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**Title:** Butanol is cytotoxic to *Lactococcus lactis* while ethanol and hexanol are cytostatic

**Running Title:** Butanol cytotoxicity in *Lactococcus lactis*

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ABSTRACT

Lactic acid bacteria currently used extensively by the dairy industry have a superior tolerance towards small chain alcohols, which makes them interesting targets for use in future bio-refineries. The mechanism underlying the alcohol tolerance of lactic acid bacteria has so far received little attention. In the present study the physiological alcohol stress response of Lactococcus lactis subsp. cremoris MG1363 towards the primary, even-chain alcohols; ethanol, butanol, and hexanol was characterized. The alcohol tolerance of L. lactis was found comparable to those reported for highly alcohol resistant lactic acid bacteria. Combined results from alcohol survival rate, live/dead staining, and a novel usage of the beta-galactosidase assay, revealed that while high concentrations of ethanol and hexanol were cytostatic to L. lactis, high concentrations of butanol were cytotoxic, causing irreparable damages to the cell membrane.

INTRODUCTION

To forsake modern society’s dependency on limited, non-renewable oil-reserves, recent approaches have aimed at constructing microbial hosts for the production of bio-alcohols as alternative fuels. Using several metabolic engineering strategies production of the bio-alcohols ethanol and butanol have been shown exfeasible in a number of organisms such as Escherichia coli, Saccharomyces cerevisiae, Pseudomonas putida, and Bacillus subtilis (1–3).

A major hurdle limiting the yield and titers of microbial bio-alcohol production is the toxicity of these compounds (4). As ethanol and butanol are both short-chain alcohols there are similarities in how microbes respond to them. Through their
hydrophobic nature, short chain alcohols disrupt the phospholipid components of the cell membrane thereby increasing membrane fluidity and destroying the integrity of the cell (5–8). A direct correlation between the potency of alcohols as biological effectors and their hydrophobicity has been found (9–11), suggesting that the alcohols primarily influence hydrophobic associations (8). Ethanol stress, the most studied alcohol stress, has been shown to increase membrane permeability allowing for the free passage of ions and charged molecules (8). High concentrations of ethanol and butanol have been shown to interfere with the cell membranes pH gradient thereby increasing acid sensitivity in S. cerevisiae and Clostridium strains, respectively (5,12,13). Introduction of saturated fatty acids have shown to counteract the increased membrane fluidity associated with long term ethanol stress (5,8). In Lactococcus lactis subsp. cremoris MG1363 an increase in membrane fluidity has likewise been observed as a consequence of sub inhibitory ethanol stress (14). To counteract long term ethanol stress L. lactis was found to up-regulate shorter chained fatty acids and down-regulate longer chained unsaturated fatty acids. The shorter chained fatty acid C16:1n-7, known to decrease hydrophobic interaction between the free acyl chains and the proteins, was found up-regulated under prolonged sub inhibitory ethanol stress (14), in accordance with the suggested influence of ethanol on hydrophobic associations (8).

In the pursuit of an optimal production strain various organisms have been screened for their alcohol tolerance (15–17), and it has here been found that lactic acid bacteria (LAB) intrinsically possess a superior tolerance towards ethanol compared to other micro-organisms (18,19). Gold et al., determined the
ethanol tolerance of 31 Lactobacillus strains, and among these 8 showed a significant growth increase in medium containing 16% ethanol (16). In comparison E. coli is known to be extremely sensitive towards ethanol with marginal growth in concentrations above 6%, and the obligate ethanol-producer Zymomonas mobilis show minimum growth above 9% ethanol (8). In searching for butanol tolerant strains, Knoshaug and Zang determined the butanol tolerance of a range of organisms deemed suitable for production of butanol (15). From a total of 10 S. cerevisiae strains, 5 E. coli, and 3 Lactobacillus strains only two lactobacilli showed an increase in optical cell density in the presence of 2.5% n-butanol. One even showed growth in the presence of 3% n-butanol.

