Middle East Journal of Applied Sciences, 4(4): 1141-1149, 2014
ISSN: 2077-4613

Screening of Fungal Candidates for Ethanol Production from Agro-Industrial Wastes

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ABSTRACT

Some samples were collected from different rotten agricultural wastes, decomposed wood and compost, to screen different isolates of fungi for their ability to produce ethanol. The main goal of this research was to obtain isolates capable of fermentation of agro-industrial wastes i.e. vinasse and whey permeate**.** One hundred and seven isolates were secured on potato dextrose agar medium. Twenty eight isolates were positive for ethanol production; among those four were the most efficient ethanol producers. They were identified morphologically and biochemically as *Fusarium oxysporum-I*, *Mucor indicus, Aspergillus versicolor-2* and *Aspergillus oryzae*. They were compared with *Fusarium oxysporum* known with its ability to produce ethanol. Upon studying the time course for ethanol production by these five fungi, the 5th day was the best time for high productivity. *M. indicus* was the superior in its efficiency (70.20%), followed by F. *oxysporum* (53.99%) from the theoretical yields. The effect of different concentrations of glucose (5, 10, 15, 20 and 25%) on ethanol production was also studied. The best concentration for high productivity was 15% by all the tested strains. The efficiency of *M. indicus* was the highest (66.34% from the theoretical yields) followed by *F. oxysporum* (52.91%) and *A. oryzae* (52.84%). The tested fungal strains were compared with *Saccharomyces cerevisiae* and *Kluyveromyces fragilis* for ethanol production from vinasse and whey, respectively, as sole carbon source. Because fungi can utilize another organic carbon such as phenolic compounds and glycerol, it was found that these strains were unique for ethanol production from vinasse. *F. oxysporum-I* was the best producer (1.93 g eth/100 ml) followed by *M. indicus* (1.89 g eth/100 ml). On glucose, *M. indicus* was the best, with an efficiency of 56.38 % from the theoretical yield. In respect to whey; the efficiency of *A. oryzae* was the highest among all the fungal strains (5.89% from the theoretical yield). But *Kluyveromyces fragilis* was found to be the most efficient in ethanol production after 24 hrs from whey.

Key words: Ethanol production, *Fusarium sp., Mucor sp., Aspergillus sp., Saccharomyces cerevisiae, vinasse,* whey, *Kluyveromyces fragilis***.**

Introduction

Increased interest on alternative fuels has been observed in the past few years, as a result of increasing energy demand and forecasted depletion of fossil resources. Global warming and the consequent need to diminish greenhouse gases emissions have encouraged the use of fuels produced from biomass, which is the only renewable carbon source that can be efficiently converted into solid, liquid or gaseous fuels.

With the rapid increase of world population and of the use of fossil fuels, together with constant climate changes, the search for sources of renewable energy has become more necessary, through the coordinated and sustainable actions, in environmental, social and economical aspects. So far, many countries have invested in research to introduce and ensure the availability of bio-ethanol in the market (Daniela *et al.,* 2011).

Ethanol is the most used liquid bio-fuel alternative to fossil fuel. Bio-ethanol fermentation is by far the largest scale microbial process (Devi and Shankar, 2009). Its production from renewable biomass has received considerable attention in recent years (Patel *et al.,* 2007). It is presently the most abundant bio-fuel for automobile transportation. In addition to being renewable, ethanol has a major advantage in that it can be easily blended with gasoline.

The sugarcane industry in Egypt is defined as an open industrial system that consumes material and energy and creates products and wastes. For sugarcane industry to become a sustainable industry or a green industry, its production process has to be closed over its lifecycle in order to reach zero waste. Its wastes, residues and by-products should not be burned or disposed but should be rather treated as raw material for other industries (Nakhla and Salah, 2014).

