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THE INFLUENCE OF COMPLEXING AGENTS ON BIO-ETHANOL PRODUCTION FROM MOLASSES

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The influence of complexing agents on bio-ethanol production from molasses has been investigated. The agents are nitrilotriacetic acid (NTA) and 8 - hydroxyquinoline. They were introduced during the inoculation, propagation and fermentation stages.The increase was between 0.5-1.0% v/v ethanol for NTA and 2.9-3.8% v/v ethanol for 8-hydroxyquinoline.These results as well as the mode of action of the agents were discussed.

Key words: Bio-ethanol, Molasses, NTA, 8-Hydroxyquinoline, *Saccharomyces cerevisiae*.

Introduction

The production of bio-ethanol is inherently associated with low yields among other problems. The sensitivity of the microbial strain to fermentation condtions like pH, substrate concentration nutrient supplementation and temperature coupled with the suceptibility of the agricultural substrate to seasonal and agronomic conditions, are some of the factors contributing to this [1-4].

In veiw of these synthetic method has nearly overtaken the traditional fermentation methods. However increasing crude oil prices, have put a severe strain on the synthetic methods and has necessicitated researches in various laboratories on ways of improving the efficiency of the fermentation routes to make it competitive.

One of those ways is the addition of complexing agents. Complexing agents have proved indispensable in various bio-processes for the production of useful chemicals from crude substrates. Potassium ferrocyanide, ethylenediaminetetraacetic and EDTA, diethylenetriaminepentaacetic acid DPTA and dihydroxyethylene glycine DHEG have been used to improve the yield of citric acid using *Aspergillus niger* [5,6]. Also EDTA have been used in the production of riboflavin by *Candida guilliermondii*. [7]. In this Laboratory, we have used potassium, ferrocyanide, EDTA, DPTA and cyclohexane-diaminetetraacetic acid CDTA to improve the yield of ethanol from molasses using *Saccharomyces cerevisiae* [8,9]. In the studies potassium ferrocyanide improved the yield of ethanol during inoculation stage but depressed the yield during propagation stage showing its inconsistency. However, CDTA exerted an all round stimulation during the three stages of the fermentation cycle.

Therefore, in continuation of our studies we have chosen to consider the effects of 2 other agents, NTA and 8-hydroxy quinoline on ethanol yield. In addition, the 4 other complexing agents earlier used will be revisited in our comparative analysis as well as for rationalizing the mode of action of the agents. It is hoped that this study would throw some light on the mode of action of the complexing agents in bio-processes as well as in identifying the agent best suited for ethanolic fermentation of molasses.

Materials and Methods

Media for fermentation. Two media were used for the fermentation. Malt agar medium contained g/l, malt extract, 30.0, mycological peptone, 5.0, agar, 20.0 pH adjusted to 4.5 with phosphoric acid. Malt extract broth had the above composition but contained no agar.

Organism used. A pure strain of *Saccharomyces cerevisiae* supplied by the Botany and Microbiology Department of the University of Ibadan, was cultivated and used for the experiments. The inoculum after growing for 18 hrs was subcultured on malt extract agar. This was used for the exepriments.

Conditions for cultivation. The culture were grown by inoculating 10ml of the malt extract broth with yeast culture and growing for 24 hrs. Temperature was 30° ± 2°. All mycological media were sterilized in the autoclave at 103kpa, 121° for 15 mins. Yeast culture was propagated by injecting 15 ml of the substrate into 10 ml of the substrate and fermented for 36 hrs.

Fermentation medium. Sugar cane molasses obtained from Bacita Sugar Company in Kwara State, Nigeria was used for the experiments. It was diluted with water in a 1 : 4 ratio to bring the sugar level to between 12-15% w/v (Invert Sugar, 4-7% w/v, Sucroce, 8% w/v). The solution was adjusted to between 4.5 - 4.8 with phosphoric acid and then sterilized by autoclaving at 103kpa, 121° for 15 mins.

Addition of complexing agents. Various concentrations of complexing agents (0-1000 ppm) were introduced into the

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cultures during inoculation, propagation and fermentation stages.

Analytical methods. Sugars were determined by the lane and Eynon constant volume titrimetric method [10]. Ethanol content in the fermented broth was determined by the dichromate oxidation method [11]. Yeast population was determined by the direct counting method using hemocytometer slide. The number of dead cells were monitored by staining with methylene blue. For this test, 0.6% w/v methylene blue was mixed with yeast culture in ratio 1:5. After 5 mins, the number of cells which took up the stain was determined [12].

