Assessment of adapted and non-adapted *Rhodosporidium toruloides* strain growth in hemicellulosic hydrolysate from spent coffee grounds

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Introduction

01.

Environmental Context

- Increasing pressure for sustainable alternatives to fossil fuels

- Spent Coffee Grounds (SCG) among the most abundant residues worldwide
(20 million tons annually)

- Agro-industrial waste as renewable resource
- Potential for biotechnological applications: biofuel, bioplastic, and industrial biomolecule production

Highlights

- Utilization of spent coffee grounds (SCG) hydrolysates as a sugar source for microbial cultivation;
- Generation of high-value-added products from residual biomass;
- Optimization of SCG pre-treatment for maximizing fermentable sugar release;
- *Rhodosporidium toruloides* UFMG-CM-Y2896: a promising oleaginous yeast for lipids production;
- Microbial adaptation strategies for enhanced tolerance to toxic and inhibitory compounds.

Research Objective

02.

01.

Evaluate SCG hemicellulosic hydrolysate as a fermentation substrate (optimization of SCG pretreatment)

02.

Compare lipid production by adapted and non-adapted *R. toruloides.*

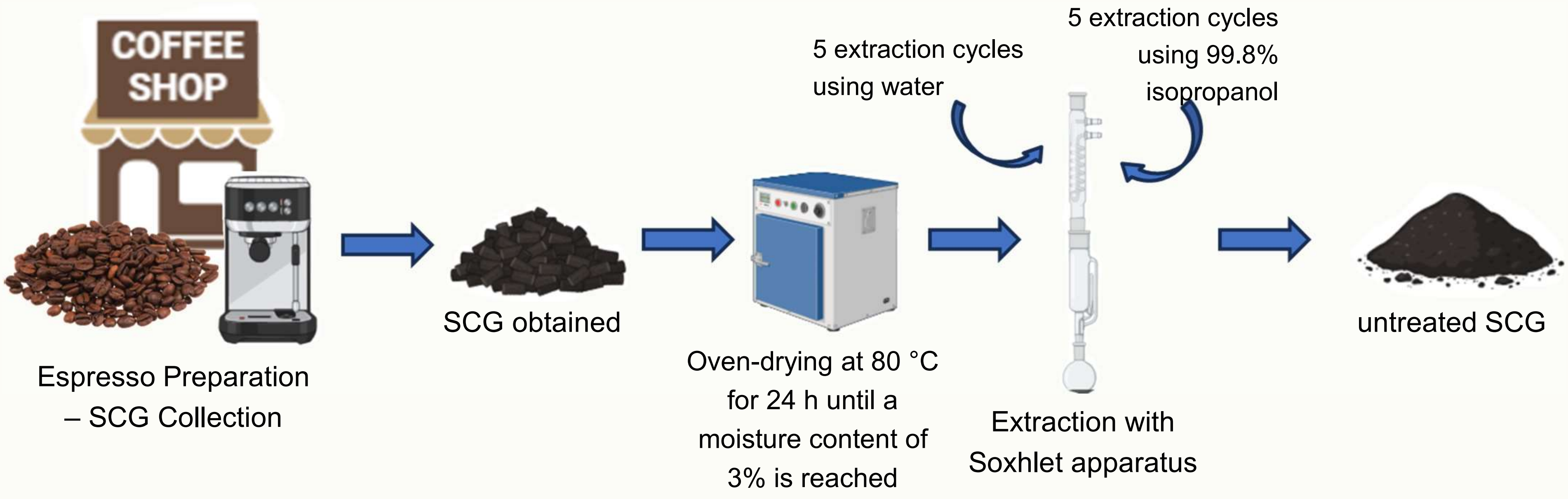
03.

Identify if adaptation improves tolerance and lipid yield.

Methodology

03.