Sugarcane is so far the most efficient raw material for bio-ethanol production (Dias *et al.*, 2010). It is processed to produce crystalline sugar, pulp and molasses. The latter are further processed by fermentation to

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ethanol, ascorbic acid or other products. After the removal of the desired product (alcohol, ascorbic acid, etc.) the remaining material is called vinasse. It represents a major environmental problem from the ethanol industry. Its production is 10 to 15 times greater than the ethanol itself. Vinasse contains 93% water, 7% solids with acid pH, high levels of salt (24,000 - 80,000 mg/l) and high concentrations of potassium, calcium, magnesium, sulfur and nitrogen. This effluent contains also organic and inorganic material. These compounds cause to vinasse to have a very high biological oxygen demand (BOD) ranging from 30 to 40.000 (Voegele, 2009). These organic matters may be a good source of carbon. It contains also an amount of fermentable sugar (1-2%). So, it is of interest to profit from this carbon source for ethanol production by some fungi other than yeasts which could be inhibited because of the presence of the phenolic compounds and the absence of reducing sugars in vinasse (Parnaudeau *et al.,* 2008). Also, an appreciable amounts of minerals and mineral salts were found in vinasse (Ahmed *et al.,* 2012), indicating that it can be used as a raw material to produce bio-ethanol by fungi and to solve the problem of environmental pollution, as a result of the high contents of BOD and COD.

Also, whey is a by-product of the cheese industry and contains mainly lactose (5%). The disposal of whey via fermentation to produce alcohol is practiced in various locations throughout the world. In the present study, we describe the ethanol production from lactose using ethanol fungal producers in comparison with *Kluyveromyces fragilis*.

The major target of the present study is to examine the ability of some isolates of fungi to produce ethanol from some agro-industrial wastes, i.e. vinasse and whey permeate.

Materials and Methods

Microorganisms

Fusarium oxysporum, *Saccharomyces cerevisiae* and *Kluyveromyces fragilis* were provided from Microbiology Department, Research Institute of Soil, Water and Environment. It was used as the standard strain in the present study. They were sub cultured every 30 days.

Isolation and identification of ethanol producing fungi

Some fungal isolates were isolated from different rotten agricultural wastes (strawberry, grapes, fig, harankash and artichoke), decomposed wood and compost, using a tenfold serial dilution-plating technique on potato dextrose agar (PDA) containing chloramphenicol (Oxoid, 1979). The culture was daily observed and successfully sub cultured onto fresh plates of PDA until pure isolates were obtained. One hundred and seven isolates were secured. The pure cultures were then transferred to PDA slants and maintained at 4 ^{0}C.

All the isolates were tested for their abilities to produce ethanol in conical flasks (250ml) containing 100ml of a fermentation medium according to Reihaneh *et al.* (2011). It was prepared by adding (g/l) yeast extract, 5; $(NH_4)_2SO_4$, 7.5; K_2HPO_4, 3.5; $CaCl_2.2H_2O$, 1; $MgSO_4.7H_2O$, 0.75 and glucose 150. Its pH was adjusted to 5. The amount of ethanol was determined according to Arnold *et al.* (1985).The superiors in their productivities (4 isolates) were identified morphologically and biochemically according to the method described by Domsch *et al.* (1980), by Plant Pathology Department (A.R.C), Giza, Egypt.

The best fungal isolates were enriched by inoculation in Mandel's medium (Patel *et al.,* 2007) at 32^{0}C, shaking at 125 rpm for 5 days. The medium contains the following reagents (g/l): urea, 0.3; glucose, 50; $MgSO_4.7H_2O$, 0.3; KH_2PO_4, 2; $CaCl_2.2H_2O$, 0.3; $(NH_4)_2$ SO_4, 1.4; Bactopeptone, 1; Tween 80, 0.1; trace elements: $FeSO_4.7H_2O$, 5mg; $MnSO_4.H_2O$, 16mg; $ZnCl_2.2H_2O$, 17mg; $CoCl_2.6H_2O$, 2 mg.

Fermentation medium for ethanol production

The enriched cultures of the best fungal strains were re-inoculated (5% v/v) into the fermentation medium according to Reihaneh *et al.* (2011). Its pH was adjusted to 5. After inoculation, it was incubated at room temperature for five to eight days. The experimental flasks were allocated in a complete randomized design with three replicates. After five to eight days, the mycelium was separated by filtration through Whatman filter paper No 1. The filtered broth was collected for determination of ethanol and reducing sugars.

Time course of ethanol production by fungi

An experiment was designed and conducted using the Reihaneh medium supplemented with 150g/l glucose (Sanaa, 2006; Tomoko, 2013) for the estimation of the time course of ethanol production. Fermentation was extended for eight days, ethanol and reducing sugars were determined daily.

Effect of glucose concentration on ethanol production

Another experiment was done on the Reihaneh fermentation medium supplemented with different concentrations of glucose (5, 10, 15, 20 and 25%) to examine the optimum concentration of glucose for ethanol production. Fermentation was extended for five days.