Although the superior alcohol tolerance of LAB have long been known (8,19) no studies have characterized the mechanism behind LAB’s superior alcohol tolerance. L. lactis subsp. cremoris MG1363 represents a suitable model organism for the analysis of alcohol tolerance in LAB because; (i) it has a simple fermentative metabolism in which glucose is converted almost exclusively into lactic acid (20), (ii) a wide range of regulatory networks has been characterized easing physiological and metabolic understanding (21,22), (iii) the genome sequence is known (23), and lastly, (iv) a large array of genetic tools are available (24–26). As a case study for its use as a biofuel producer, L. Lactis's ethanol production pathway was changed from lactic acid to ethanol, reaching yields comparable to those reported for optimized S. cerevisiae strains (27).

In the present study, novel insight into the alcohol tolerance of LAB is achieved through characterization of growth physiology, cell survival, and membrane
integrity of *Lactococcus lactis* subsp. *cremoris* MG1363 when grown in the presence of the short primary alcohols ethanol, butanol, and hexanol.

**METHODS**

*Strains and culture conditions:* *Lactococcus lactis* subsp. *cremoris* MG1363, a plasmid-free derivative of *L. lactis* subsp. *cremoris* NCDO712 (28) was used throughout. PRJ4621 is a derivative of MG1363 containing the plasmid pAK80 with the synthetic promoter CP25 in front of lacLM from *Leuconostoc lactis* (29).

*L. lactis* were grown in the chemically defined SA medium (30) modified by exclusion of acetate and supplemented with 2 μg ml⁻¹ lipoic acid and 1% glucose (GSAL) and incubated at 30°C without aeration but with slow stirring to ensure homogeneity. Medium with excess buffering capacity was achieved by supplementing with twice the amount of the pH buffer morpholinepropanesulfonic acid (MOPS) as in regular GSAL (GSALMOPS).

To ensure statistical significance all growth experiments were performed with a minimum of three biological replicas.

Alcohol stress response: The alcohol stress response of *L. lactis* was determined by adding exogenous alcohol to cultures that had grown exponentially for at least ten generations, ensuring a balanced pre-stress phenotype. By exposing growing cultures (OD₄₅₀ ~ 0.4 ml⁻¹) to a range of ethanol (25 – 115 mg ml⁻¹), butanol (5 – 25 mg ml⁻¹), or hexanol (1 – 3 mg ml⁻¹) concentrations the tolerance range of *L. lactis* towards the specific alcohols was determined. The alcohol stress response was quantified through growth rates (h⁻¹), final cell density
yields (OD$_{450}$), and pH. Amount of product produced and substrate consumed was quantified by HPLC following overnight stress inoculation.

Quantification of extracellular glucose and end products: To determine the extracellular metabolite levels in the growth medium, culture samples were filtrated through a 0.22 μm filter. Glucose, lactate, acetate, formate, and ethanol were separated using an Ultimate 3000 high-pressure liquid chromatography system (Dionex, Sunnyvale, CA) equipped with an Aminex HPX-87H column heated (Bio-Rad, Hercules, CA) and a Shodex RI-101 detector (Showa Denko KK, Tokoy, Japan). The column temperature was set to 60°C and 5mM H$_2$SO$_4$ was used a mobile phase with a flow rate of 0.6 ml min$^{-1}$.

Acid sensitivity assay: Cultures grown in GSAL or in GSAL$_{MOPS}$ were subjected to a range of exogenous ethanol (25 – 115 mg ml$^{-1}$), butanol (5 – 20 mg ml$^{-1}$), and hexanol (1 – 3 mg ml$^{-1}$) concentrations. Differences between final pH’s and between final yields of alcohol-stressed cultures grown in GSAL$_{MOPS}$ to alcohol-stressed cultures grown in GSAL were used to quantify acid sensitivity.