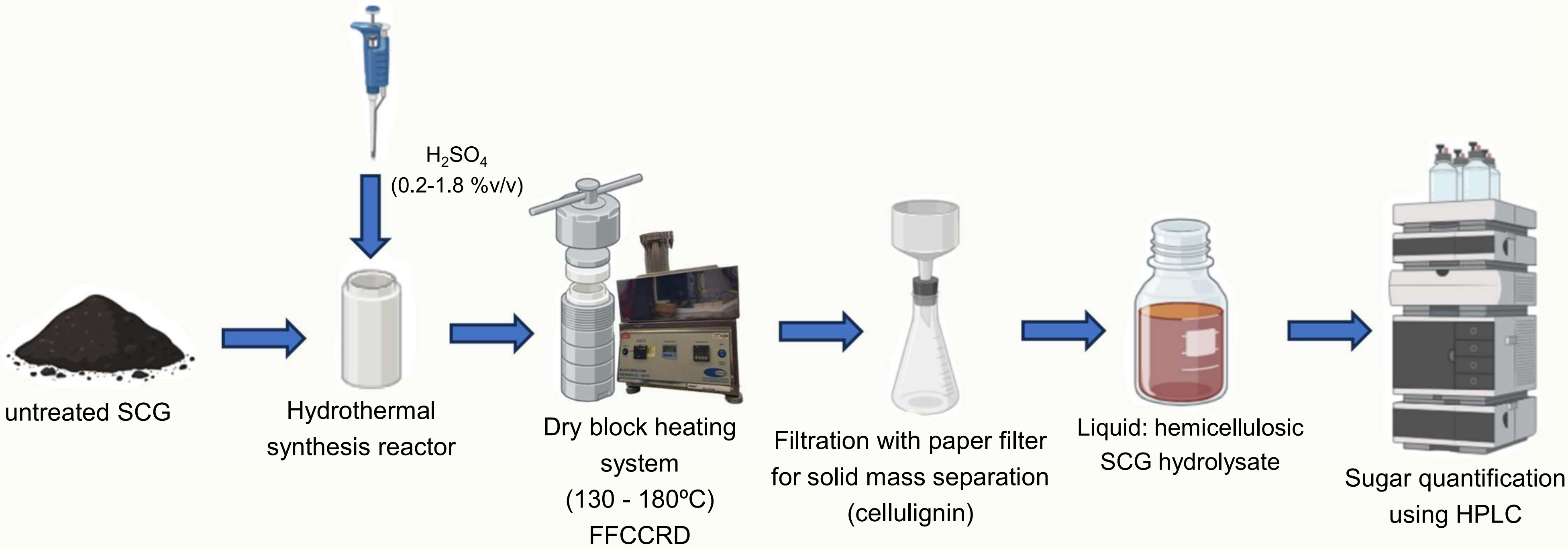
Collection and Preparation of Biomass



• After the removal of extractive compounds, the material was identified as **untreated SCG**. The characterization of untreated SCG was performed using the standard NREL method [1].

[1] Sluiter A, Hames B, Ruiz R, et al (2008) Determination of structural carbohydrates and lignin in biomass. Laboratory analytical procedure 1617:1–16

Pretreatment of SCG Biomass



- The response variable was the total sugar concentration obtained in the liquid fraction (sum of the concentrations of **cellobiose, glucose, xylose, galactose, arabinose, and mannose**).

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Table 1 Coded levels and real levels of fractional-factorial central composite rotational design (FFCCRD) (24-1) for evaluating the extraction of monosaccharides in the diluted acid pretreatment process of spent coffee grounds.

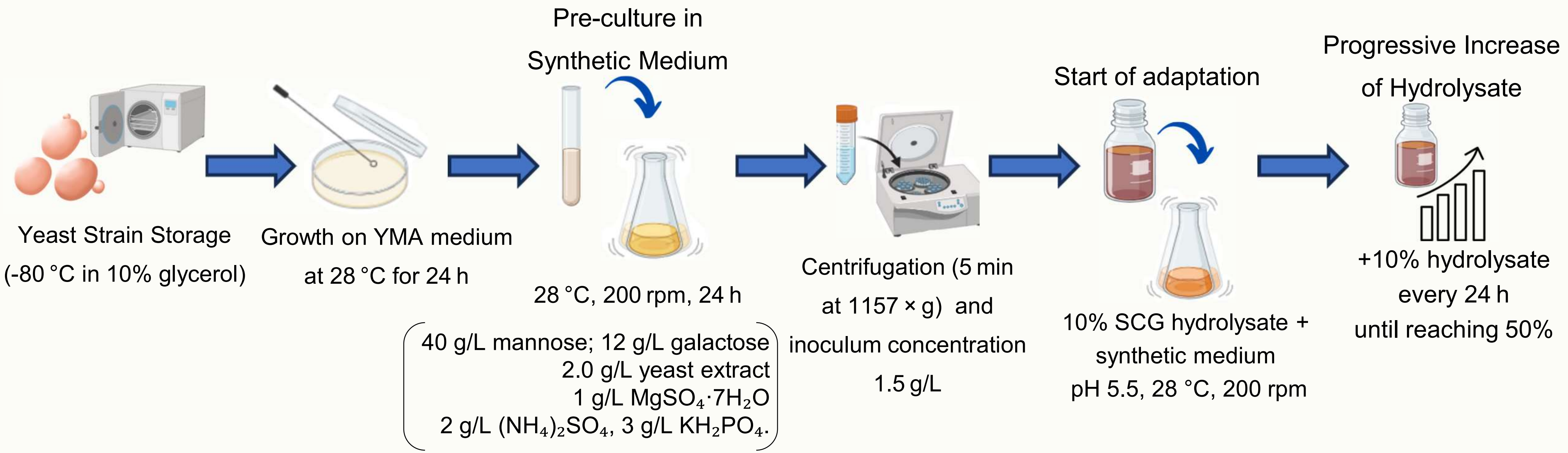
Experiments	Coded Variables			
	X_1	X_2	X_3	X_4
1	-1	-1	-1	-1
2	1	-1	-1	1
3	-1	1	-1	1
4	1	1	-1	-1
5	-1	-1	1	1
6	1	-1	1	-1
7	1	1	1	1
8	1	1	1	1
9	0	0	0	0
10	0	0	0	0
11	0	0	0	0
12	-1.68	0	0	0
13	1.68	0	0	0
14	0	-1.68	0	0
15	0	1.68	0	0
16	0	0	-1.68	0
17	0	0	1.68	0
18	0	0	0	-1.68
19	0	0	0	1.68

