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EFFECT OF IRON AND EDTA ON ETHYL ACETATE ACCUMULATION IN *CANDIDA UTILIS*

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SUMMARY

Production of economically-recoverable products from dilute sugar or ethanol is of practical importance. Conversion of glucose to ethyl acetate by *Candida utilis* was inhibited by $FeCl_3$ supplementation as low as 10 uM. EDTA added at the onset of growth on glucose relieved such an inhibition and also caused faster and greater amounts of accumulation of the ester. Addition of EDTA during conversion of ethanol to ethyl acetate showed little effect. EDTA may affect cell permeability and/or oxidative metabolism. For continual ethyl acetate production iron limitation must be maintained during as well as before ethanol utilization.

INTRODUCTION

The recovery of dilute ethanol by distillation is not economical. The conversion of ethanol to products (e.g. ethyl acetate) which can be economically recovered and are of practical importance would be an attractive alternative. Ethyl acetate is amenable to simple extraction with solvents (e.g. gasoline), and has potential to be used as an octane-enhancing gasoline additive as well as a solvent and chemical feedstock.

We have previously shown that *Candida utilis* produces ethyl acetate from dilute ethanol if a growth medium is not supplemented with iron (Armstrong *et al.*, 1984). In general yeasts catabolize ethanol as follows: ethanol ---→ acetaldehyde ---→ acetic acid ---→ acetyl CoA, and the supply and consumption of acetyl CoA effects ethyl acetate formation (Nordstrom, 1962, 1963). It has been shown that acetyl CoA reacts with ethanol to form ethyl acetate (Howard and Anderson, 1976; Thomas and Dawson, 1978). Alcohol acetyltransferase responsible for this catalysis has been characterized and its activity is not affected by iron (Yoshioka and Hashimoto, 1981). It has been speculated that under iron limitation acetyl CoA is poorly oxidized through the TCA cycle and accumulating acetyl CoA reacts with ethanol forming ethyl acetate (Thomas and Dawson, 1978). The present communication describes the effect of iron and EDTA* on ethyl acetate accumulation in *C. utilis*.

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*EDTA = Ethylenediaminetetraacetic acid.

MATERIALS AND METHODS

Candida utilis (ATCC 9950) was grown in a sealed 250-ml Wheaton serum bottle at 28 C with shaking at 150 rpm in the minimal-salts medium of Thomas and Dawson (1978) with or without $FeCl_3$ supplementation. Glucose (20 g/L) was used as a carbon source and the medium was adjusted to pH 6.0. The medium was inoculated with 0.01 volume of an overnight culture. The degree of aeration was adjusted by varying the ratio of headspace/culture volume (H/C). A higher H/C indicates greater aeration. Cell mass density was measured by absorbance at 620 nm (A_{620}). Glucose was assayed by the dinitrosalicylate method (Miller, 1959). All products were confirmed by GC-mass spectrometry and assayed by gas chromatography. Iron was assayed by the dipyridyl method (Herbert, 1971).

RESULTS AND DISCUSSION

We have previously shown that ethyl acetate did not accumulate in a culture of *C. utilis* grown on glucose if the medium is supplemented with 100 uM $FeCl_3$ (Armstrong *et al.*, 1984). Figure 1 shows that considerable inhibition of ester accumulation occurred even when as low as 10 uM of $FeCl_3$ was supplied. Iron supplementation had little effect on cell mass growth. Our medium without iron supplementation (which was made of analytical grade chemicals) was found to contain 1.25 uM iron. An industrial medium made of technical grade chemicals could contain inhibitory concentrations of iron and would require chelation (or removal) of iron for ester production. Thus the effect of EDTA added at the time of inoculation was studied in glucose medium supplemented with 100 uM $FeCl_3$. Figure 2B confirms that supplementation of 100 uM $FeCl_3$ completely inhibited ethyl acetate accumulation. However EDTA, when added along with $FeCl_3$, relieved such an inhibition (Fig. 2C). The maximum level of the ester accumulated was similar to that in the culture without supplementation of iron and EDTA.