Survival rate under alcohol stress: The survival rate of *L. lactis* when exposed to the individual alcohols was determined by measuring the survival in colony forming units (CFU) after 120 min of alcohol stress. Concentrations used; ethanol (55 – 120 mg ml$^{-1}$), butanol (10 – 25 mg ml$^{-1}$), and hexanol (2 – 3 mg ml$^{-1}$). Aliquots were removed at 0 (reference), 10, 20, 40, 80, and 120 min after induction of alcohol stress, and the first six serial dilutions were plated on GM17 agar and incubated at 30°C over night (31). The relative increase in CFU compared to the reference was calculated.
**Quantification of the Live/dead ratio:** The live/dead ratio of alcohol stressed *L. lactis* was determined in-situ using the BacLight Live/dead Bacterial Viability Kits, L7012, provided by Molecular Probes and imaged using Zeiss Axioplan Fluorescence Microscope with a RS Photometrics CoolSNAP Camera. The filter had an excitation of 450-490 nm with long pass 520 nm enabling simultaneously visualization of SYTO 9 and propidium iodide dye. Exponentially growing *L. lactis* was harvested and re-suspended in cold GSAL medium to yield a final OD$_{450}$~ 1.5. Aliquots of the harvested cells were exposed to ethanol, butanol, or hexanol for 5 min on ice before being mixed with the staining agents SYTO 9 and propidium iodide dye and visualized by microscopy. A minimum of 3 samples of at least 500 fluorescent cells each were counted for each condition and used to determine the live/dead ratio. For further details regarding the Live/dead kit used see the manufactures product manual, Molecular Probes (Live/dead BacLight Bacterial Viability Kit: https://tools.lifetechnologies.com/content/sfs/manuals/mp07007.pdf).

**Assay of cell permeability towards ONPG.** The amount of ortho-nitrophenylgalactoside (ONPG) passing the membrane of PRJ4621 cells was measured as the rate of ortho-nitrophenol (ONP) formation inside the cells. The strain PRJ4621 has a high constitutive β-Galactosidase activity, and the rate of ONP formation was determined using the standard protocol described by Miller with the following modifications (32). Exponentially growing cells OD$_{450}$~ 0.4 ml$^{-1}$ were either harvested for β-Galactosidase assay or incubated at 30°C for 1 hour with ethanol (120 mg ml$^{-1}$), butanol (25 mg ml$^{-1}$), or hexanol (2.5 mg ml$^{-1}$). The non-stressed harvested cells, were washed and lysed with chloroform, left
untreated or subjected to either ethanol (120 mg ml⁻¹), butanol (25 mg ml⁻¹), or hexanol (3 mg ml⁻¹) for 10 min at 30°C prior to addition of ONPG and subsequent quantification of β-Galactosidase activity. The alcohol stressed cells were harvested and washed followed by treatment with chloroform or left untreated for 10 min at 30°C. ONPG was hereafter added and the rate of ONP formation was quantified.

RESULTS AND DISCUSSION

Tolerance of L. lactis towards primary alcohols categorizes L. lactis as a highly butanol tolerant strain

The tolerance range of Lactococcus lactis subsp. cremoris MG1363 against the primary alcohols ethanol, butanol, and hexanol was determined by exposing exponentially growing L. lactis to a broad range of exogenous alcohol concentrations (Fig. 1). All growth experiments were performed in the chemically defined SA medium (30) supplemented with 1% glucose (GSAL), to avoid evoking the purine sensitive phenotype of L. lactis (33). Fig. 1a, 1b, and 1c shows the growth curves obtained for the first 100 min after addition of increasing concentrations of ethanol, butanol, or hexanol. The specific growth rates obtained under alcohol stress as a function of the final alcohol concentration in the growth medium, revealed, as expected, an inverse correlation between growth rate and alcohol concentration (Fig. 1d, 1e, and 1f).