Variables/Levels	**-1.68**	**-1**	**0**	**1**	**1.68**
Temperature, °C (X_1)	130	140	155	170	180
H_2SO_4, %v/v (X_2)	0.2	0.5	1	1.5	1.8
Reaction time, min (X_3)	30	40	55	70	80
Solid-liquid ratio (X_4)	01:06	01:08	01:11	01:14	01:16

- 2^{4-1} fractional-factorial central composite rotational design (FFCCRD), evaluating 4 independent variables: solid-liquid ratio, temperature, acid concentration, and reaction time, totaling 19 experiments (Table 1).

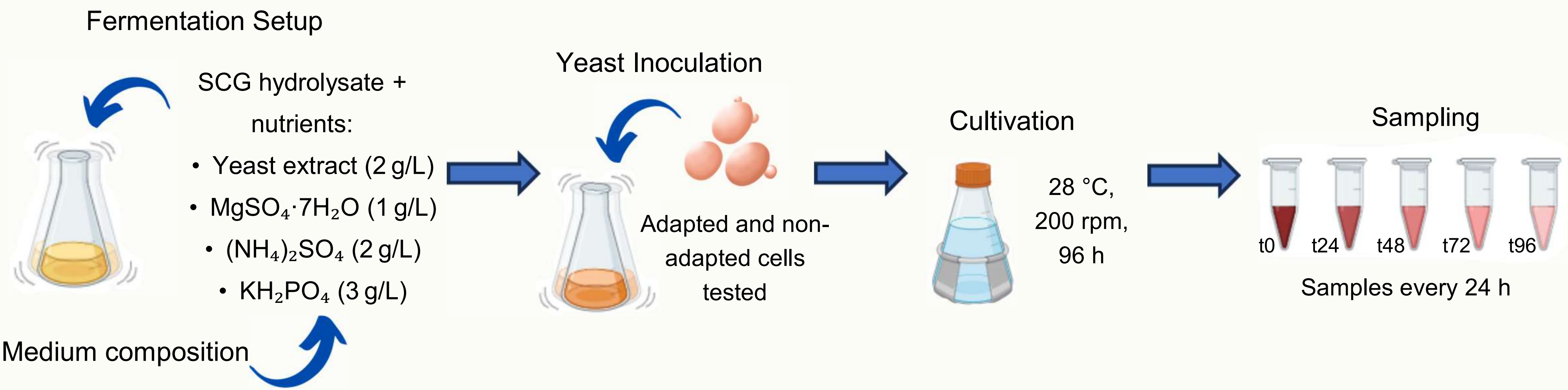
- The condition that resulted in hydrolysate with a higher concentration of fermentable sugars was chosen and used in the fermentation stage.

Adaptation of *R. toruloides* to SCG Hydrolysate



- Cells were gradually exposed to increasing concentrations (10–50%) of SCG hydrolysate to enhance tolerance to inhibitors. The adapted strain was then used in fermentation assays.

Fermentation – Lipid Production



- Control experiments with non-adapted *R. toruloides* cells were also performed.
- Cell growth, lipid yield, and productivity were evaluated

Analytical Methods and Statistic Analysis

Microbial Lipid Extraction

- Microbial lipid was obtained using the extraction method founded and adapted by Folch et al. (1957) and quantified through gravimetry, as described by Almeida et al. (2023):

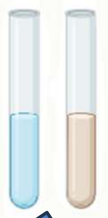
mixture of chloroform:methanol (2:1)

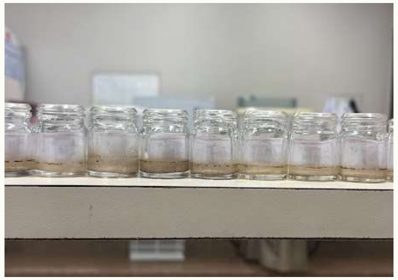
incubated in a thermostatic bath at 20 °C for 24 h

Samples were incubated in an ultrasonic bath for 10 minutes

vortexed for 1 minute, and 2 mL of NaCl (1M) was added to each tube

The resulting mixture was centrifuged at 3000 rpm for 5 min to separate the two phases: **aqueous and organic phases**.