Fermentative utilization of two agro-industrial wastes as cheap carbon source

Vinasse was obtained from Egyptian Sugar and Integrated Industry Company (ESIIC), El-Hawamdia, Giza. It was analyzed in Soils, Water and Environ. Res. Inst., Agric. Res. Center (Table 1) and used as a fermentation medium for examining ethanol production by 4 isolates of fungi compared with *Saccharomyces cerevisiae*.

Table 1: Analysis of vinasse from cane molasses

Parameters	Vinasse from cane molasses
Total solids	8.6%
Fermentable sugars	1.3-2%
Total sugars	8.5 %
Organic matter	74.88 %
Organic carbon	43.43 %
Total nitrogen	0.84 %
Total phosphorus	0.59 %
Total potassium	1.62 %
pH	4.46–4.80
Phenols	(3.4%)
Vinasse yield	12–20 l/l eth.

Whey permeate was obtained from Egypt Milk Company, El-Sawah, Egypt. It contains 5% lactose. It was used as a fermentation medium after adding 10% lactose to obtain 15% sugar concentration for examining ethanol production by the 4 isolates of fungi compared with *Kluyveromyces fragilis***.** It was compared with Reihaneh fermentation medium supplemented with 15% glucose (control). All fermentation batches were performed in an incubator at 32^0 C.

Samples were taken after 24 hours of fermentation by the yeast strains and after 5 days of fermentation by fungi to determine reducing sugar and ethanol production kinetics [alcohol (P), yield, and efficiency of ethanol from the theoretical yield] where:

P = alcohol (g/100 ml)

$$yield\ of\ alcohol\ (\%) = \left[\frac{ethanol\ (^{g}/_{100ml})}{consumed\ sugar\ (^{g}/_{100ml})}\right] \times 100$$

$$Efficiency\ from\ the\ theoretical\ yield\ (\%) = \left[\frac{ethanol\ (^{g}/_{100ml})}{consumed\ sugar\ (^{g}/_{100ml}) \times 0.511}\right] \times 100$$

Analytical methods

Reducing sugars was determined by Di-nitro-salicylic acid (DNS) method (Miller, 1959). Ethanol was measured by spectro-photometric dichromate methods according to Arnold *et al.* (1985).

Results and Discussion

Fermentation processes are used extensively in the biotechnology, pharmaceuticals, beverage industries and food. Typically, fermentation utilizes microorganisms to produce a desired product from a substrate. Fuel alcohol is an example of the hundreds of biochemical produced by fermentation. In many cases, fermentation is the most cost effective means to manufacture products.

Isolation and screening of ethanol producing fungi

In this study, one hundred and seven isolates of fungi were obtained from different rotten agricultural wastes, compost and decomposed wood. All were tested for their ability to produce ethanol.

Twenty-eight isolates showed positive results for their productivity (Fig.1). Four isolates were chosen and identified as best producers of ethanol. They belong to *Fusarium oxysporum-I, Mucor indicus, Aspergillus versicolor-2* and *Aspergillus oryzae.* These strains were compared with a strain of *Fusarium oxysporum* known for its ability to produce ethanol from glucose. It is observed from Fig (1) that *M. indicus* followed by *F. oxysporum* were the superior in production of alcohol. They produced 4.63 and 4.23 g eth/100ml, respectively. Abedinifar *et al*. (2009) reported that *M. indicus* is able to produce ethanol from hexoses, i.e. glucose.