Another interesting effect of EDTA can be seen by comparing Fig. 2A with Fig. 2C. Without EDTA ethyl acetate and acetic acid began to accumulate only after glucose was used up and the accumulated ethanol began to be utilized, whereas the addition of EDTA caused ethyl acetate accumulation during glucose utilization and even before ethanol accumulation. The level of ethanol accumulated was considerably reduced. Thus use of EDTA would be beneficial in the fermentation of glucose to ethyl acetate, because it causes rapid ester accumulation even in the presence of glucose provided a high level of aeration was used. The following speculations on the effect of EDTA are possible. In the absence of EDTA, ethanol is readily excreted to an extracellular fluid. When glucose is depleted, utilization of extracellular ethanol is induced and ethyl acetate intracellularly formed is excreted to the medium. When ethanol is depleted, utilization of extracellular ester is induced. EDTA is known to affect membrane permeability in various microorganisms. It is therefore postulated that EDTA removes these membrane regulations by increasing membrane permeability. Since ethanol produced permeates through the membrane, it is immediately converted to ethyl acetate. Since the ester can also permeate through the membrane, an intracellular genetic system is exposed to higher levels of the ester, and induces the esterase; thus accounting for the formation of acetic acid at the expense of ethyl acetate which was evident after 4 d. Carbon balance indicates that approximately 92% of the carbon from the ester entered acetic acid. Thus EDTA may have potential to be used in the fermentation of glucose to acetic acid by this yeast.

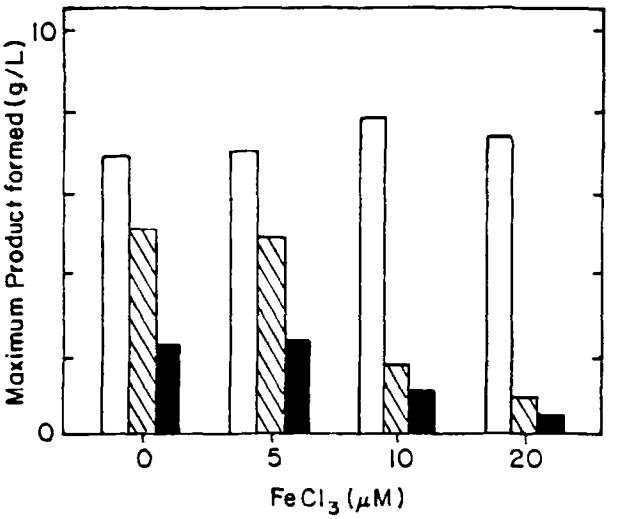

Fig. 1. Effect of $FeCl_3$ on ethyl acetate accumulation. *Candida utilis* was grown in glucose (20 g/L) medium supplemented with different concentrations of $FeCl_3$ under a high level of aeration (H/C=4) (Armstrong *et al.*, 1984). Products were analyzed at the point of maximum accumulation (ethanol, 1 d; ethyl acetate, 4 d). (□) ethanol; (▨) ethyl acetate; (■) acetic acid (assayed at 4 d). The medium without $FeCl_3$ supplementation was found to contain 1.25 uM iron.