Place organic phase in fume hood + desiccator→ Wait until solvent dries (dry weight)→ **Lipid obtained**

Almeida ELM, Ventorim RZ, Ferreira MAM, et al (2023) New Papiliotrema laurentii UFV-1 strains with improved acetic acid tolerance selected by adaptive laboratory evolution. Fungal Genetics and Biology 164:103765. https://doi.org/10.1016/J.FGB.2022.103765

Analytical Methods and Statistic Analysis

- The **lipid content** (%), **lipid concentration** (mg·L^{-1}), **lipid productivity** (mg·L^{-1}·h^{-1}) and **lipid yield** (g.g^{-1}) were determined using the respective equations:

$$Lipid\ content\ (\%) = \frac{final\ lipid\ mass\ (mg)}{dry\ biomass\ mass\ (mg)} \times 100 \qquad (1)$$

$$Lipid\ titer\ (g \cdot L^{-1}) = Final\ fermentation\ biomass\ conc.(g/L) \times Lipid\ content\ (\%) \qquad (2)$$

$$Lipid\ productivity\ (g \cdot L^{-1} \cdot h^{-1}) = \frac{Lipid\ titer\ (g \cdot L^{-1})}{fermentation\ time\ (h)} \qquad (3)$$

$$Lipid\ yield\ (g.g^{-1}) = \frac{lipid\ titer\ (g.L^{-1})}{a_i - a_f} \qquad (4)$$

Almeida ELM, Ventorim RZ, Ferreira MAM, et al (2023) New Papiliotrema laurentii UFV-1 strains with improved acetic acid tolerance selected by adaptive laboratory evolution. Fungal Genetics and Biology 164:103765. https://doi.org/10.1016/J.FGB.2022.103765

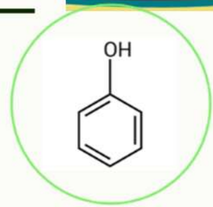
Analytical Methods and Statistic Analysis

01. 

Concentration of sugars was quantified using HPLC equipped with HPX-87P column and a refractive index detector using distilled water as mobile phase.

02.

Cellular growth was assessed by measuring OD600 nm and then converted into biomass concentration using a utilization of a calibration curve.

03. 

Total phenol content was measured by the colorimetric method of Folin-Ciocalteu (Singleton et al. 1999), with modifications

04. **Statistical analysis:** analysis of variance (ANOVA) and multiple regression methods with the STATISTICA software

Almeida ELM, Ventorim RZ, Ferreira MAM, et al (2023) New Papiliotrema laurentii UFV-1 strains with improved acetic acid tolerance selected by adaptive laboratory evolution. Fungal Genetics and Biology 164:103765. https://doi.org/10.1016/J.FGB.2022.103765

Results

04.

Characterization of Untreated SCG

- High hemicellulose content (34.68%), with mannose as the main monosaccharide.
- Similar composition to Ballesteros et al. (2014) in hemicellulose, cellulose, and total lignin.
- Lower galactose (5.92% vs. 16.43%) and soluble lignin (2.97% vs. 6.31%) compared to literature.
- Variations attributed to SCG origin, coffee type, and analytical methods.

Table 2 Characterization of SCG

SCG fraction	Present work	Ballesteros et al. 2014
Hemicellulose (%)	34.68 ± 4.31	39.10 ± 1.94
Mannose (g L^{-1})	17.45 ± 1.52	19.07 ± 0.85
Galactose (g L^{-1})	5.92 ± 0.49	16.43 ± 1.66
Arabinose (g L^{-1})	1.88 ± 0.19	3.60 ± 0.52
Xylose (g L^{-1})	9.43 ± 2.99	ND
Total lignin (%)	22.22 ± 0.12	23.90 ± 1.70
Insoluble lignin	19.25 ± 0.33	17.59 ± 1.56
Soluble lignin	2.97 ± 0.40	6.31 ± 0.37
Extractives (%)	21.75 ± 1.96	NA
Cellulose (%)	19.67 ± 1.56	12.40 ± 0.79
Acetyl (%)	1.23 ± 0.28	NA
Ashes (%)	0.45 ± 0 .02	1.30 ± 0.10

Ballesteros LF, Teixeira JA, Mussatto SI (2014a) Chemical, Functional, and Structural Properties of Spent Coffee Grounds and Coffee Silverskin. Food Bioproc Tech 7:3493–3503. https://doi.org/10.1007/s11947-014-1349-z

Pretreatment of Biomass – Results and Optimization

- Total sugar yields varied from 10.50 to 59.19 g·L⁻¹ among the 19 FFCCRD assays (Table 3).