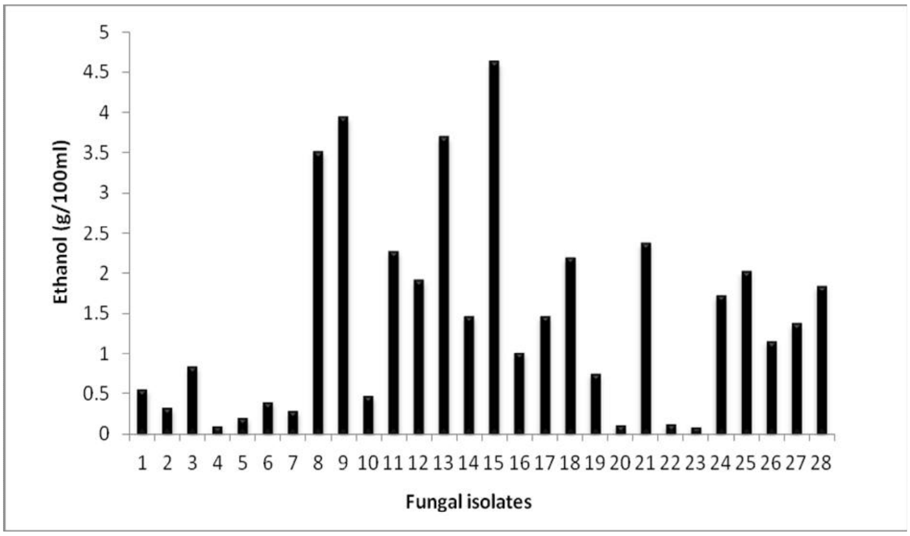

Fig 1: Ethanol production from glucose (15 gl100ml) using different fungal isolates
(8): *Aspergillus versicolor* (9): *Aspergillus oryzae*
(13): *Fusarium oxysporum-I* (15): *Mucor indicus*

Time course of ethanol production by fungi

In order to study the time course of ethanol production, the selected fungal strains were grown in Reihaneh fermentation medium containing 15% sugar. Tomoko *et al.* (2013) reported that the fungus *Flammulina velutipes* produced 40- 60g/l ethanol from high substrate concentration (15% sugar). Results in Table (2a) showed that, in general, consumption of sugar and maximum ethanol production were observed mostly within 5 days. There was a significant difference in the production of ethanol by *M. indicus* which is the highest (4.99 g eth/100ml) and *A. oryzae* (3.98 g eth/100 ml). Also, results in Table (2b) showed that there is a significant difference between the efficiency of *M. indicus* (70.2%) and *F. oxysporum* (53.99%) but there is no significant difference between the last one and *A. oryzae* which achieved 53.6% of the theoretical yields. Gianni *et al.* (2011) mentioned that *Fusarium oxysporum* produced ethanol after 6 days of fermentation with an efficiency of 42% of the theoretical yields. Also, Sorahi *et al.* (2009) reported that Zygomycetes filamentous fungi such as *Mucor indicus* have been characterized for ethanol production. They mentioned that *M. indicus* seems to be a good alternative to baker's yeast in production of ethanol from lignocelluloses and rice straw in particular.

Table 2a: Time courses of the production of ethanol

Time (days)	*F. oxysporum*		*F. oxysporum*-I		*M. indicus*		*A. versicolor*-2		*A. oryzae*	
	Consumed sugar (g/100ml)	Ethanol Production (g/100 ml)	Consumed sugar (g/100ml)	Ethanol Production (g/100 ml)	Consumed sugar (g/100ml)	Ethanol Production (g/100 ml)	Consumed sugar (g/100ml)	Ethanol Production (g/100 ml)	Consumed sugar (g/100ml)	Ethanol Production (g/100 ml)
1	12.21	1.39	12.11	0.96	11.98	1.09	13.11	1.93	12.97	1.00
2	12.62	1.64	12.54	1.31	12.16	1.29	13.47	2.00	13.19	1.00
3	13.33	2.04	13.43	1.89	12.29	1.58	13.68	2.22	13.21	1.50
4	13.69	2.36	13.82	2.25	12.31	2.83	14.06	2.25	14.34	2.23
5	14.10	3.89	14.15	3.75	13.91	4.99	14.21	3.64	14.53	3.98
6	14.10	3.12	14.15	2.34	13.91	2.46	14.21	2.42	14.53	2.26
7	14.10	2.84	14.15	2.37	13.91	2.45	14.21	2.42	14.53	1.45
8	14.10	2.81	14.15	2.37	13.91	2.46	14.21	2.42	14.53	1.45
LSD (0.05)	0.018	0.073	0.037	0.051	0.058	0.086	0.190	0.091	0.254	0.077

