

Ethanol Tolerance of *Saccharomyces cerevisiae*, L1400 (Exp. #2)

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Purpose

To determine the level of complete inhibition and the fermentation rates of L1400 in corn fiber media with increasing levels of exogenous ethanol at 0, 5, 6, 8, and 10% (w/w).

Background

The ability of L1400 to ferment in the presence of exogenous ethanol is an important consideration in the development of several current and future processes. Two important development aspects are recycling of spent simultaneous saccharification and fermentation (SSF) broth and the continuous addition of inoculum to fermentations. Recycling spent media will reduce costs by increasing biomass yields and ethanol concentration thereby optimizing downstream processing and improving enzyme utilization. Continuous addition of inoculum will provide an active yeast population capable of fermenting glucose under adverse conditions. Determining the ability of the nonadapted yeast to ferment in a high ethanol environment will permit setting practical limits for recycled ethanol that are not severely inhibitory.

This experiment was designed utilizing information from published literature and previous experiments performed by NREL and Amoco CRADA team members.¹⁻⁵

Materials and Methods

Yeast strain

The organism used in these studies was *Saccharomyces cerevisiae* Labatt 1400 strain and is a spheroplast fusion product of the polyploid brewing strain *Saccharomyces uvarum*, strain 21 and a genetically constructed diploid *Saccharomyces diastaticus*, strain 1384⁶. The seed vials were prepared by growing in YEPD media for 14 hours then diluting 1/2 with a 40% (w/v) glycerol solution and quick freezing. The vials contained 7×10^7 cells/ml. The organism was supplied by Amoco Corporation.

Preparation of Inocula

A one ml frozen vial stored at -70° C was thawed at room temperature and inoculated into 200 ml YEP with 2% (w/w) glucose media. The inoculum was incubated at 30° C in a rotary shaker (150 r.p.m.) for 24 hours.

Growth Media

The corn fiber media was prepared by adding 2% (w/w) corn steep liquor (CPN) to pretreated corn fiber. The pretreated corn fiber was prepared by Amoco Corporation. The media was then adjusted to a pH of 5.00 which required 37 grams of calcium hydroxide (reagent grade, GFS Chemicals) for 2136 grams of corn fiber. The amount of corn fiber media added was 77.4 grams to each of fifteen flasks and

autoclaved at **121** C for 15 minutes. After cooling, 0.82 ml (500 units or 200 units/g oligomeric sugar) amyloglucosidase (Sigma) and 0.75 ml (62 **IFPU** or **20 IFPU/g** cellulose) cellulase enzyme (Iogen) was added to each flask, mixed well, and incubated for 24 hours.

Growth Conditions

The **SSFs** were carried out at 30° C with 100 ml of corn fiber media in 250 ml baffled shake flasks capped with anaerobic gas locks. Triplicates for each of five levels of exogenous ethanol 0, 5, 6, 8, and 10% (w/w) were fermented in a rotary shaker set at 150 r.p.m.

Analytical techniques

Glucose and ethanol were determined using a Hewlett Packard 1090 HPLC equipped with a 1047 IR detector and a HPX-87H column. Column temperature was 85° C. Samples were centrifuged, sterile filtered (0.2 μ) and then diluted 1/5 with deionized water. **Seven** samples were taken at **various** times during the 208 hour fermentations (see Data, pages 1 and 2).