- Highest experimental yield:
  - 59.19 g·L⁻¹ in Assay 18

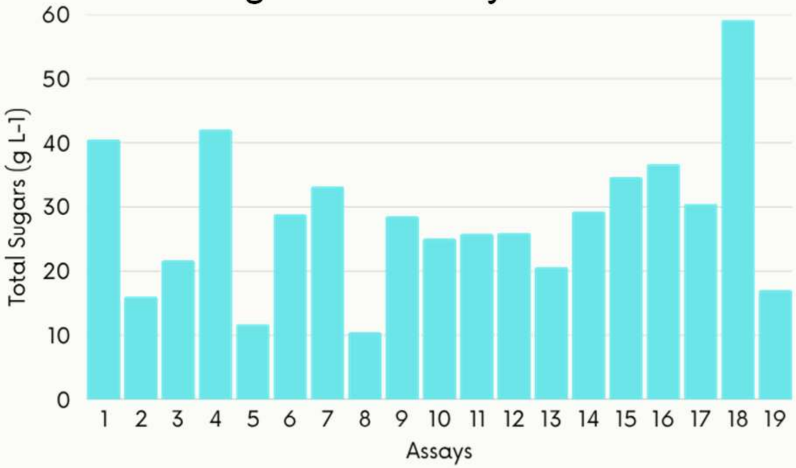


Table 3 Total sum of sugars concentration after pretreatment of SCG according to fractional-factorial central composite rotational design (FFCCRD) (2^{4-1})

Assays	Temperature (°C)	H_2SO_4 (%v/v)	Reaction time (min)	Solid-liquid ratio	Total Sugars (g L^{-1})
1	140	0.50	40.0	8.00	40.55
2	170	0.50	40.0	14.00	16.01
3	140	1.50	40.0	14.00	21.69
4	170	1.50	40.0	8.00	42.11
5	140	0.50	70.0	14.00	11.73
6	170	0.50	70.0	8.00	28.92
7	140	1.50	70.0	8.00	33.18
8	170	1.50	70.0	14.00	10.50
9	155	1.00	55.0	11.00	28.58
10	155	1.00	55.0	11.00	25.12
11	155	1.00	55.0	11.00	25.86
12	130	1.00	55.0	11.00	25.99
13	180	1.00	55.0	11.00	20.66
14	155	0.15	55.0	11.00	29.32
15	155	1.80	55.0	11.00	34.65
16	155	1.00	30.0	11.00	36.68
17	155	1.00	80.0	11.00	30.50
18	155	1.00	55.0	6.00	59.19
19	155	1.00	55.0	16.00	17.07

Pretreatment of Biomass – Optimization

Predicted optimal condition (STATISTICA model):

Temperature: **159 °C**

H_2SO_4 concentration: **1.8%** (v/v)

Reaction time: **37 minutes**

S/L ratio: **1:6**

- Selected to balance sugar release and inhibitor formation.
- Optimization confirms the importance of temperature and S/L ratio in depolymerizing SCG [2 , 3].
- Statistical modeling helps identify robust, scalable conditions — even if not directly tested [4].

[2] Behera S, Arora R, Nandhagopal N, Kumar S (2014) Importance of chemical pretreatment for bioconversion of lignocellulosic biomass. Renewable and Sustainable Energy Reviews 36:91–106.
[3] Świątek K, Gaag S, Klier A, et al (2020) Acid Hydrolysis of Lignocellulosic Biomass: Sugars and Furfurals Formation. Catalysts 10:437. https://doi.org/10.3390/catal10040437
[4] Kumar AK, Sharma S (2017) Recent updates on different methods of pretreatment of lignocellulosic feedstocks: a review. Bioresour Bioprocess 4:7. https://doi.org/10.1186/s40643-017-0137-9

Pretreatment of Biomass – Effect of Parameters and Key Observations

Effect of Variables:

- **S/L Ratio** was the most significant factor (Fig. 1):
  - Lower S/L (1:6) enhanced contact between biomass and acid, boosting sugar release.
  - Higher S/L values reduced efficiency due to dilution effects [5].
- **Temperature**:
  - 159 °C near the upper limit favored hemicellulose and cellulose solubilization.
  - Excessive heat may cause sugar degradation to furfural and HMF [6; 7].
- **H_2SO_4 Concentration**:
  - 1.8% found sufficient for catalysis without excess degradation or corrosion [8].
- **Reaction Time**:
  - 37 min balanced hydrolysis efficiency and minimized thermal degradation [3].

