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RESEARCH PROJECT

From gene to gummy: Engineering *Pichia pastoris* for high-yield production of food-grade hydroxylated gelatin

Doctoral thesis proposal integrating genetic engineering and bioprocess optimization for enhanced recombinant gelatin production yield through precision fermentation

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BACKGROUND

Traditional animal-derived gelatin associated challenges[1]:
- **Batch variability**
- **Pathogen risks** (ex. BSE)
- **Ethical concerns** (vegan, vegetarian, halal and kosher diets)
- **Environmental harm**

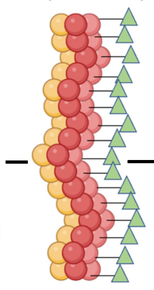
*Despite these issues, the global gelatin market is expected to grow to **$13 billion** by 2030*

SOLUTION

Pichia pastoris *(Komagataella phaffii)* offers an animal-free, sustainable, scalable and safe (GRAS & QPS) platform for recombinant gelatin production

Well stablished for type I and III collagen expression[2]

Gly-Pro-Hyp



Prolyl 4-hydroxylase (P4H) co-expression

Hydroxylated → Gelling and rheological properties

However…

Functional hydroxylated gelatin is hard to express at high yields (reported levels up to **0.6 g/L**)[3] and industial-scale gelatin production requires **3-14 g/L** accumulation[4]

Non-Hydroxylated → Lacks gelling properties

= not suitable for food applications

PROJECT TIMELINE

= 70 **Deep 24 well plate** screening
= 30 **Shake flask** cultivations
= 25 **(1L)** fermentation runs
= 2 **(50L) PPP** runs

Build pGEL-PAOX1 and pP4H-PCAT/PFLD1

Select top performers

Strain optimization approaches

Base strain and construct engineering

1ST YEAR

Bioprocess optimization

Test fermentation conditons DSP testing at (1L) scale

2ND YEAR

Scale up and validation

3RD YEAR

LCA + Patent application

Final assesment

Quality validation 50L fermentations+ DSP

4TH YEAR

OBJECTIVES

Main goal: To develop an optimized *P.pastoris* platform for scalable production of **10 g/L** hydroxylated food-grade gelatin at **50 L scale**.

Key objectives:
- To engineer high expression strains via target genetic blocks.
- To optimize the fermentation process to maximize yield and scale-up.
- To design an EFSA-compliant bioprocess for confectionery applications.

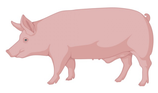
GMO-free, DNA-free, antibiotic marker-free, methanol-free

HYPOTHESIS

This study hypothesizes that engineering *P.pastoris*, optimizing fermentation conditions and carfully designing DSP units will **boost extracelular yield and functionality of recombinant hydroxylated gelatin.**

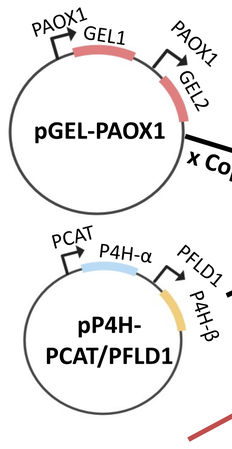
PROJECT METHODOLOGY

1) Construct design and strain optimization approaches

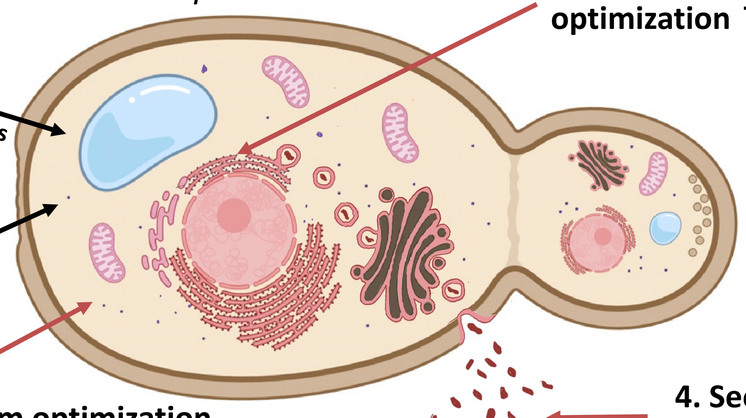


Collagen

GEL1 (0.9 kb, pI 7.01)
GEL2 (1.4 kb, pI 8.09)



Mut^S *P.pastoris* strain



1. Transcription optimization

- Promoter tuning
  - PAOX1 for high gelatin expression
  - Balancing α:β ratio to enhance P4H folding
- ↑pGEL-PAOX1 gene dosage
  = Testing multi-copy variants to boost yield without overloading metabolism
- Codon Optimization — *S.scofa* → ***P.pastoris***
- Antibiotic marker-free screening
  = Homology directed integrations + CRISPR/Cas9

2. NAD(P)H metabolism optimization

- ↑NADH supply → ***↑SOL3, ZEF1*** =↑PPP flux
- ↑ATP avaliability → ***↑MDH1*** =↑TCA flux

