Cost-competitive γ-PGA production from low costs feedstock using an engineered *Bacillus subtilis* lab strain

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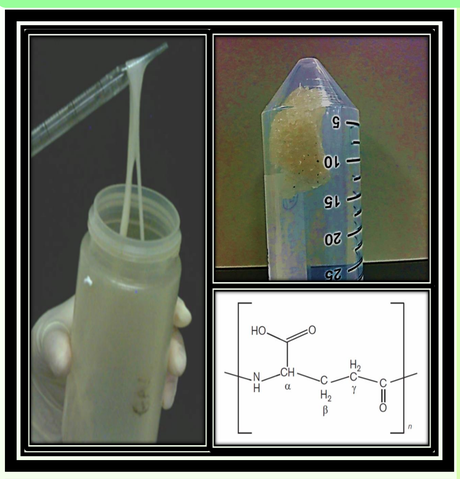
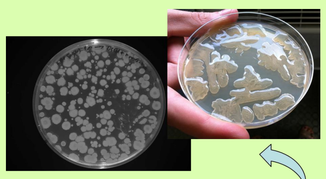
Background



- ✓ **Poly γ-glutamic acid** (γ-PGA) is a natural, water soluble, edible and anionic homopolyamide made up by thousands repetitions of glutamic acid units connected by amide linkages between α-amino and γ-carboxylic acid groups.
- ✓ Due to this particular property and the biological safety of the molecule, a large number of industrial applications based on γ-PGA have been developed such as **drug carriers, heavy metal absorbers, flocculant agents** and **additive in cosmetic and food industries**. [1]



- ✓ Different bacterial species belonging to the genus ***Bacillus*** naturally produce this polymer.
  In our lab, a domestic strain (a 168 derivative) of *B. subtilis* was engineered to become **high-yield producer** (PB 5383: *swrA*⁺, *degU*32(Hy)) [2].

- ✓ **Rice straw** (RS) is the most abundant rice production waste. RS is typically formed by the stalk of the rice plant that is left over on the field upon harvesting of the rice grain.
- ✓ Is a fibrous solid waste rich in **cellulose**, **hemicellulose**, **lignin** and **silica** [3].
- ✓ RS is normally disposed via open burning in the field. Such practice leads to energy loss and poses environmental and health threats. Several alternatives for RS valorization process were proposed. Among these, **fermentation** represents an interesting option.
- ✓ Silica can be inhibitory on bacterial growth. However, an asset of our project forecasts a first step of silica recovery which conveniently removes SiO_2 from RS before saccharification takes place. The pure SiO_2 constitutes an inorganic valorization product co-derived from RS which can also be used in a large number of industrial applications.

AIM OF THE STUDY: obtaining a **CBP producer strain**, i.e. a cellulolytic strain of *B. subtilis* able to use rice straw as the only carbon source in aerobic fermentation for γ-PGA production

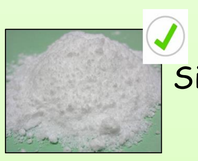
1 – Process scheme

Three types of rice straw pre-treatments for xylans recovery were evaluated:

A. **2h washing** with 40 ml of tap H_2O at **room T** for every grams of rice straw.
B. **2h washing** with 40 ml of tap H_2O at **100° C** for every grams of rice straw
C. **20 minutes** washing with tap H_2O at **160° C + microwave** for every grams of rice straw

Solid fraction → **SILICA EXTRACTION:** 2h, 100° C, 10% (w/w) NaOH

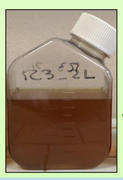
→ filtration + precipitation →



Silica powder

Solid residue

Liquid fraction →



Xylan-rich (C_5) fraction

Cellulose-rich (C_6) fraction

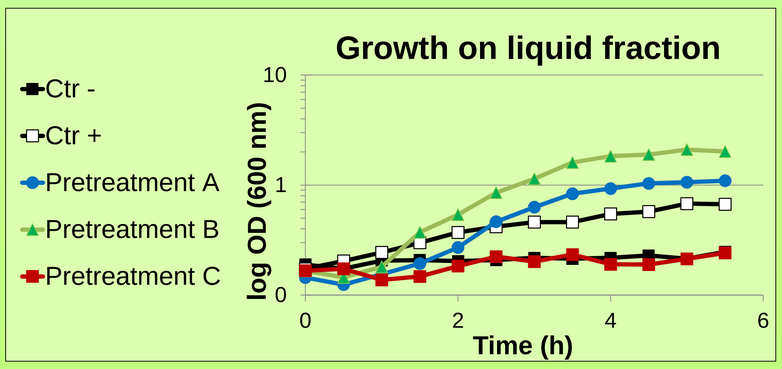
C_5 & C_6 fermentation

High MW γ-PGA

2 – Preliminary growth tests and silica recovery

- ✓ 50 ml of liquid fraction as the ONLY carbon source in minimal medium
- ✓ Domesticated strain
- ✓ Sugar content (by DNS assay) [7]