As a measure of the tolerance of L. lactis towards the individual alcohols, the alcohol concentration that resulted in 50% decrease in the specific growth rate was determined. This was observed for ethanol at 55 mg ml⁻¹, butanol at 10 mg ml⁻¹, and hexanol at 1.5 mg ml⁻¹, corresponding to (7%, 1.2%, and 0.2% (v/v),
respectively). At these concentrations a constant flux of glucose consumption could be detected (data not shown) confirming that the vitality of the bacteria was intact.

When the final optical density was plotted as a function of alcohol concentrations (Fig. 2a, 2b, and 2c) a 50% decrease in final yield was observed for ethanol, butanol, and hexanol at 75, 15, and 2.5 mg ml\(^{-1}\), corresponding to 10%, 2%, and 0.3% (v/v), respectively, indicating a high level of alcohol resistance.

**Alcohol does not induce increased acid sensitivity**

To assess if alcohol stress causes increased acid sensitivity in *L. lactis*, a growth-based assay was devised, in which the buffering capacity of the medium could be increased by doubling the concentration of the buffering agent. This assay was used to distinguish between two contrasting models; one where the alcohol stress causes an increased acid sensitivity (model 1), or one where the alcohol stress does not cause an increased acid sensitivity (model 2). If the final OD was determined by a pH threshold (model 1), then a doubling of the buffering capacity of the medium would result in a doubling of the final OD of the culture reaching the same pH threshold. If, in contrast, the final OD was determined by other alcohol induced thresholds (model 2), then a doubling of the buffering capacity would have no affect on the final yield, but would result in a higher final pH.

In Fig. 3a, 3b, and 3c a set of growth experiments has been represented in two types of plots. The first type of plots shows the increase in final pH upon doubling the buffering capacity of the medium (left axis) as a function of alcohol
concentration. Since unstressed cultures use the excess buffering capacity to increase biomass production and continue fermentation until the normal pH threshold is reached, doubling the buffering capacity only insignificantly increased the pH level (0.1 pH unit), as shown in Fig. 3a, 3b, and 3c for the unstressed conditions (0 mg ml$^{-1}$). The excess biomass production upon doubling the buffering capacity of the medium (right axis) is shown as a function of alcohol concentration in the second type of plots. Plots from all three alcohol stress conditions showed that higher alcohol concentrations follow model 2, since the increase in biomass yield is reduced to zero and the final pH is elevated. Butanol at 15 mg ml$^{-1}$ has the most severe effect on the increase in final pH (1.6 pH units).

Growth cessation was not caused by sugar starvation, as an excess of glucose was found to be present in the medium (data not shown). Furthermore, the higher pH observed at higher alcohol concentrations was not accompanied by a shift in the general metabolism since the cultures performed homolactic fermentation throughout the experiment (data not shown).

**Butanol is cytotoxic while ethanol and hexanol is cytostatic to *L. lactis***

Alcohol could be either cytostatic or cytotoxic to the lactococcal cell. If alcohol is cytostatic it inhibits cell growth as long as the stress is applied, but the inhibition is reversed when the stress disappears. In contrary, if alcohol is cytotoxic, the inhibition is not reversed. The survival rate of *L. lactis* exposed to the three alcohols was determined by quantifying the number of colony forming units (CFU) at various time points before and after the onset of alcohol stress. Only alcohol concentrations that had been found to inhibit growth to less than half-
maximal growth rates were used. Fig. 4a, 4b, and 4c show the growth in optical density before and after addition of ethanol, butanol, or hexanol, respectively. Fig. 4d, 4e, and 4f show the relative CFU determination (survival) of *L. lactis* exposed to ethanol, butanol, or hexanol.