Results and Discussion

In an ideal continuous fermentation there would be one initial inoculation to provide cell mass throughout the fermentation and the substrate would be completely converted to ethanol in a reasonable time. **An SSF** would require a long fermentation period and an absence of inhibitors to reach complete substrate conversion. **An SSF** converts cellulose to liberate glucose at a very slow rate and therefore produces little energy for biomass production and maintenance so that in the presence of fermentative inhibitors most organisms are unable to remain fermentatively active. This extremely slow rate of cell generation may require a continuous inoculum and the removal of some of the inhibitory components in the spent media. Recycling media components that still have usefulness after the initial fermentation such as cellulase, biomass, and nutrients may help improve cost efficiency. Performance evaluation of cultures subjected to a sudden increase in ethanol, as would be experienced by a continuous inoculum, becomes important in optimizing an **SSF**. In this work, increasing levels of ethanol were tested in shake flasks and resulted in lower productivity rates and complete inhibition above a concentration of **6%** (w/w). There was about a **45** hour delay in the glucose consumption rate for fermentations containing **5%** (w/w) ethanol compared to the controls which had no ethanol added (see graph one). The controls went to completion after 208 hours utilizing all available glucose. The fermentations at **5%** (w/w) ethanol did not complete fermentation leaving about **2%**(w/v) residual glucose. The fermentations with **6%** (w/w) and **8%** (w/w) ethanol consumed glucose at a depressed rate for the first 16.5 hours but then glucose conversion ceased. The fermentations at 10% (w/w) ethanol showed a

gradual increase in glucose throughout the fermentation **period** and **was** probably due to a continuation of enzyme activity and a lack of fermentative activity.

The controls produced ethanol immediately with a productivity rate of 0.93 g/L h at which time the rate began to decline and level off (see graph two). The controls produced about 24 g/l ethanol during the course of the fermentation which represents a **71.9%** theoretical yield. The **5%** ethanol flasks showed very little activity for 40 hours and then the productivity rate increased to a maximum of 0.33 grams/L h between the period from **64** to **88** hours with the total ethanol being produced at 16 g/l and a 49.1% theoretical yield. Above the **5%** ethanol concentration there was no ethanol production and the ethanol gradually decreased during the fermentations. This gradual decrease in ethanol may be due to evaporation and binding from increased surface area of corn fiber media. The upper limit of ethanol tolerance was somewhere between 5 % (w/v) and 6% (w/v) ethanol. The tolerance of ethanol increases if the ethanol is produced endogenously rather than added exogenously. The upper limit of tolerance for produced ethanol has been reported to be around 11%(w/v)². Fermentations that reach higher ethanol concentrations such as these are carried out in an environment with high carbon availability which provides for maintenance of older cells and the generation of younger, more metabolically active cells. Higher concentration of cells, even though less active, may still provide an efficient conversion of glucose to ethanol and these recycled cells may experience less inhibition due to sudden environmental changes. The level of complete inhibition may be of little

concern because it is **likely** that **the** limitation of solids loading will not permit ethanol levels to reach higher than **6%** (w/v) unless **a** liquid recycling stream is used.

