Development of co-culture between *Ralstonia eutropha* and *Saccharomyces cerevisiae* to produce ethanol from glucose in a carbon neutral process.

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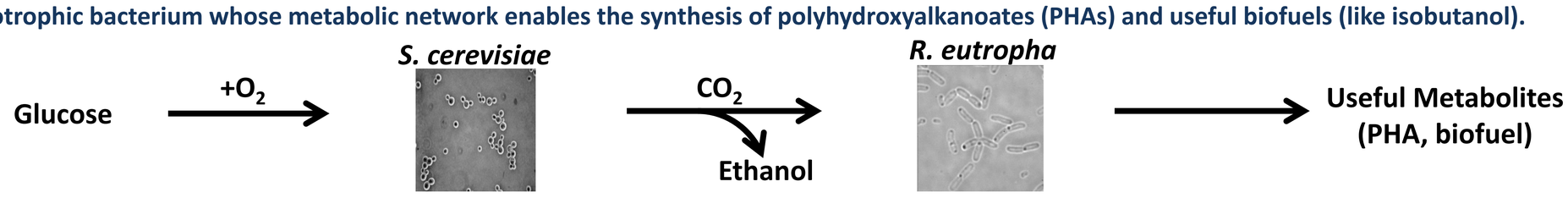
Introduction

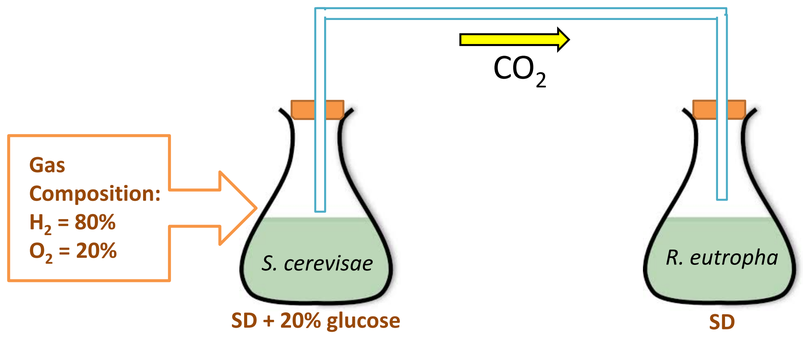
We are interested in the co-culture of two microorganisms, the yeast *Saccharomyces cerevisiae* and *Ralstonia eutropha*. *S. cerevisiae* is a very attractive cell factory, as it is very well suited for industrial production of a range of products due to its robustness and tolerance towards industrial conditions and *R. eutropha* is a facultative chemolithoautotrophic bacterium whose metabolic network enables the synthesis of polyhydroxyalkanoates (PHAs) and useful biofuels (like isobutanol).



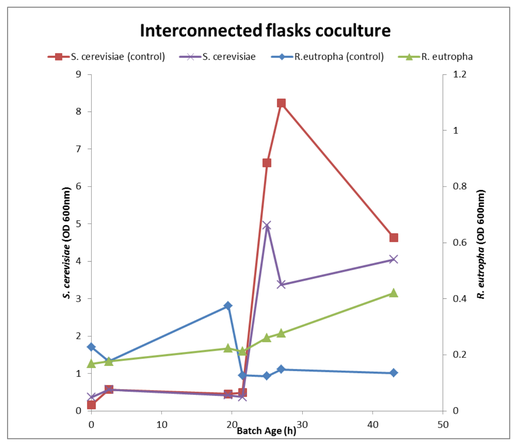
The goal of this project is to set up a bioreactor system which is able to sustain *Ralstonia eutropha* and *Saccharomyces cerevisiae* in a co-culture in SD minimal media with 20% glucose. By fermentation of the glucose, *S. cerevisiae* is able to produce ethanol and CO_2, which is used by *R. eutropha* to grow autotrophically. By controlling growth conditions in this process, ethanol is produced from glucose and other useful metabolites like PHB or biofuel can be produced from CO_2 in a carbon neutral process.

Experimental Approach

1. **Selection of a suitable growth medium for co-culture of *R. eutropha* and *S. cerevisiae***
   - Mineral Salt Media (MSM) and Synthetic Dextrose (SD) medium were compared ➡ **SD was selected** as both species was able to grow if supplemented with the appropriate carbon source.
2. **Physiological compatibility between both microorganisms was tested**
3. **Flask scale approach for a carbon neutral process** ➡ Two flasks with 100 mL of SD (supplemented with glucose 20% [*w*/*v*] for *S. cerevisiae* and no carbon source for *R. eutropha*) were interconnected to allow CO_2 transfer.
   - A flask of *R. eutropha* in SD media with glucose and no CO_2 was used as control.
   - Growth curve of all flasks was followed by spectrophotometry at 600nm.
   - Ethanol production was detected by High Performance Liquid Chromatography (HPLC).