Results and Discussion

The effect of NTA and 8-hydroxy quinoline on the yield of ethanol is depicted in Fig. 1 and 2 respectively.

When added during inoculation stage, the concentration of ethanol increased by 1.0% v/v more than control cultures with the addition of 200ppm NTA. Whereas during propagation stage, there was 0.9% v/v increase in the concentration of ethanol with the addition of 100ppm NTA. However, during fermentation stage., 300ppm NTA increased the concentration of ethanol by 0.5% v/v more than control cultures.

The addition of 8-hydroxy quinoline also resulted in increased production of ethanol. 8-Hydroxy quinoline (900ppm) increased the concentration of ethanol by 3% v/v more than control cultures when it was introduced at the time of inoculation of yeast. During propagation stage, the concentration increased by 3.8% v/v more than control cultures, with the addition of 500ppm 8-hydroxy quinoline. During fermentation stage, there was an increase of 2.9% v/v ethanol when 700ppm of the chelating agent was used.

The comparative analysis of these agents with others earlier studied is presented in Table 1. From the Table, 8-hydroxy quinoline produced the greatest stimulation during the three stages of fermentation cycle, while NTA recorded the lowest stimulation in ethanol production.

Mode of action. One of the possible modes of action of the complexing agents is in terms of the favourable adjustement of metals present in the fermentation medium.

The effect of these various metals on the growth and accumulation of ethanol has been investigated [13]. Mn, Fe, Cu, Zn and Pb were indentified as toxic metals while K, Na, Ca and Mg supported the growth as well as the secretion of ethanol. Since all these metals are present in the fermentations medium, the formation of their complexes is governed by the various stability constants. The Table of stability constants is presented by Bjerrum *et al.* [14]. From the Table, it seems the metals which have earlier been found to be toxic to microorganism have higher stability constants allowing for easier removal from the fermentation medium. NTA which is the least active of the complexing agents have the lowest stability constants. This may be responsible for the slight stimulation in the production of ethanol.

The effects of the complexing agents are also applicable in terms of the population of yeast cells. The population of the organism was monitored during the 3 stages of fermentation cycle. The results is presented in Tables 2 and 3. In all the cultures, there were slight increases in the population of the micro organism during the inoculation stage. This was expected since it was the stage when the organism acclamatizes to the broth medium. The critical stage during the production was the propagation stage. The number of cell accumulated during this stage determines the concentration of ethanol in the final fermentation media. NTA which was the least active of

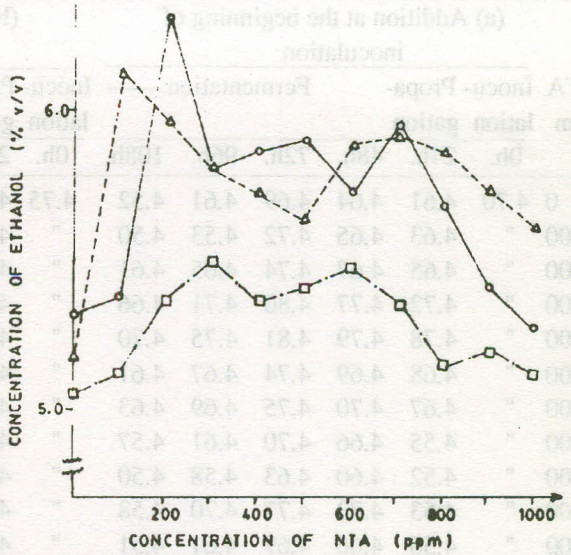

Fig. 1. Effect of NTA addition during —O—O— innoculation, —Δ—Δ— propagation and □—□ —□ —fermentation stages on ethanolic fermentation of molasses.