Conclusions

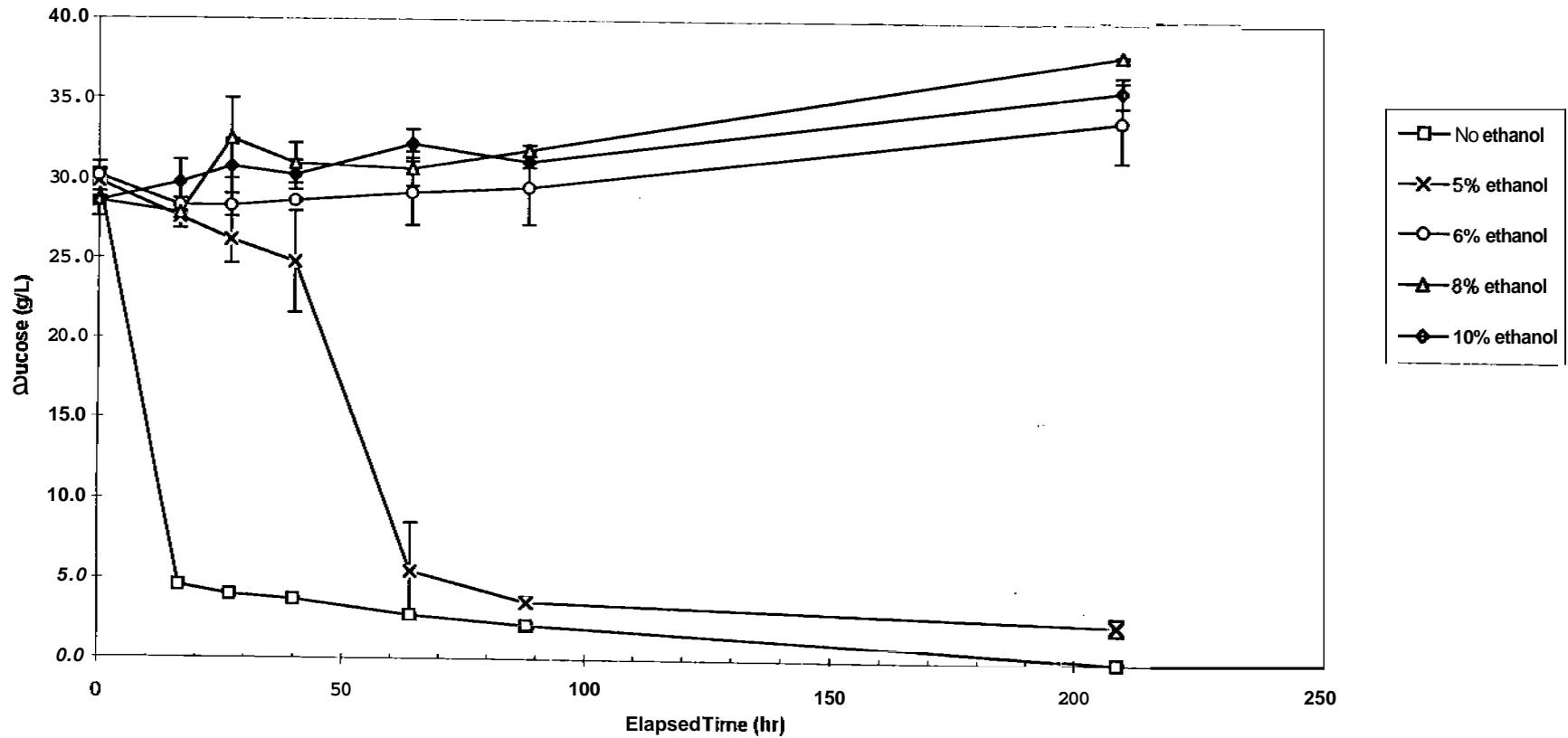
The level of complete fermentative inhibition occurred between **5%** (w/w) and **6%** (w/w) exogenous ethanol and supports the findings of the Amoco **group**.⁴ A significant lag in fermentation exists for fermentations with added ethanol and the productivity rate at **5%** (w/w) ethanol fell to one third that of the controls. Methods of increasing the tolerance limits such **as** nutrient supplementation, inoculum propagation, and fermentation parameters should be investigated if higher levels of ethanol are needed to improve recoverability and cost effectiveness. A more definitive investigation of the area between 0% and **5%** ethanol may uncover more productive levels of operation.