- Pretreatment A : 0,45 mg gluc. eq/ml
- Pretreatment B : 0,39 mg gluc. eq/ml
- Pretreatment C : 0,93 mg gluc. eq/ml
- Ctr⁺ : 0,39 mg/ml of pure glucose
- Ctr⁻ : no sugar



- ➢ The amount of reducing sugars is enough to sustain growth up to 24 hr
- ➢ With Pretreatment A and B bacteria growth better than the Ctr⁺ with glucose → a supplementary C source from RS is provided
- ➢ Pretreatment B gives the best C source
- ➢ Despite the higher sugar content no growth is observed with pretreatment C.

↓

Caramelization & Furfurals

Preliminary Silica Recovery Yields	% (w/w)
Pretreatment A	9,0
Pretreatment B	9,4
Pretreatment C	7,4
Literature [6]	13,7

3 – Strain optimization

- ✓ Two endogenous genes with low expression levels were identified:

I. ***bglC*** (endo/exo 1-3/1-4 β-glucanase)
II. ***xynA*** (endo 1-4 β-xylanase)

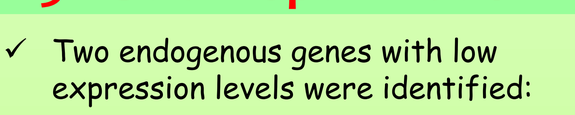
Their *sigA*-dependent promoters were characterized at the nucleotide level and optimized towards the consensus to improve transcription of the genes.

The Gibson Assembly tool [4] with the pMAD suicide vector [5] was used to achieve this goal.

- **Chromosomal region:**

- **Optimization:**

(green → optimized sequence)
(red → synthetic insertion)

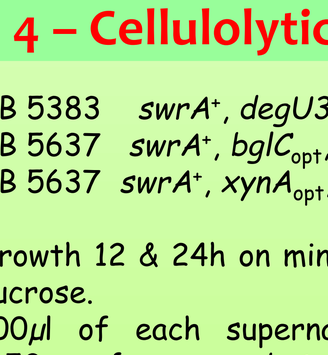


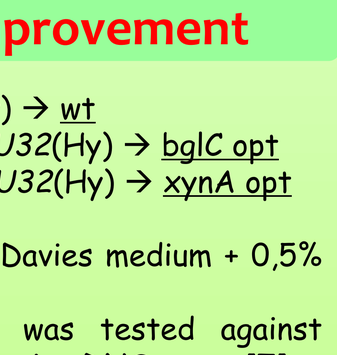
- ✓ PB 5637: *swrA*⁺, *bglC*$_{opt}$, *degU32*(Hy)
- ✓ PB 5638: *swrA*⁺, *xynA*$_{opt}$, *degU*(Hy)

4 – Cellulolytic improvement

- ✓ PB 5383 *swrA*⁺, *degU32*(Hy) → wt
- ✓ PB 5637 *swrA*⁺, *bglC*$_{opt}$, *degU32*(Hy) → bglC opt
- ✓ PB 5637 *swrA*⁺, *xynA*$_{opt}$, *degU32*(Hy) → xynA opt

1) Growth 12 & 24h on minimal Davies medium + 0,5% sucrose.
2) 100μl of each supernatant was tested against 3,52mg of proper substrates using DNS assay [7]
   - Carboxymetilcellulose (CMC) for "*bglC*" gene
   - Xylan for "*xynA*" gene
3) The enzymatic units released by the optimized strains were compared to the activity of the wt





5 – Conclusions

- ➢ Cellulolytic activity on standard substrates was successfully improved in both "optimized" strains.
- ➢ A cheap and "industrial-suitable" RS pre-treatment was established to extract C_6 and C_5 fractions.
- ➢ ~70% silica recovery compared to literature data.

6 – Future perspectives

- ➢ Double "*bglC xynA*" mutant.
- ➢ γ-PGA fermentation tests with treated liquid and solid RS fractions.
- ➢ 5 Liters-fermenter scale up.

References

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