study, it has been observed that the presence of sodium chloride at 0.5 M (3%) concentration did not affect the consumption of glucose and xylose when compared to their control. They have also stated that the metabolic ethanol yields were higher in the presence of 0.5 M salt (0.77 to 0.84) compared to the control (0.81) and there was no consistent trend observed on increasing the salt concentration.

Table 1. Effect of NaCl (3%) on the ethanol production by *S. cerevisiae* NCIM 3570

Time (h)	Glucose (%) measured in spent broth		Glucose (%) consumed by yeast cells		Measured ethanol (%)		Ethanol (%) produced during intervals		Theoretical Ethanol		Ethanol Yield	
	FW	SW	FW	SW	FW	SW	FW	SW	FW	SW	FW	SW
0	2.86	3.15	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
5	1.16	1.53	1.71	1.61	0.10	0.09	0.10	0.09	0.87	0.82	0.11	0.11
12	0.00	0.04	1.15	1.50	0.17	0.13	0.07	0.04	0.59	0.76	0.11	0.05
24	0.00	0.00	0.00	0.03	0.15	0.14	0.00	0.01	0.00	0.02	0.00	0.62
48	0.00	0.00	0.00	0.00	0.12	0.15	0.00	0.00	0.00	0.00	0.00	0.00
72	0.01	0.01	0.00	-0.01	0.07	0.09	0.00	0.00	0.00	0.00	0.00	0.00
96	0.00	0.00	0.01	0.01	0.00	0.00	0.00	0.00	0.01	0.01	0.00	0.00
120	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Table 2. Effect of NaCl (3%) on the ethanol production by *P. stipitis* NCIM 3498 in media with glucose

Time (h)	Glucose (%) measured in spent broth		Glucose (%) consumed by yeast cells		Measured ethanol (%)		Ethanol (%) produced during intervals		Theoretical Ethanol		Ethanol Yield	
	FW	SW	FW	SW	FW	SW	FW	SW	FW	SW	FW	SW
0	2.78	2.51	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
5	1.38	1.40	1.40	1.11	0.01	0.11	0.01	0.11	0.71	0.57	0.02	0.19
12	0.00	1.24	1.37	0.16	0.06	0.19	0.05	0.08	0.70	0.08	0.07	0.97
24	0.00	0.00	0.00	1.24	0.03	0.66	0.00	0.47	0.00	0.63	0.00	0.74
48	0.00	0.00	0.00	0.00	0.02	0.49	0.00	0.00	0.00	0.00	0.00	0.00
72	0.00	0.00	0.00	0.00	0.01	0.31	0.00	0.00	0.00	0.00	0.00	0.00
96	0.00	0.00	0.00	0.00	0.00	0.42	0.00	0.11	0.00	0.00	0.00	0.00
120	0.00	0.00	0.00	0.00	0.00	0.15	0.00	0.00	0.00	0.00	0.00	0.00

Table 3. Effect of NaCl (3%) on the ethanol production by *P. stipitis* NCIM 3498 in media with xylose

Time (h)	Xylose (%) measured in spent broth		Xylose (%) consumed by yeast cells		Measured ethanol (%)		Ethanol (%) produced during intervals		Theoretical Ethanol		Ethanol Yield	
	FW	SW	FW	SW	FW	SW	FW	SW	FW	SW	FW	SW
0	1.92	2.11	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
5	1.76	1.33	0.16	0.78	0.00	0.00	0.00	0.00	0.08	0.40	0.00	0.00
12	1.90	1.54	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
24	0.86	1.55	1.04	0.00	0.07	0.00	0.07	0.00	0.53	0.00	0.13	0.00
48	0.10	1.30	0.76	0.25	0.30	0.10	0.23	0.10	0.39	0.13	0.60	0.83
72	0.03	0.07	0.07	1.23	0.05	0.23	0.00	0.12	0.04	0.63	0.00	0.20
96	0.05	0.02	0.00	0.05	0.03	0.20	0.00	0.00	0.00	0.02	0.00	0.00
120	0.10	0.00	0.00	0.02	0.04	0.00	0.00	0.00	0.00	0.01	0.00	0.00