Bibliography

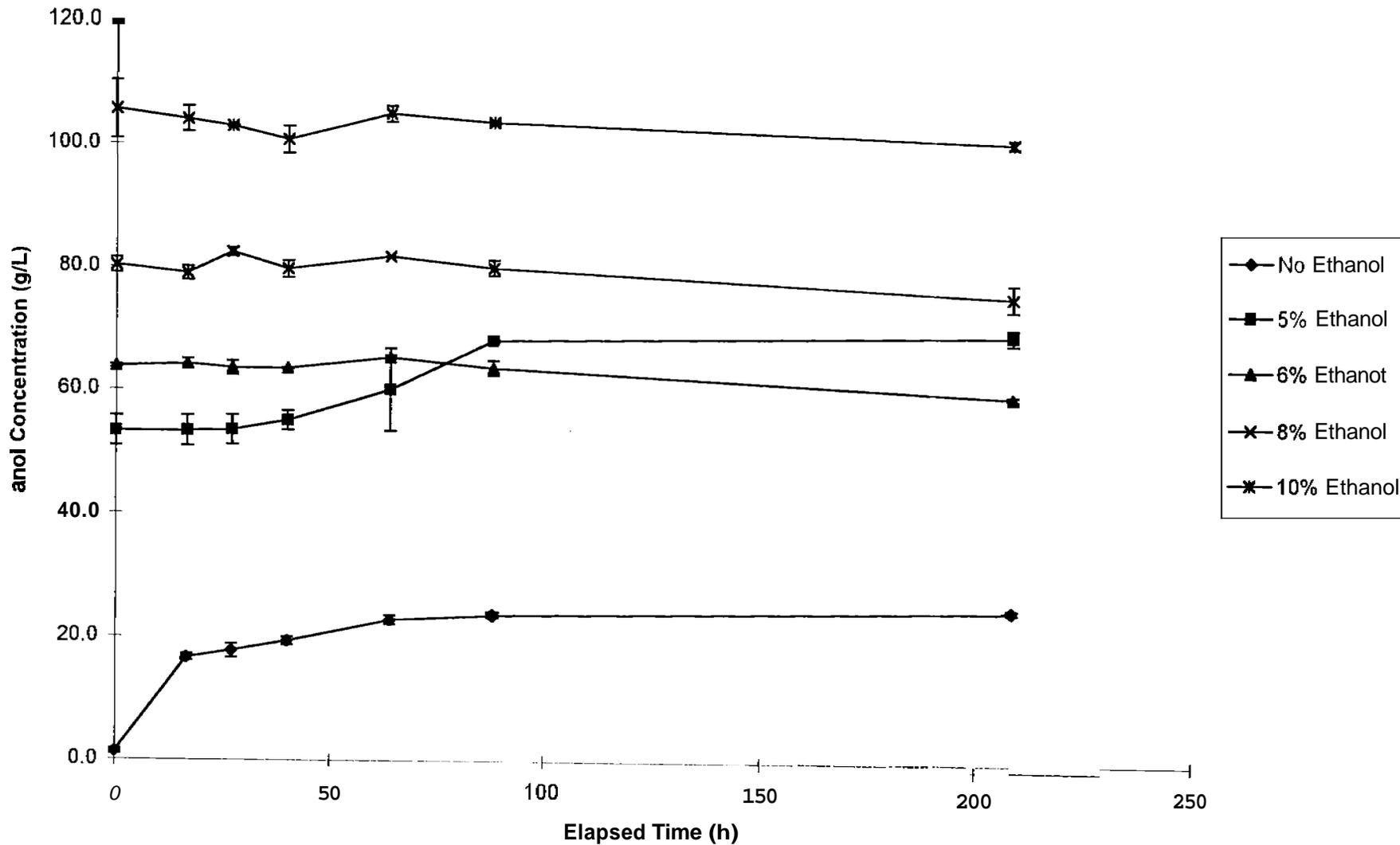
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Glucose Concentration

Glucose consumption



Ethanol Tolerance w/ L1400



Glucose Data

Time sample	Elapsed Time	Flask1	Flask2	Flask3	AVG	STDDEV
T0	0	29.7	28.5	31	29.7	1.3
T1	16.5	4.7	4.5	4.5	4.6	0.1
T2	27	4	4.1	3.8	4.0	0.2
T3	40	3.6	3.7	3.8	3.7	0.1
T4	64	2.7	2.8	2.6	2.7	0.1
T5	88	1.9	2.2	2.2	2.1	0.2
T6	208.5	0	0	0	0.0	0.0

Time sample	Elapsed Time	Flask 4	Flask 5	Flask 6	AVG	STDDEV
T0	0	29.5	30.5	29.3	29.8	0.6
T1	16.5	27	28.4	27.3	27.6	0.7
T2	27	25.6	27.8	25	26.1	1.5
T3	40	24.2	28.2	21.9	24.8	3.2
T4	64	3.3	28.2	7.6	5.5	3.0
T5	88	3.8	28.8	3.3	3.6	0.4
T6	208.5	2.7	33.2	2	2.4	0.5

Time sample	Elapsed Time	Flask 7	Flask 8	Flask 9	AVG	STDDEV
T0	0	30.5	30.2	29.7	30.1	0.4
T1	16.5	28.7	28.3	27.9	28.3	0.4
T2	27	28.1	27.7	29.1	28.3	0.7
T3	40	28.7	28.5	28.6	28.6	0.1
T4	64	30.5	26.8	30	29.1	2.0
T5	88	30.9	26.8	30.7	29.5	2.3
T6	208.5	35.2	31	35.4	33.9	2.5