Table 2b: Time courses of the production of ethanol

Time (days)	*F. oxysporum*		*F. oxysporum*-I		*M. indicus*		*A. versicolor*-2		*A. oryzae*	
	Yield (%)	Efficiency (%)	Yield (%)	Efficiency (%)	Yield (%)	Efficiency (%)	Yield (%)	Efficiency (%)	Yield (%)	Efficiency (%)
1	11.38	22.28	7.93	15.51	9.10	17.81	14.72	28.81	7.71	15.09
2	13.00	25.43	10.45	20.44	10.61	20.76	14.85	29.06	7.58	14.84
3	15.30	29.95	14.07	27.54	12.86	25.16	16.23	31.76	11.36	22.22
4	17.24	33.74	16.28	31.86	22.99	44.99	16.00	31.32	15.55	30.43
5	27.59	53.99	26.50	51.86	35.87	70.20	25.62	50.13	27.39	53.60
6	22.64	44.31	18.00	35.23	20.13	39.40	18.15	35.53	17.24	33.74
7	22.29	43.62	18.57	36.35	20.20	39.53	18.09	35.39	11.02	21.56
8	22.06	43.16	18.72	36.63	20.26	39.65	18.18	35.58	11.04	21.61
LSD (0.05)	0.551	1.078	0.408	0.799	0.650	1.269	0.751	1.470	0.569	1.120

Effect of different concentrations of sugar on ethanol production

The effect of different sugar concentrations (5, 10, 15, 20 and 25%) on ethanol production was investigated by all the tested fungi. Results in Table (3) showed that there was a significant difference between the concentration of 15% and all the other concentration of glucose for all the tested strains. *M. indicus* was the superior in its production. It consumed 12.89 g sugar/100 ml from the 15% to produce 4.37 g eth/100 ml. It is shown that the effect of 15% sugar on the efficiency of *M. indicus* was the best one which was 66.34% from the theoretical yields. It is followed by the strain *F. oxysporum* and *A. oryzae* which consumed 14.98 and 14.48 g sugar/100 ml to produce 4.05 and 3.91 g eth/100 ml, respectively, achieving an efficiency of 52.91 and 52.84 % from the theoretical yields, respectively. Tomoko *et al.* (2013) reported that the maximum ethanol conversion rate from 15% sugar was 63% by the fungus *Flammulina velutipes*. Abedinifar *et al*. (2009) reported that ethanol yield and productivity of *Mucor* strains from hexoses i.e. glucose are as high as *Saccharomyces cerevisiae.* That is the reason why it is one of the candidates for ethanol production.

Table 3: Effect of different concentrations of sugar on ethanol production from isolate fungal strains

Conc. of sugar (%)	Consumed sugar (g/100ml)	Ethanol kinetics production		
		g/100 ml	Yield (%)	Efficiency (%)
F. oxysporum				
5	4.80	1.07	22.29	43.62
10	9.28	1.94	20.91	40.91
15	14.98	4.05	27.04	52.91
20	13.46	2.72	20.21	39.55
25	24.95	1.67	6.69	13.10
LSD (0.05)	0.051	0.053	0.483	0.947
F. oxysporum-I				
5	4.69	1.04	22.17	43.39
10	9.05	2.27	25.08	49.09
15	14.31	3.73	26.07	51.01
20	17.95	2.14	11.92	23.33
25	23.35	2.79	11.95	23.38
LSD (0.05)	0.033	0.041	0.757	1.483
M. indicus				
5	4.74	0.99	20.89	40.87
10	8.05	1.80	22.36	43.76
15	12.89	4.37	33.90	66.34
20	18.97	3.57	18.82	36.83
25	21.74	2.04	9.38	18.36
LSD (0.05)	0.027	0.044	0.683	1.333
A. versicolor-2				
5	4.77	1.06	22.22	43.49
10	8.07	1.57	19.45	38.07
15	14.05	3.43	24.41	47.77
20	18.21	2.61	14.33	28.05
25	21.27	2.68	12.60	24.66
LSD (0.05)	3.034	0.017	0.245	0.469
A. oryzae				
5	4.74	0.90	18.99	37.16
10	9.84	1.83	18.60	36.39
15	14.48	3.91	27.00	52.84
20	15.75	2.78	17.65	34.54
25	24.28	2.78	11.45	22.41
LSD (0.05)	0.011	0.036	0.314	0.612