Interestingly, it could be noted from all curves in Fig. 4d, 4e, and 4f that even though a steady increase in OD$_{450}$ was observed for all of the tested alcohol concentrations (Fig. 4a, 4b, and 4c), none of the cultures showed any significant increase in CFU. Cell clumping and chain formation are known to underestimate the number of CFU under heat stress of *L. lactis* (34), and phase-contrast microscopy revealed that *L. lactis* forms chains when dividing during ethanol, butanol, and hexanol stress (data not shown). As shown in Fig. 4e, high butanol concentrations caused severe cell death with a fast death rate at a concentration of 25 mg ml$^{-1}$. In contrast, high concentrations of ethanol (Fig. 4d), and hexanol (Fig. 4f) did not lead to cell death, suggesting that butanol is cytotoxic to *L. lactis* whereas both ethanol and hexanol are cytostatic. It should be noted that no decrease in optical density was observed for butanol treated *L. lactis* cells, thus butanol is not suspected to cause cell lysis.

To substantiate the claim that high butanol concentrations are cytostatic, the cytotoxicity of the three alcohols was investigated *in situ* using live/dead staining. Live/dead staining uses a double labeling technique with the green-fluorescent SYTO 9 and the red-fluorescent propidium iodide (PI). PI is frequently used in lactococcal research to distinguish dead from living cells, as only cells with irreparable damaged membranes are stained (35–37), while SYTO 9 stains the DNA of both living and dead cells. Exponentially growing cells
in GSAL medium were harvested and subjected to ethanol, butanol, or hexanol at 120, 25, or 3 mg ml\(^{-1}\) for 5 min on ice, respectively, before addition of the live/dead stain. Fluorescence microscopy imaging was subsequently used to determine the ratio of dead vs. live cells (Fig. 5).

By inspection of the staining pattern in Fig. 5, it became clear that the ratio of dead cells was higher after butanol addition than after addition of ethanol or hexanol, in accordance with the results from the survival determination. Quantification of the live/dead-ratio of *L. lactis* showed that ethanol at 120 mg ml\(^{-1}\) and hexanol at 3 mg ml\(^{-1}\) slightly lowered the percentage of live cells from 100% in untreated cells to 86% ± 8% and 91% ± 2%, respectively. In contrast, butanol at 25 mg ml\(^{-1}\) decreased the fraction of living cells to 35% ± 8%. Even when the butanol concentration was reduced to 21 mg ml\(^{-1}\), a low survival rate was observed (25% ± 17%), confirming that high butanol concentrations are toxic towards *L. lactis*.

To confirm the observation that higher concentrations of butanol causes damage to the cell membrane, the ability of ortho-nitro phenyl galactoside (ONPG) to cross the membrane under ethanol, butanol, and hexanol stress was determined. An *L. lactis* strain PRJ4621, with a high constitutive β-Galactosidase activity, was used for detection of intracellular ONPG by conversion to ONP. Chloroform is usually used to permeabilize cells prior to determination of β-Galactosidase activity in bacteria (32), and Fig. 6 shows that non-permeabilized cells produce very little ONP. Exposure to chloroform for 10 min resulted in high ONP production (1000 miller units). Treatment with ethanol at 120 mg ml\(^{-1}\) for 10 min had no effect, while exposure to butanol or hexanol for 10 min (at 25 mg ml\(^{-1}\)
and 2.5 mg ml\(^{-1}\), respectively) resulted in permeabilization close to that of chloroform (Fig. 6). The obtained β-Galactosidase activity in PRJ4625 was comparable to those observed in the literature (29).