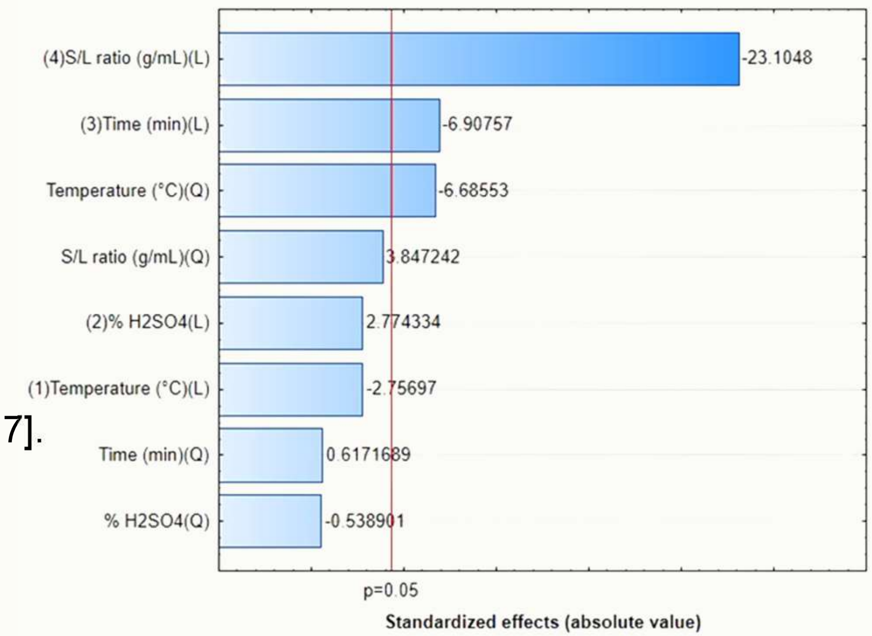


Fig. 1 Pareto chart for standardized effects of factors studied in the pretreatment of spent coffee grounds (SCG).

[3] Świątek K, Gaag S, Klier A, et al (2020) Acid Hydrolysis of Lignocellulosic Biomass: Sugars and Furfurals Formation. Catalysts 10:437. https://doi.org/10.3390/catal10040437

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Pretreatment of Biomass – Effect of Parameters and Key Observations

- Confirmed interaction effects between variables.
- Intermediate temperature and acid, combined with moderate reaction time and lower S/L, maximize yield.
- Emphasized importance of process balance to prevent byproduct formation and inhibitor generation.

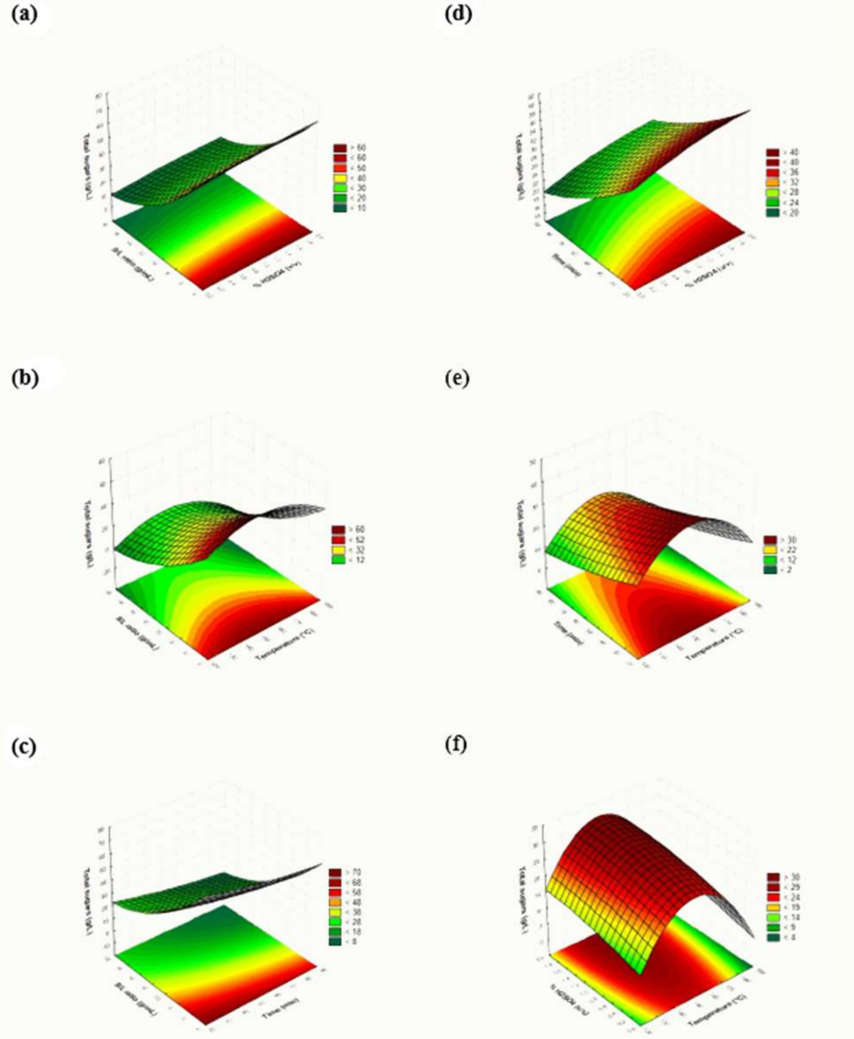
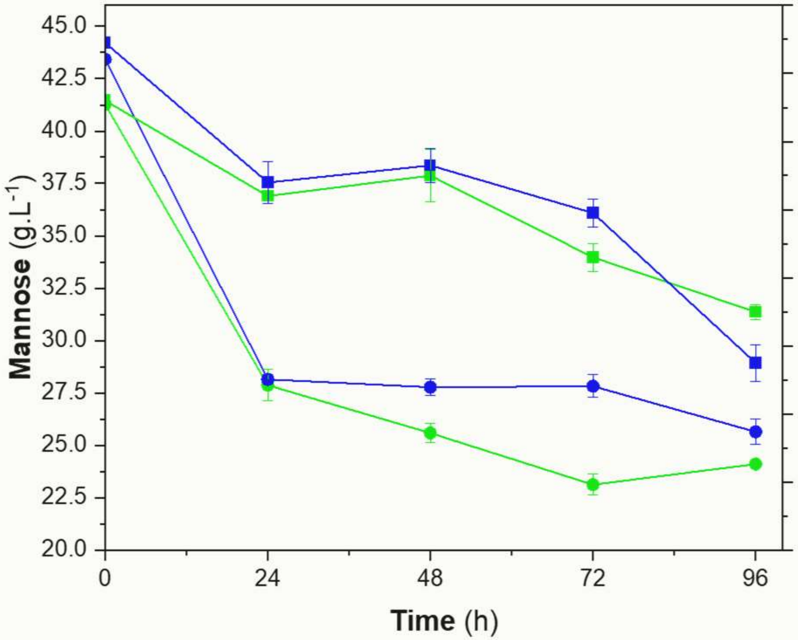