Utilization of whey and vinasse as the sole carbon source for ethanol production

Małgorzata and Wojciech (2007) mentioned that the lactose-rich permeate can be a suitable substrate for alcohol fermentation. So, it was a comparison between glucose and whey as sole carbon source for ethanol production by isolated fungal strains. Results in Table (4) suggested that there was a significant difference in the production of ethanol from both of them by all the tested strains of fungi. *M. indicus* was the best isolate in its production when grown on glucose. It consumed 14.96 g glucose/ 100 ml to produce 4.31 g eth/100 ml with an efficiency of 56.38 % from the theoretical yields, followed by *F. oxysporum-I* which achieved an efficiency of 53.5%, from the theoretical yields. In respect of whey, results revealed that the whey isn't a good carbon source for ethanol production by fungi. But it was seen a significant difference between *A. oryzae* and all the other tested fungal strains in their productivity when grown on whey as it consumed 14.95 g lactose/ 100 ml to produce 0.45 g eth/ 100 ml giving an efficiency of 5.89% from the theoretical yields. *A. oryzae* has β-galactosidases which can hydrolysis lactose of whey *(*Shakeel and Qayyum, 2012*)*. Fig (2) represent a comparison between fungal strains and *Kluyveromyces fragillis* in their production of ethanol when grown on whey permeate medium (supplemented with 10% lactose). It suggested that the yeast strain was the superior in its productivity producing 4.49 g eth/100 ml after 24 hrs with an efficiency of 61.1% from the theoretical yields. Banat and Marchant (1995) mentioned that *Kluyveromyces fragilis* can grow and ferment lactose producing high alcohol concentrations from whey permeates. Shakeel and Qayyum (2012) reported that β- galactosidase from *Kluveromyces fragilis* converted 96% lactose from whey permeate.

Table 4: Ethanol production by fungal strains grown on glucose and whey fermentation media

Carbon source	*F. oxysporum*			
	Consumed sugar (g/100ml)	g/100 ml	Yield (%)	Efficiency (%)
Glucose	14.94	3.97	26.57	52.00
Whey	14.91	0.30	2.01	3.94
LSD (0.05)	0	0.063	0.386	0.737
F. oxysporum-I				
Glucose	14.96	4.09	27.34	53.50
Whey	14.93	0.32	2.14	4.19
LSD (0.05)	0	0.144	1.019	1.99
M. indicus				
Glucose	14.96	4.31	28.81	56.38
Whey	14.96	0.13	0.87	1.70
LSD (0.05)	0.035	0	0.098	0.225
A. versicolor-2				
Glucose	14.95	3.39	22.68	44.37
Whey	14.95	0.28	1.87	3.67
LSD (0.05)	0.088	0.093	0.708	1.398
A. oryzae				
Glucose	14.93	3.42	22.91	44.83
Whey	14.95	0.45	3.01	5.89
LSD (0.05)	0	0.152	1.053	2.043

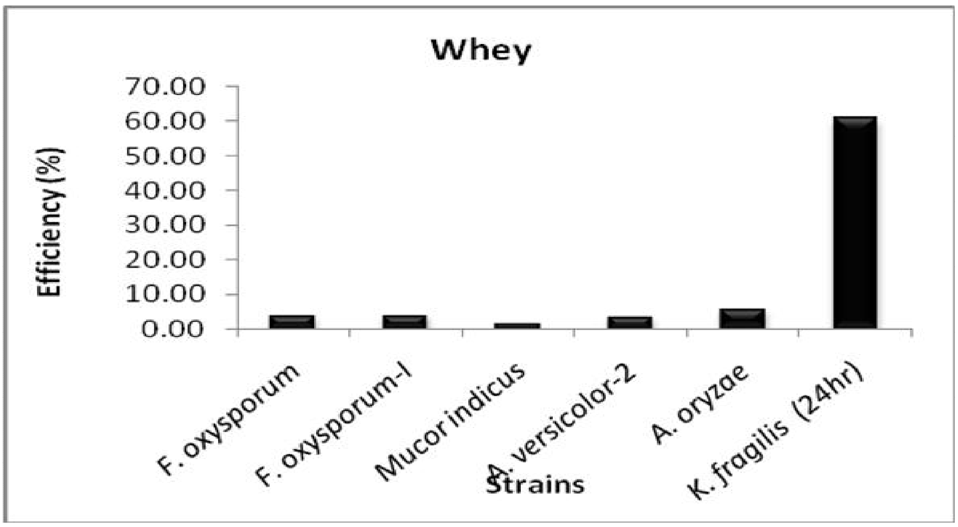

Fig.2: Comparison between efficiencies of ethanol production by isolated fungal strains and *Kluyveromyces fragilis* from whey permeate (15% lactose)