Time sample	Elapsed Time	Flask 10	Flask 11	Flask 12	AVG	STDDEV
T0	0	29.4	28.7	27.5	28.5	1.0
T1	16.5	27.9	27.8	27.7	27.8	0.1
T2	27	30	32.4	35	32.5	2.5
T3	40	30.8	29.8	32.3	31.0	1.3
T4	64	31.3	29.4	31.2	30.6	1.1
T5	88	31.7	32.2	31.6	31.8	0.3
T6	208.5	37.9	37.9	38	37.9	0.1

Time sample	Elapsed Time	Flask 13	Flask 14	Flask 15	AVG	STDDEV
T0	0	28.3	28.5	28.8	28.5	0.3
T1	16.5	30	28.2	31	29.7	1.4
T2	27	29.8	29.7	32.7	30.7	1.7
T3	40	29.7	29.7	31.3	30.2	0.9
T4	64	32.9	32.5	31.2	32.2	0.9
T5	88	31.3	31.3	30.7	31.1	0.3
T6	208.5	36.7	35.7	34.8	35.7	1.0

Ethanol Data

Time sample	Elapsed Time	Flask 1	Flask 2	Flask 3	AVG	STDDEV
T0	0	1.4	1.7	0.9	1.3	0.4
T1	16.5	17.2	16.2	16.5	16.6	0.5
T2	27	18.8	17.8	16.6	17.7	1.1
T3	40	20	18.7	19.2	19.3	0.7
T4	64	23.6	22.6	22.2	22.8	0.7
T5	88	24.1	23.5	23.2	23.6	0.5
T6	208.5	25	24.4	24.5	24.6	0.3
Time sample	Elapsed Time	Flask 4	Flask 5	Flask 6	AVG	STDDEV
T0	0	51.3	56	52.6	53.3	2.4
T1	16.5	51	55.9	53.2	53.4	2.5
T2	27	52.1	56.3	52.2	53.5	2.4
T3	40	53.3	56.2	55.7	55.1	1.6
T4	64	23.7	55.4	64.8	60.1	6.6
T5	88	67.9	56.7	68.2	68.1	0.2
T6	208.5	68.3	54.7	70.1	69.2	1.3
sample	Time	Flask 7	Flask 8	Flask 9	AVG	STDDEV
T0	0	63.5	56.1	63.9	63.7	0.3
T1	16.5	64.7	54.8	63.5	64.1	0.8
T2	27	64.3	56.2	62.7	63.5	1.1
T3	40	63.4	41.5	63.5	63.5	0.1
T4	64	65.1	58.8	65.5	65.3	0.3
T5	88	62.8	57.9	64.5	63.7	1.2
T6	208.5	59.1	55.9	59.6	59.4	0.4
Time sample	Elapsed Time	Flask 10	Flask 11	Flask 12	AVG	STDDEV
T0	0	81.6	79.5	79.5	80.2	1.2
T1	16.5	79.8	77.7	79.4	79.0	1.1
T2	27	82.9	82	53.7	82.5	0.6
T3	40	79.8	78.3	81	79.7	1.4
T4	64	81.8	75.1	81.7	81.8	0.1
T5	88	81.1	78.6	80.3	80.0	1.3
T6	208.5	77.2	73.1	76.4	75.6	2.2
Time sample	Elapsed Time	Flask 13	Flask 14	Flask 15	AVG	STDDEV
T0	0	102.2	79.7	108.8	105.5	4.7
T1	16.5	102.7	103	106.3	104.0	2.0
T2	27	103	102.8	79.1	102.9	0.1
T3	40	101.1	102.7	98.4	100.7	2.2
T4	64	105.4	105.9	103.5	104.9	1.3
T5	88	69.4	103.9	103.4	103.7	0.4
T6	208.5	101.3	101	100	100.8	0.7