Surprisingly, both butanol and hexanol caused permeabilization of the membrane towards ONPG, since only butanol was found to be cytotoxic. It was therefore hypothesized that the damage by hexanol could be reversible and thus cytostatic, while the damage by butanol was irreversible leading to cytotoxicity. To test this, exponentially growing cells of PRJ4621 were first subjected to ethanol, butanol, or hexanol (120, 25, or 2.5 mg ml\(^{-1}\) respectively) for 1 hour. The stressed cells were then harvested, washed, and assayed for production of ONP. The maximal ONP production rate was found for each condition by assaying a chloroform permeabilized sample. The relative ONP production rate between untreated and chloroform permeabilized cells for each stress condition is shown in Fig. 7. For unstressed cells the intracellular ONP production rate was approximately 60-fold elevated by chloroform permeabilization. In contrast, for butanol treated cells the chloroform permeabilization only result in 5-fold elevation of the ONP production rate, showing that the cells are already highly permeable to ONPG. It is evident from Fig. 7 that the hexanol permeabilization is partially reversed, since chloroform is needed for ONPG to enter the cell. The 28-fold elevated ONP production between chloroform treated and non-treated hexanol stressed cells is almost half of the observed 59-fold change for the control condition, which supports the suggestion that butanol is cytotoxic because it creates irreversible damage to the membrane that results in increased permeability to small impermeable molecules. Due to the irreversible nature of
butanol induced membrane disruption, it could be hypothesized that butanol forms stable pores in the membrane, while the larger hexanol form chains that align with the phospholipids. Upon removing hexanol stress, the aligned hexanol chains may distribute evenly resulting in a partial reversal of the permeabilization.

CONCLUSION

Combined results from the alcohol survival rate, live/dead staining through SYTO 9 and PI, and novel usage of the β-galactosidase assay, revealed that while high concentrations of ethanol and hexanol were cytostatic to L. lactis, high concentrations of butanol were cytotoxic through disruption of the cell membrane.

For the future industrial use of LAB as butanol producers, the cytotoxic effect of butanol needs to be addressed before selecting production strains. In this regard, L. lactis is unsuitable unless the cytotoxicity of butanol is diminished. Adaptive laboratory evolution has been initiated to provide butanol resistant strains for the further characterization of a L. lactis strain with improved butanol tolerance.


Figure 1: Stress response of *L. lactis* towards exogenous ethanol, butanol, and hexanol. (a-c) Growth of *L. lactis* in GSAL medium in the presence of various concentrations of (a) ethanol, (b) butanol, and (c) hexanol was monitored by OD$_{450}$. Exogenous alcohol was added in mid-exponential phase (OD$_{450}$ ~0.4 ml$^{-1}$). (d-f) Growth rates, $\mu$ (h$^{-1}$), were calculated from the growth curves (a-c) and plotted against the final concentrations of (d) ethanol, (e) butanol, and (f) hexanol. (a) Concentrations of ethanol in mg ml$^{-1}$: 25 [filled circles], 55 [open circles], 75 [filled squares], 95 [open squares], and 115 [filled triangles]. (b) Concentrations of butanol in mg ml$^{-1}$: 5 [filled circles], 10 [open circles], 15 [filled squares], 20 [open squares], and 25 [filled triangles]. (c) Concentrations of hexanol in mg ml$^{-1}$: 1.0 [filled circles], 1.5 [open circles], 2.0 [filled squares], 2.5 [open squares], and 3.0 [filled triangles].

Figure 2: Final yield as defined by OD$_{450}$ of *L. lactis* grown in GSAL as a function of increased ethanol (a), butanol (b), and hexanol (c) concentrations. Exogenous alcohol was added in mid-exponential phase (OD$_{450}$ ~0.4 ml$^{-1}$), and final yield was measured after overnight incubation.

Figure 3: Acid sensitivity assay of alcohol stressed *L. lactis*. Exogenous alcohol was added in mid-exponential phase (OD$_{450}$ ~0.4 ml$^{-1}$). Final yield, as defined by OD$_{450}$ and pH were measured after overnight incubation of *L. lactis* grown in regular GSAL medium and in GSAL with doubled buffering capacity. Left axis, relative increase in final pH [filled circles]; and right axis, relative increase in final yield [open circles] by doubling the buffering capacity of the medium as a function of increased concentrations of ethanol (a), butanol (b), and hexanol (c).