Fig 2 Response surface plots showing the effects of process variables on total sugar release: (a) Solid-liquid ratio vs. H_2SO_4 (% v/v); (b) Solid-liquid ratio vs. Temperature; (c) Solid-liquid ratio vs. Reaction time; (d) Reaction time vs. H_2SO_4 (% v/v); (e) Reaction time vs. Temperature; (f) H_2SO_4 (% v/v) vs. Temperature.

Fermentative Performance: Sugar Consumption

- Fermentation performed with **adapted** and **non-adapted** *R. toruloides* in SCG hydrolysate.
- **Hexose consumption (glucose and mannose):**
  - Efficient uptake by both strains.
  - Glucose decreased from ~2.0 to ~0.9 $g \cdot L^{-1}$ over 96 h.
  - Mannose consumption was gradual (~41% for both).
  - No significant difference between strains ($p < 0.05$).
- P**entose consumption (arabinose and galactose):**
  - Low assimilation (<15%) for both strains.
  - Likely inhibited by total phenols (1.52 $g \cdot L^{-1}$ in hydrolysate).
  - Phenolic compounds impair sugar metabolism via ROS production [9 , 10].

Fig. 3 - Mannose (circle) and glucose (square) consumption from fermentations of SCG hydrolysate by adapted (green symbol) and non-adapted (blue symbol) *R. toruloides* UFMG-CM-Y2896 cells.



[9] Wang S, Sun X, Yuan Q (2018) Strategies for enhancing microbial tolerance to inhibitors for biofuel production: A review. Bioresour Technol 258:302–309. https://doi.org/10.1016/J.BIORTECH.2018.03.064

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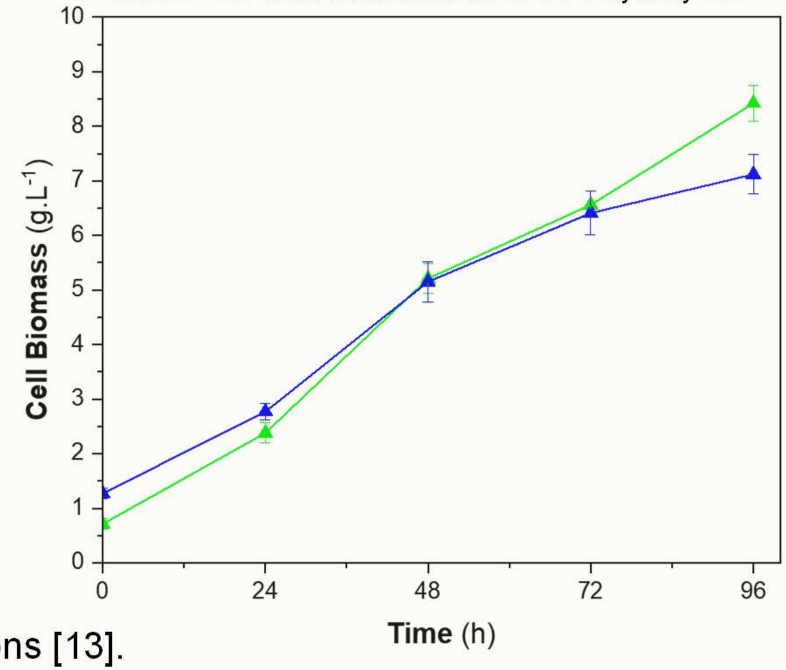
Fermentative Performance: Biomass Growth

- **Cell growth profile:**
  - No lag phase or diauxic behavior observed.
  - Biomass reached ~7.0 – 8.5 $g \cdot L^{-1}$ after 96 h.
  - pH remained stable (~5.5) throughout fermentation.
  - Adapted and non-adapted strains showed similar performance.
- **Technical conclusion:**
  - <u>Adaptation did not improve sugar uptake or biomass yield.</u>
  - <u>Process can be simplified by removing the adaptation step, reducing time and cost.</u>
- **Supported by previous studies:**
  - *R. toruloides* prefers hexoses in lignocellulosic hydrolysates [12 , 12].
  - No significant benefits from prior adaptation reported in similar fermentations [13].