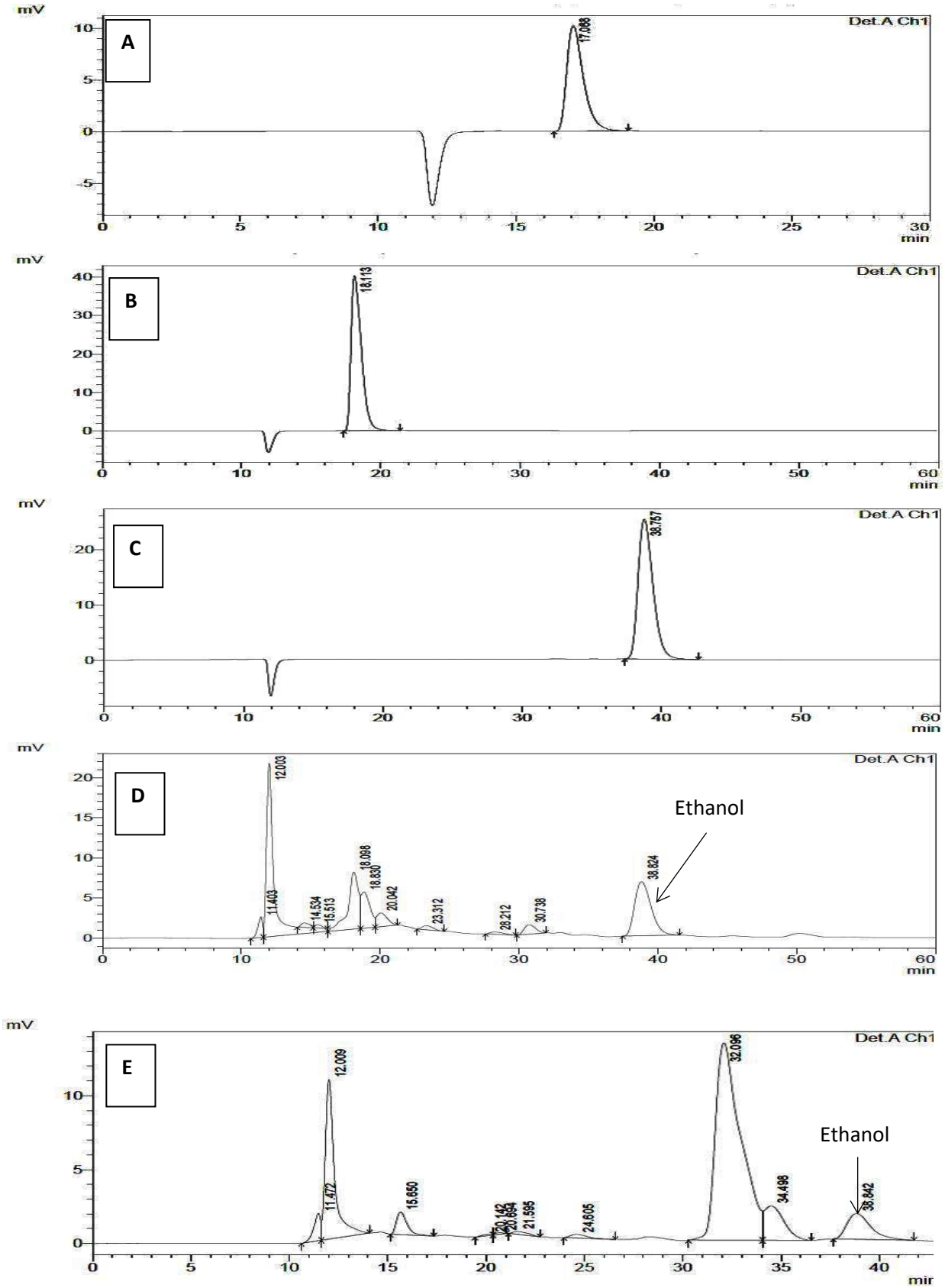

Figure.7.HPLC chromatograms of glucose (A), xylose (B), ethanol (C), and fermented media in the absence (D) and presence (E) of 0.5 M sodium chloride

4. Conclusion

Salt water, that represents the seawater, was investigated as an alternative to the freshwater in bioethanol production by *S. cerevisiae* and *P. stipitis*. The study revealed that the growth and ethanol production *S. cerevisiae and P. stipitis* were not affected by the presence of salt in the medium. Compared to *S. cerevisiae, P. stipitis* performed better both regarding growth and ethanol production in salt water. It is advantageous to use *P. stipitis* over *S. cerevisiae* due to its ability to grow well and ferment both glucose and xylose in salt water. The experiments are carried out for one time, and the results are subjected to check the repeatability.

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