Vinasse is the main effluent of ethanol production coming from sugar cane or from sugar beet. It is the final by-product of the biomass distillation, mainly for the production of ethanol. Due to the large quantities of vinasse produced, alternative treatments and uses have been developed, such as recycling of vinasse in fermentation, concentration by evaporation, and yeast and energy production (Christofoletti *et al.,* 2013; Fadel *et al.,* 2014). So, its production is one of the most significant and challenging issues in the industrial production of ethanol due to pollution problems. So, vinasse is used as sole carbon source for ethanol production by the isolated fungal strains compared with *Saccharomyces cerevisiae* which is known for its high productivity of alcohol. Results in Fig (3), reported that, the fungus strains was unique in its production of ethanol when grown on vinasse medium. The strain *F. oxysporum-I* was the best producer followed by *M. indicus* which produced 1.93 and 1.89 g eth/100 ml, respectively. While it was shown that the yeast strain can't grow well, consequently, its ethanol production was nil. This is because of the presence of the phenolic compounds which is a big inhibitor for the growth of the yeast and the absence of reducing sugars in vinasse. (Parnaudeau *et al.,* 2008).

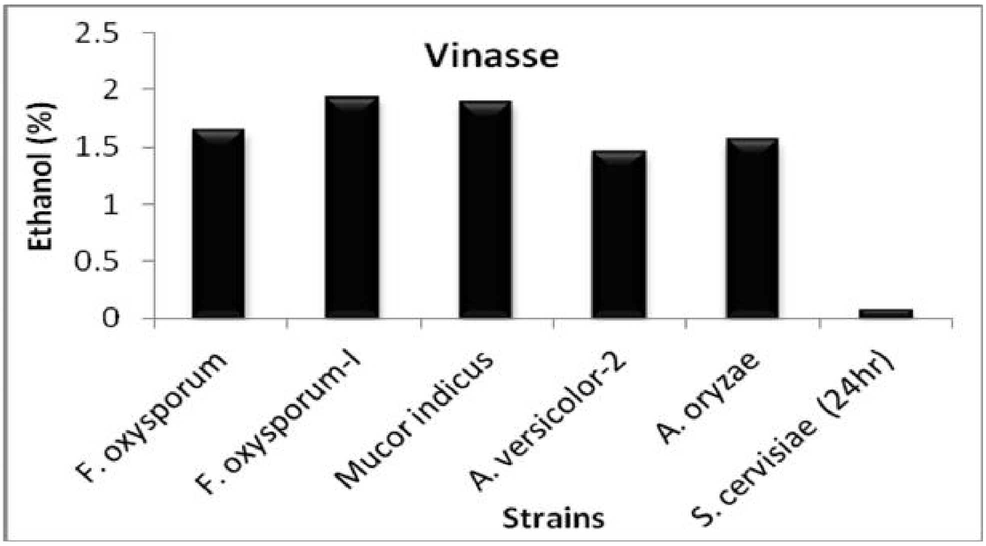

Fig. 3: Comparison between ethanol productions from vinasse by isolated fungal strains and *S. cerevisiae*

Navarro and Sepulveda (2000) and Fadel *et al.* (2014) used vinasse instead of water in the preparation of a fermentation medium. Available literature suggests that the major organic components of sugar cane vinasse are glycerol, lactic acid, ethanol, and acetic acid (Decloux and Bories, 2002; Parnaudeau *et al.,* 2008). Cheng *et al.* (2013) reported that fungus can convert glycerol to valuable chemicals. They also mentioned that waste glycerol represents a carbon source that is widely available at relatively low-cost and potentially suitable for many applications. In addition to these low molecular weight compounds, vinasse may contain melanoidins and phenolic compounds, which can inhibit or reduce the activity of microorganisms (Parnaudeau *et al.,* 2008) except fungi which can grow and resist these compounds (Aggelis *et al.,* 2003; Ahmadi *et al.,* 2006). Vinasse also contains different carbohydrates. Subsequently, fungi can secret enzymes for its hydrolysis producing ethanol. Also, fungi can benefit from nutrients present in the vinasse.

Conclusion

Fifteen percent sugar found to be the best concentration for ethanol production by fungi after five days of fermentation. Fungi can be used for ethanol production in some cases instead of yeast, especially from vinasse which is a big inhibitor for the growth of yeasts because of the presence of phenolic compounds and glycerol, also, the absence of reducing sugars in vinasse. *Mucor indicus* seems to be a good alternative to *Saccharomyces cerevisiae* in production of ethanol.

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