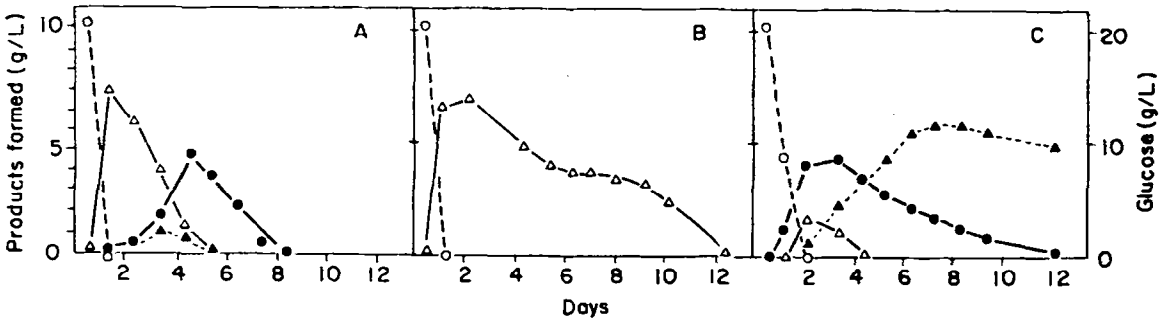

Fig. 2. Effect of EDTA on ethyl acetate accumulation. *Candida utilis* was grown in glucose (20 g/L) medium supplemented with: no $FeCl_3$ (part A), 100 uM $FeCl_3$ (part B) or 100 uM $FeCl_3$ plus 0.1% EDTA.2Na.$2H_2O$ (part C). A high level of aeration (H/C=4) was used. (o) glucose; (Δ) ethanol; (●) ethyl acetate; (▲) acetic acid. After 1 day incubation, cell density (A_{620}) was about 1.0 for A and B and 0.5 for C.

To study the effect of timing of EDTA addition, EDTA was added to cultures (without $FeCl_3$ supplementation) at three different times, and products from glucose were measured (Fig. 3). A lower level of aeration was used here to facilitate the delineation of product relationships (Armstrong *et al.*, 1984). Under this condition, ethanol accumulation clearly preceded ethyl acetate accumulation even in the presence of EDTA. Although EDTA added at the onset of growth on glucose caused faster and greater accumulation of ethyl acetate, it showed little effect on ester accumulation if added during growth on ethanol. Growth on glucose in the presence of EDTA may cause increased membrane permeability as speculated earlier as well as deficiency of acetyl CoA oxidizing capacity.