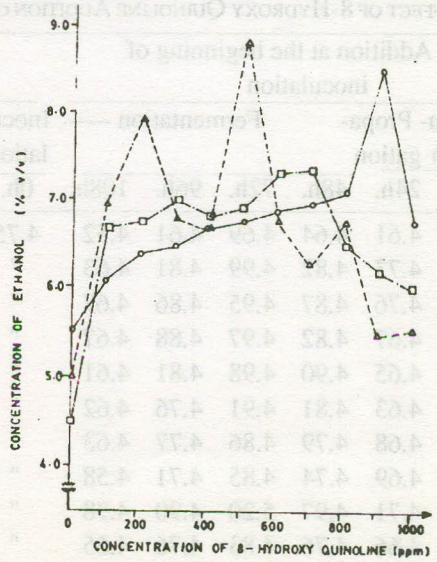

Fig. 2. Effect of 8-hydroxyquinoline addition during —O—O— inoculation, —Δ—Δ— propagation and —□—□—□— fermentation stages on ethanolic fermentation of molasses.

TABLE 1. STIMULATING EFFECTS OF COMPLEXING AGENTS ON THE ETHANOLIC FERMENTATION OF MOLASSES.

Chelating agents	Inoculation			Propagation			Fermentation		
	Conc. (ppm)	Stimulation	Effect*	Conc. (ppm)	Stimulation	Effect	Conc. (ppm)	Stimulation	Effect
**Hexacyanoferrate (II)	100	+	+3.0	300	–	-4.0	100	+	+0.7
**EDTA	400	+	+0.7	200	+	+1.5	700	+	+0.8
**CDTA	600	+	+1.5	1000	+	+2.3	700	+	+1.3
**DPTA	800	+	+2.9	200	+	+1.1	200	+	+0.2
8-Hydroxy quinoline	900	+	+3.0	500	+	+3.8	700	+	+2.9
NTA	200	+	+1.0	100	+	+0.9	300	+	+0.5

** Indicates the chelating agents earlier used. * Indicates % v/v ethanol production more than the control. + Inicates stimulation in ethanol prodduction. – Indicates decrease in ethanol production.

TABLE 2. EFFECT OF NTA ADDITION ON THE POPULATION OF YEAST CELLS (x 10^5 CELLS/ml) USING CANE MOLASSES.

NTA ppm	(a) Addition at the beginning of inoculation						(b) Addition at the beginning of propagation						(c) Addition at the beginning of fermentation					
	Inoculation	Propagation	Fermentation				Inoculation	Propagation	Fermentation				Inoculation	Propagation	Fermentation			
	0h.	24h.	48h.	72h.	96h.	108h.	0h.	24h.	48h.	72h.	96h.	108h.	0h.	24h.	48h.	72h.	96h.	108h
0	4.70	4.61	4.64	4.69	4.61	4.52	4.75	4.72	4.75	4.80	4.76	4.62	4.79	4.77	4.81	4.83	4.77	4.65
100	"	4.63	4.65	4.72	4.53	4.50	"	4.71	4.76	4.78	4.75	4.67	"	4.78	4.80	4.82	4.73	4.67
200	"	4.65	4.67	4.74	4.65	4.61	"	4.77	4.81	4.88	4.80	4.69	"	4.81	4.89	4.90	4.80	4.65
300	"	4.72	4.77	4.80	4.71	4.66	"	4.73	4.88	4.90	4.72	4.63	"	4.68	4.81	4.87	4.76	4.68
400	"	4.78	4.79	4.81	4.75	4.70	"	4.68	4.89	4.93	4.82	4.75	"	4.74	4.83	4.85	4.70	4.57
500	"	4.68	4.69	4.74	4.67	4.61	"	4.63	4.73	4.78	4.69	4.63	"	4.67	4.81	4.86	4.76	4.62
600	"	4.67	4.70	4.75	4.69	4.63	"	4.58	4.63	4.67	4.59	4.51	"	4.62	4.71	4.78	4.63	4.58
700	"	4.55	4.66	4.70	4.61	4.57	"	4.62	4.68	4.70	4.62	4.53	"	4.65	4.69	4.75	4.71	4.53
800	"	4.52	4.60	4.63	4.58	4.50	"	4.55	4.59	4.65	4.60	4.51	"	4.69	4.72	4.78	4.68	4.57
900	"	4.53	4.73	4.77	4.70	4.58	"	4.61	4.70	4.73	4.61	4.55	"	4.61	4.70	4.73	4.61	4.55
1000	"	4.56	4.66	4.69	4.61	4.51	"	4.60	4.65	4.72	4.62	4.58	"	4.73	4.78	4.79	4.69	4.61

TABLE 3. EFFECT OF 8-HYDROXY QUINOLINE ADDITION ON THE POPULATION OF YEAST CELLS (x 10^5 CELLS/ml) USING CANE MOLASSES.