3. Translation optimization

- ↑P4H hydroxylation
  - ***↑HEM 1,2,3,13*** =↑cytocrome activity
  - ***↑SOD1, CAT*** = ↓oxidative stress
- ↑ER folding capacity → ***↑HAC1*** **UPR** → ↑Kre2p, ↑PDI

4. Secretion optimization

- Signal peptide
  - **α-MF** =post-translational translocation
  - **Ost1 + α-MF pro** =co-translational translocation
- ↑Protein secretion
  - ***ΔPRB1*** = ↓ proteolysis
  - ***ΔVPS21*** = ↑ER-to-Golgi vacuole transport

2) Upstream Processing (USP)

Stages for recombinant protein production in *P.pastoris:*

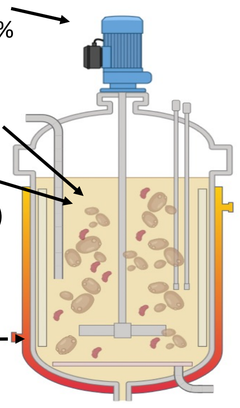
Glycerol batch phase
↓
Glycerol Fed-Batch phase ← ↓Glycerol feed = Derepress PAOX1
↓
Methanol induction phase
↓
Production phase

Constant *glycerol:methanol* ratio

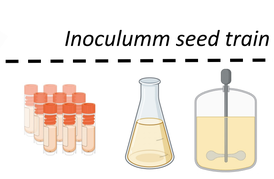
↑Airation =DO above 20-30%

↑Protein yield =↑*glycerol: methanol* ratio

↓Proteolysis =Low pH (pH=5) + Casamino acid



Inoculumm seed train



T=28,3ºC

Harvesting: **Centrifugation**

3) Downstream Processing (DSP)

- Biomass
- Supernatant — *Extracellular protein*
  → **TFF** (Tangential Flow Filtration) — *Concentration + Diafiltration* (Methanol, DNA, cellular debris…)
  → **AEX**(Anion Exchange Chromatography) — *Purification* (Host proteins)
  → Gelatin → **Filtration** → **Pasteurization** (HTST) → **Spray drying** → Recombinant gelatin precision ingredient → Food formulation

POLISHING

Sucrose 86.5%
Gelatin (250 Bloom) 9.0%
Fumaric Acid 2,5%
Sodium citrate 1.2%

EXPECTED RESULTS

- High gelatin extracellular expression levels (10 g/L) through scale up.
- Reduced metabolic burden.
- Enhanced secretion.
- Compliance with EFSA regulations on food-grade additives.

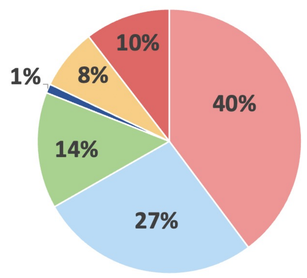
= Baseline process for scaling up protein production to industrial levels.

Table 1. Expected gelatin propierties.

Gelatin properties	Expected values
Proline hydroxylation levels (%)	40-60
Circular dichroism results	Triple helical structure
Bloom strength	200-250
Isoelectric point	7-9
Viscosity (mP)	15-755
Moisture (%)	8-13
Density (kg/L)	1,3-1,4

ESTIMATED ECONOMIC DIMENSION

The overall estimated cost associated to this PhD project is **207.790 €**



- Personnel remunerations
- Materials and consumables
- Scientific-technical external services
- Attendance at conferences and seminars
- Publication and dissemination costs

→ *DIRECT COSTS*

- Overheads corresponing to 21% PhD project

→ *INDIRECT COSTS*

DISSEMINATION PLAN

Academic dissemination → Attendance at conferences → Industrial networking → Technology transfer

Microbial Cell Factories
frontiers in Microbiology

ECB 25 — esib EUROPEAN SUMMIT OF INDUSTRIAL BIOTECHNOLOGY

GELITA GELTOR

Proofs of Concept
UAB Universitat Autònoma de Barcelona

MAIN REFERENCES

1. Song, X., Chu, T., Shi, W., & He, J. (2024). Expression, characterization, and application of human-like recombinant gelatin. Bioresources and Bioprocessing, 11(1), 69.
2. de Moura Campos, S., dos Santos Costa, G., Karp, S. G., Thomaz-Soccol, V., & Soccol, C. R. (2025). Innovations and challenges in collagen and gelatin production through precision fermentation. *World Journal of Microbiology and Biotechnology, 41*(2), 63.
3. Myllyharju, J., Nokelainen, M., Vuorela, A., & Kivirikko, K. I. (2000). Expression of recombinant human type I-III collagens in the yeast Pichia pastoris. *Biochemical Society Transactions, 28*(4), 353-357.
4. Báez, J., Olsen, D., & Polarek, J. W. (2005). Recombinant microbial systems for the production of human collagen and gelatin. Applied microbiology and biotechnology, 69, 245-252.