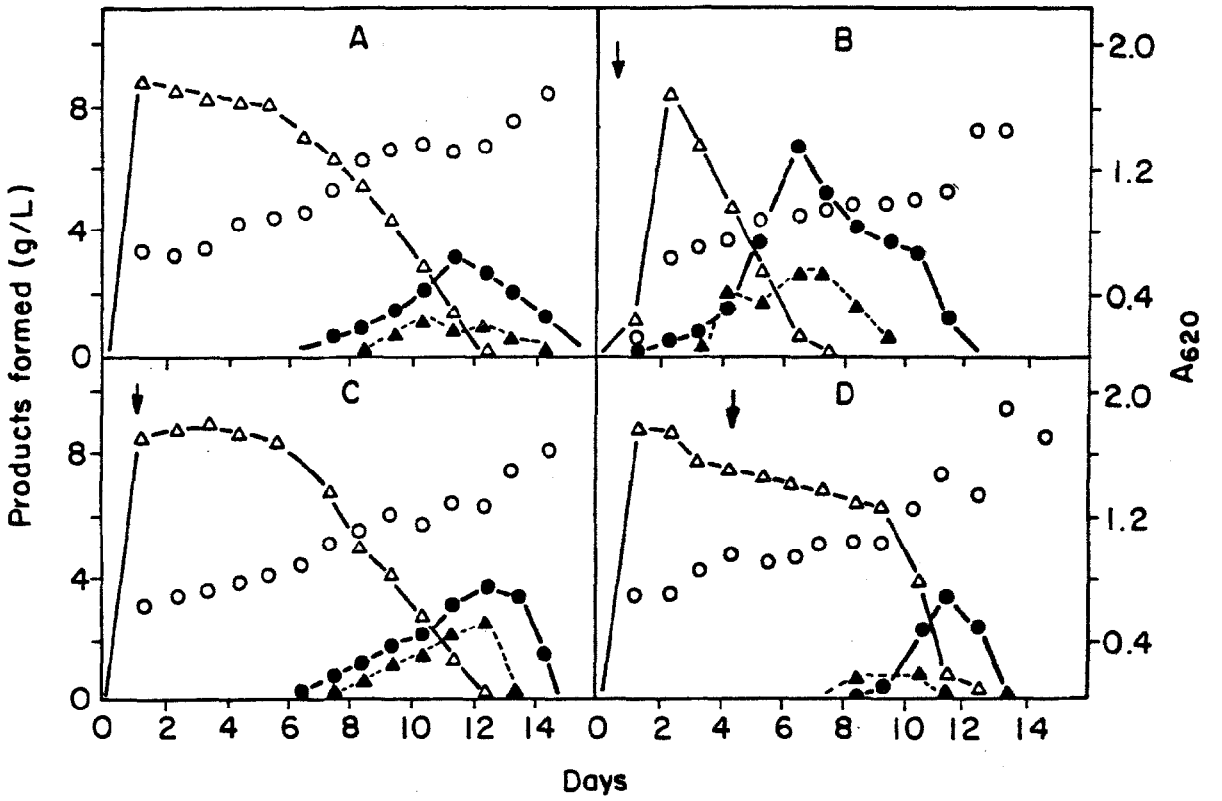

Fig. 3. Effect of timing of EDTA addition on ethyl acetate accumulation. *Candida utilis* was grown in glucose (20 g/L) medium without $FeCl_3$ supplementation. A low level of aeration (H/C=1.5) was used. EDTA.2Na.$2H_2O$ was added at different times as indicated by the arrows. Part A received no EDTA. (o) A_{620}; (Δ) ethanol; (●) ethyl acetate; (▲) acetic acid.

In the absence of EDTA, *C. utilis* membrane integrity would be normal. Therefore we speculate that ethyl acetate accumulation by the cells grown without $FeCl_3$ supplementation (Fig. 1A) is primarily due to underdevelopment of acetyl CoA oxidizing capacity under iron limitation. Such cells can be continuously used in repeated batch production of ethyl acetate from ethanol. Thus it is relevant to examine how their ethyl acetate-producing capacity is affected by $FeCl_3$ in ethanol medium. Cells were grown in glucose medium without $FeCl_3$ supplementation until cells began to convert accumulated ethanol to ethyl acetate. Cells were then suspended in ethanol medium with and without $FeCl_3$ supplementation. Figure 4 shows that the effect of iron supplementation was not seen during 9 h but became evident at 24 h when ethyl acetate accumulation was significantly reduced and little acetic acid accumulated. Since inhibition by iron was not immediate, we speculate that inhibition results from development of the acetyl CoA oxidizing system after iron supplementation.

The present study shows that for continual ethyl acetate production, iron limitation must be maintained during as well as before ethanol utilization and that EDTA can counteract iron inhibition.

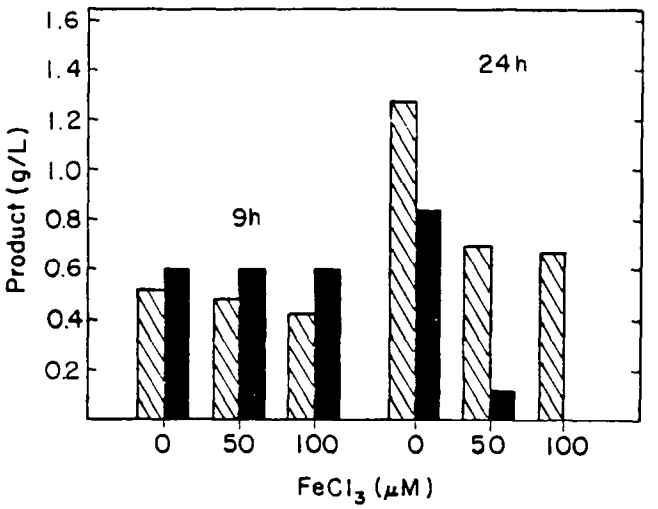

Fig. 4. Effect of $FeCl_3$ addition on ethyl acetate accumulation by *C. utilis* grown without $FeCl_3$ supplementation. *Candida utilis* was grown in glucose medium without $FeCl_3$ supplementation until cells began to produce ethyl acetate. The cells were washed and resuspended in medium containing 10 g/L ethanol and no glucose to a final cell density of A_{620} = 1.6. The cells were grown under a low level of aeration (H/C=1.5), and ethyl acetate (▨) and acetic acid (■) were measured after 9 h and 24 h. $FeCl_3$ supplementation showed little effect on growth. Cell density (A_{620}) was 2.3 at 9 h and 3.2 at 24 h.

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