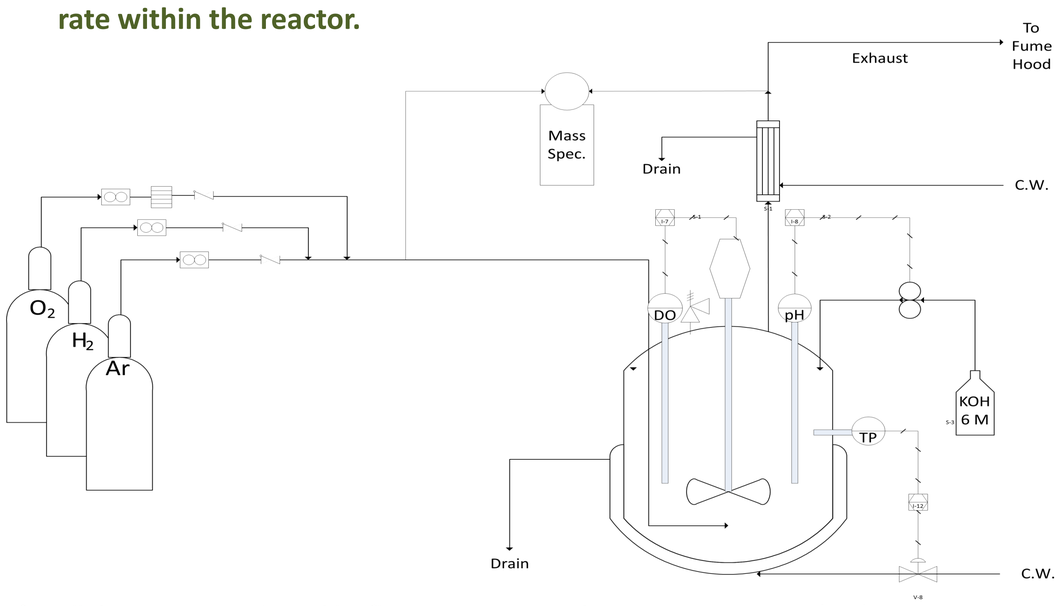


- Ethanol and CO_2 was produced from glucose.
- *R. eutropha* does not grow in SD medium with glucose.
- *R. eutropha* was able to grow autotrophically on CO_2 produce by *S. cerevisiae*.
- Growth can be measured by spectrophotometry at 600nm.



Reactor Scale Up

Using data obtained from preliminary experiments, a 2L bioreactor was set up and used to control the growth conditions and gas uptake rate within the reactor.

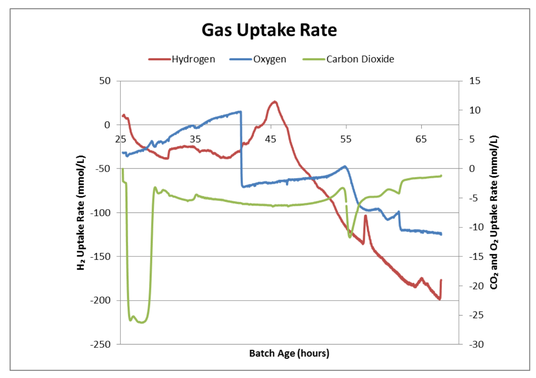


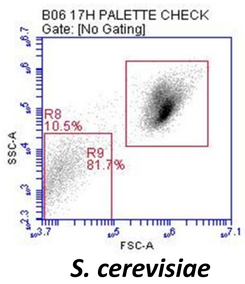
- All data were monitored by flow cytometer and mass spectrometer (MS)
- Initial gas feed composition: O2 (5%), H2 (90%), Ar (5%)
- Total gas flow rate was 200 mL/ min, 1vvm
- Dissolved oxygen controlled at 25 % with stirring control (200 – 1200 rpm)
- Temperature was controlled at 30 °C, pressure at 1 atm and pH at 4.6
- The medium was SD with glucose 20 % (*w*/*v*) and growth was followed during ~ 68 hours
- Additional 10% glucose shot was given at 25 hours to replenish glucose levels for *S. cerevisiae*

Results

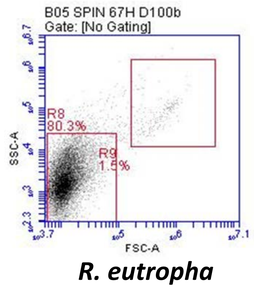
Inoculation of *S. cerevisiae* was followed by a high density inoculum of *R. eutropha* once the CO_2 concentration in the reactor was 5%

- During first 24 hours of culture, only *S. cerevisiae* was detected.
- Decrease of gas uptake at 45-47 hours implied growth of *R. eutropha.* Uses hydrogen oxidation for CO_2 fixation
- Fluctuation on gas readings was detected during first 24 h of the culture (data not shown)

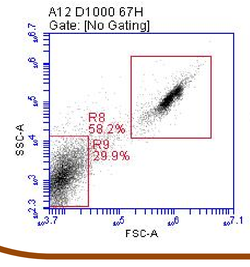




S. cerevisiae



R. eutropha



Co-culture

- *S. cerevisiae* is separated from *R. eutropha* by centrifuge for 5 minutes at 2000 rpm.
- Clear difference between populations
- Species were identified by forward scatter, FSC-A. (~13000 for *R. eutropha* and ~750000 for *S. cerevisiae*)
- Number of events at *R. eutropha* region increased after 47 hours, when *S. cerevisiae* enter stationary phase
- Confirmation of MS data for gas uptake rates.

Conclusions

1. **CO_2 and ethanol were produced by *S. cerevisiae* from glucose and *R. eutropha* was able to fixate it and grow ➡ Carbon neutral process was achieved.**
2. **Both populations can be co-cultured, followed and analyzed by flow cytometer and MS.**
3. **Further engineering improvements are needed to control gas rates for more conclusive data through MS and the long lag phase of *R. eutropha* since inoculation (~ 24 hours) needs to be reduced.**

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