Figure 4: Relative survival of *L. lactis* grown in GSAL after addition of ethanol (a, d), butanol (b, e), or hexanol (c, f). Exogenous alcohol was added in mid-exponential phase (OD$_{450}$ ~0.4 ml$^{-1}$, reference time point). Relative survival was determined by measuring changes in colony forming units (cfu) following the onset of stress as compared to the reference condition. (a, d) Concentrations of ethanol in mg ml$^{-1}$: 55 [filled circles], 75 [open circles], 95 [filled squares], and 120 [open squares]. (b, e) Concentrations of butanol in mg ml$^{-1}$: 10 [filled circles], 20 [open circles], 22.5 [filled squares], and 25 [open squares]. (c, f) Concentrations of hexanol in mg ml$^{-1}$: 2.0 [filled circles], 2.5 [open circles], 2.75 [filled squares], and 3.0 [open squares].

Figure 5: In situ live/dead imaging of *L. lactis* (a), or *L. lactis* exposed to 120 mg ml$^{-1}$ of ethanol (b), 25 mg ml$^{-1}$ of butanol (c), or 3 mg ml$^{-1}$ of hexanol (d). Exponential growing cells was harvested and subjected to ethanol, butanol, or hexanol stress before staining with SYTO 9 and propidium iodide. Cells with physically intact membranes are shown in green while cells with damaged membranes are shown in red.
Figure 6: Intracellular production of ONP from ONPG in a Lac+ derivative of *L. lactis*. Exponentially growing cells of a *L. lactis* Lac+ derivative (PRJ4621) were harvested and permeabilized with chloroform, ethanol (120 mg ml⁻¹), butanol (25 mg ml⁻¹), or hexanol (2.5 mg ml⁻¹) following by determination of ONP production from ONPG.

Figure 7: Relative ONP production rate in a Lac+ derivative of *L. lactis*. Exponentially growing cells of a *L. lactis* Lac+ derivative (PRJ4621) were subjected to either non-stressed, ethanol (120 mg ml⁻¹), butanol (25 mg ml⁻¹), or hexanol (2.5 mg ml⁻¹) stress for one hour following which the cells were either permeabilized with chloroform or not, and ONP production from ONPG was determined. The relative difference in ONP production between the chloroform permeabilized and non-permeabilized cells is shown.
Fig. 1

Graphs showing the effect of concentration of ethanol, butanol, and hexanol on the rate of production of a certain compound over time.
Fig. 2

(a) Final Yield (OD$_{450}$) vs. Concentration ethanol (mg ml$^{-1}$)

(b) Final Yield (OD$_{450}$) vs. Concentration butanol (mg ml$^{-1}$)

(c) Final Yield (OD$_{450}$) vs. Concentration hexanol (mg ml$^{-1}$)
Fig. 3

- **Figure a**: Effect of ethanol concentration on final pH and yield.
  - Concentration ethanol (mg ml⁻¹) vs. increase in final yield (OD₄₅₀).
  - Concentration ethanol (mg ml⁻¹) vs. increase in final pH.
  - Control line shows no effect.

- **Figure b**: Effect of butanol concentration on final pH and yield.
  - Concentration butanol (mg ml⁻¹) vs. increase in final yield (OD₄₅₀).
  - Concentration butanol (mg ml⁻¹) vs. increase in final pH.
  - Control line shows no effect.

- **Figure c**: Effect of hexanol concentration on final pH and yield.
  - Concentration hexanol (mg ml⁻¹) vs. increase in final yield (OD₄₅₀).
  - Concentration hexanol (mg ml⁻¹) vs. increase in final pH.
  - Control line shows no effect.
Fig. 5
Fig. 6

![Graph showing ONP production rate (Miller Units) for different compounds with and without Chloroform.](Fig 6.pptx)
Fig. 7

The figure shows a bar chart illustrating the relative ONP production rate for different conditions:

- **Control** shows the highest ONP production rate, followed by
- **Ethanol**
- **Butanol**
- **Hexanol**

The y-axis represents the relative ONP production rate, while the x-axis lists the conditions: Control, Ethanol, Butanol, and Hexanol.