8-Hydroxy-quinoline ppm	(a) Addition at the beginning of inoculation						(b) Addition at the beginning of propagation						(c) Addition at the beginning of fermentation					
	Inoculation	Propagation	Fermentation				Inoculation	Propagation	Fermentation				Inoculation	Propagation	Fermentation			
	0h.	24h.	48h.	72h.	96h.	108h.	0h.	24h.	48h.	72h.	96h.	108h.	0h.	24h.	48h.	72h.	96h.	108h.
0	4.70	4.61	4.64	4.69	4.61	4.52	4.75	4.72	4.75	4.80	4.76	4.62	4.79	4.77	4.81	4.83	4.77	4.65
100	"	4.73	4.82	4.99	4.81	4.63	"	4.76	4.80	4.97	4.87	4.59	"	4.76	4.82	5.03	4.88	4.61
200	"	4.76	4.87	4.95	4.86	4.67	"	4.77	4.89	4.96	4.88	4.62	"	4.78	4.86	5.17	4.87	4.65
300	"	4.67	4.82	4.97	4.88	4.67	"	4.69	4.98	5.05	5.17	4.78	"	4.68	4.89	5.06	4.81	4.62
400	"	4.65	4.90	4.98	4.81	4.61	"	4.71	4.87	4.92	4.72	4.61	"	4.80	4.90	5.00	4.82	4.63
500	"	4.63	4.81	4.91	4.76	4.62	"	4.65	4.88	5.17	4.99	4.72	"	4.73	4.88	4.07	4.79	4.61
600	"	4.68	4.79	4.86	4.77	4.63	"	4.72	4.79	4.98	4.73	4.57	"	4.75	4.87	4.92	4.68	4.51
700	"	4.69	4.74	4.85	4.71	4.58	"	4.73	4.82	4.92	4.81	4.61	"	4.77	4.92	5.12	4.78	4.67
800	"	4.71	4.97	5.20	4.90	4.78	"	4.68	4.85	4.96	4.82	4.59	"	4.72	4.85	4.91	4.76	4.65
900	"	4.66	4.76	4.83	4.76	4.65	"	4.78	4.77	4.89	4.77	4.60	"	4.74	4.86	4.92	4.62	4.55
1000	"	4.68	4.78	4.88	4.78	4.63	"	4.67	4.78	4.87	4.70	4.58	"	4.69	4.79	4.88	4.73	4.58

the complexing agents recorded the lowest population of yeast cell. The number of cells was between (4.5 – 4.8) x 10^5 cells/ml. Whereas 8-hydroxy quinoline which was the most active recorded the highest population of yeast cell during fermentation. The number of cells recorded was between (4.7-5.2) x 10^5 cells/ml.

Out of all the complexing agents tested only potassium ferrocyanide exerted a toxic effect on the yeast cells. The number of cells decreased from (4.8 - 4.3) x 10^5 cells/ml during propagation stage. This might be responsible for the depression of ethanol by 4% v/v. Whereas during inoculation and fermentation stages, there were increases in the population of the micro-organism as well as the concentration of ethanol.

One of the possible explanations is that the lag phase which preceeds the exponential growth phase, preludes the adaptation of the yeast to the ferrocyanide with the result that the toxic effect of ferrocyanide will be tolerated in future course. Obviously the overall effect of ferrocyanide depends on the trade off between the beneficial effect of the ferrocyanide in metal level adjustment and its deleterous effect.

The lag phase is a period of adaptation when the yeast was shifted from the environment of the malt extract agar to a new environment containing the toxic ferrocyanide. There was the need for reorganization of both its micro and macro cell components which may involve the synthesis and repression of enzymes. The addition of the toxic ferrocyanide during the exponential growth phase produced more inhibitory effect because steady state conditions were distrupted and consequently sufficient biomass could not be attained before the fermentation stage.

Conclusion

The complexing agents is selectively removing toxic metals in the medium, thereby improving the growth of the micro-organism. The ability to remove these metals however depends on the stability constants of the complexes produce between the toxic metals and the complexing agents.

Therefore in view of the non-toxicity of 8-hydroxy quinoline to the micro-organism and the effectiveness of toxic metal removal, leading to stimulation in ethanol concentration during the three stages of the fermentation cycle, the use of the agent is recommended ethanolic fermentation of molasses.

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