Fig. 4 - Cell growth of adapted (green symbol) and non-adapted (blue symbol) *R. toruloides* UFMG-CM-Y2896 cells from fermentations of SCG hydrolysate



[11] Yaegashi J, Kirby J, Ito M, et al (2017) Rhodosporidium toruloides: a new platform organism for conversion of lignocellulose into terpene biofuels and bioproducts. Biotechnol Biofuels 10:241. https://doi.org/10.1186/s13068-017-0927-5

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Fermentative Performance: Lipid Production

- No significant difference ($p < 0.05$) was observed between adapted and non-adapted cells for lipid titer, productivity (Q_P), and yield ($Y_{P/S}$).
- **Adaptation did not improve lipid performance**, unlike in de Almeida et al. (2024) with sugarcane bagasse hydrolysate.
- Both strains reached similar titers (~4.1–4.3 $g \cdot L^{-1}$) and yields (~0.23–0.24 $g \cdot g^{-1}$), despite 1.52 $g \cdot L^{-1}$ of total phenols.
- Compared to other studies:
  - Di Fidio et al. (2024): Q_P ~0.024 $g \cdot L^{-1} \cdot h^{-1}$
  - Almeida et al. (2023): $Y_{P/S} = 0.131$ $g \cdot g^{-1}$
  - This study: 2–3× higher Q_P and ~1.8× higher yield
- Confirms the robustness of *R. toruloides* in non-detoxified SCG hydrolysate for lipid production.

Table 4 - Performance parameters for lipid production during fermentation of spent coffee grounds hydrolysate by adapted and non-adapted *R. toruloides* UFMG-CM-Y2896 cells. The reported values include lipid titter, lipid productivity (Q_P) and yield ($Y_{P/S}$)

	Time (h)	Lipid Titer ($g\ L^{-1}$)	Lipid Productivity ($g\ L^{-1}\ h^{-1}$)	Lipid Yield ($g\ g^{-1}$)
Adapted	24	1.125 ± 0.085	0.046 ± 0.006	0.079 ± 0.018
	48	2.586 ± 0.236	0.053 ± 0.004	0.164 ± 0.021
	72	3.157 ± 0.079	0.043 ± 0.001	0.169 ± 0.001
	96	4.271 ± 0.397	0.044 ± 0.004	0.241 ± 0.021
Non-Adapted	24	1.838 ± 0.305	0.076 ± 0.005	0.115 ± 0.021
	48	2.909 ± 0.342	0.060 ± 0.007	0.191 ± 0.071
	72	3.678 ± 0.206	0.051 ± 0.002	0.232 ± 0.047
	96	4.099 ± 0.004	0.042 ± 0.001	0.219 ± 0.003

The results shown are means of two replicates followed by the standard deviation.

de Almeida SGC, Souza JP, Fogarin HM, et al (2024) Assessment of lipid synthesis from sugarcane biomass by adaptive strains of Rhodosporidium toruloides. Biomass Convers Biorefin. https://doi.org/10.1007/s13399-024-05499-0

Conclusion

05.

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- This study demonstrated the feasibility of utilizing spent coffee grounds (SCG) as a lignocellulosic feedstock to produce fermentable hydrolysates.

- The optimization of the acid pretreatment process identified key operational parameters—temperature, sulfuric acid concentration, reaction time, and solid-to-liquid ratio—that yielded a hydrolysate rich in fermentable sugars (59.19 $g \cdot L^{-1}$), predominantly mannose and galactose.

The SCG hydrolysate, without a detoxification step, served as a fermentation medium for *Rhodosporidium toruloides* UFMG-CM-Y2896.

No significant differences were observed in the fermentative performance between adapted and non-adapted yeast cells, both of which exhibited effective growth in the hydrolysate and preferential consumption of glucose and mannose, resulting in lipid accumulation of approximately 4.0 $g \cdot L^{-1}$.

The finds, although preliminary, support the development of strategies to valorization and enhance the applicability of SCG as a raw material in sustainable biorefineries and the robust performance of *R. toruloides* in non-detoxified SCG hydrolysate supports its application in streamlined, cost-effective fermentation processes for value-added lipids synthesis.

Future Work

06.

- Test performance in detoxified vs. non-detoxified conditions;
- Use of fed-batch or continuous reactors;
- Explore co-fermentation with other microorganisms;
- Techno-economic analysis for scale-up.

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