RED TEMÁTICA DE BIOENERGÍA

Production of cellulases in the framework of biorefineries

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The increasing energetic problem

Fossil fuels are ending

Pollution and climate change

Biodegradable and sustainable

Renewable energy source

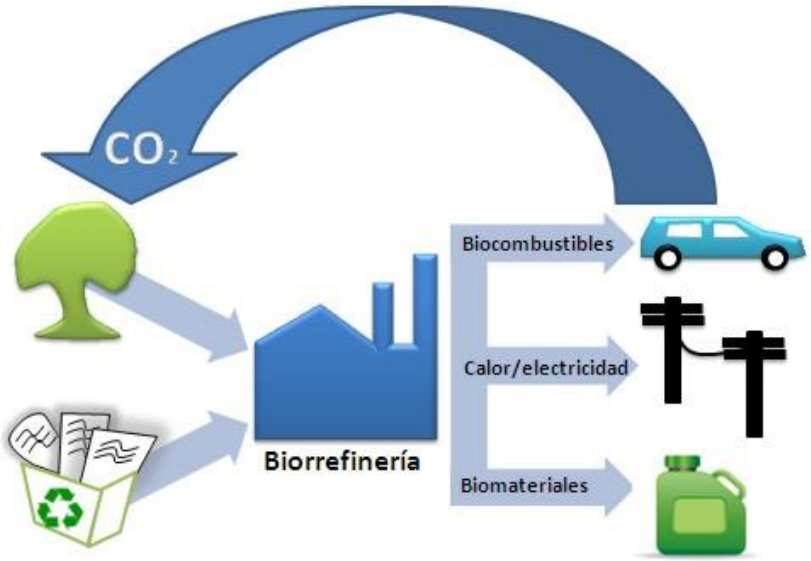
Biofuels are the best alternative

Low emissions of greenhouse gases

Bioethanol

Puppan (2002)

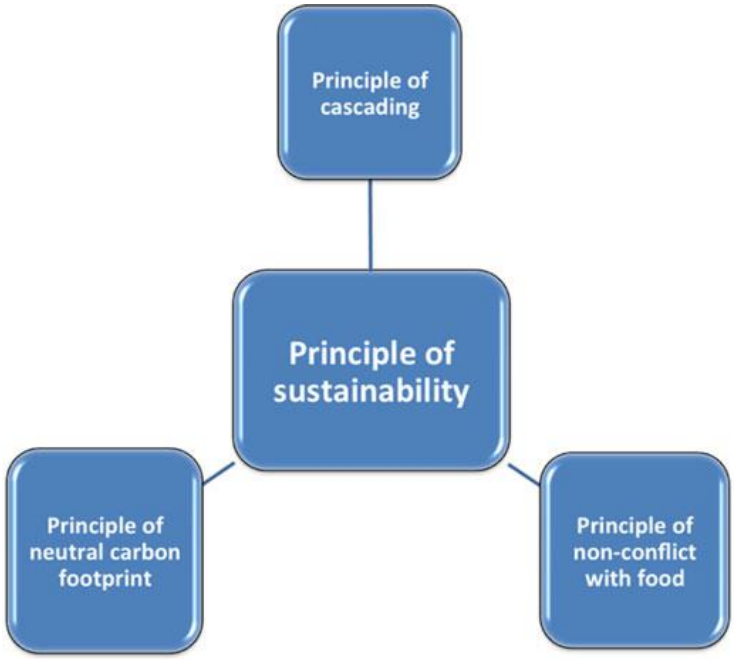
BIOREFINERY

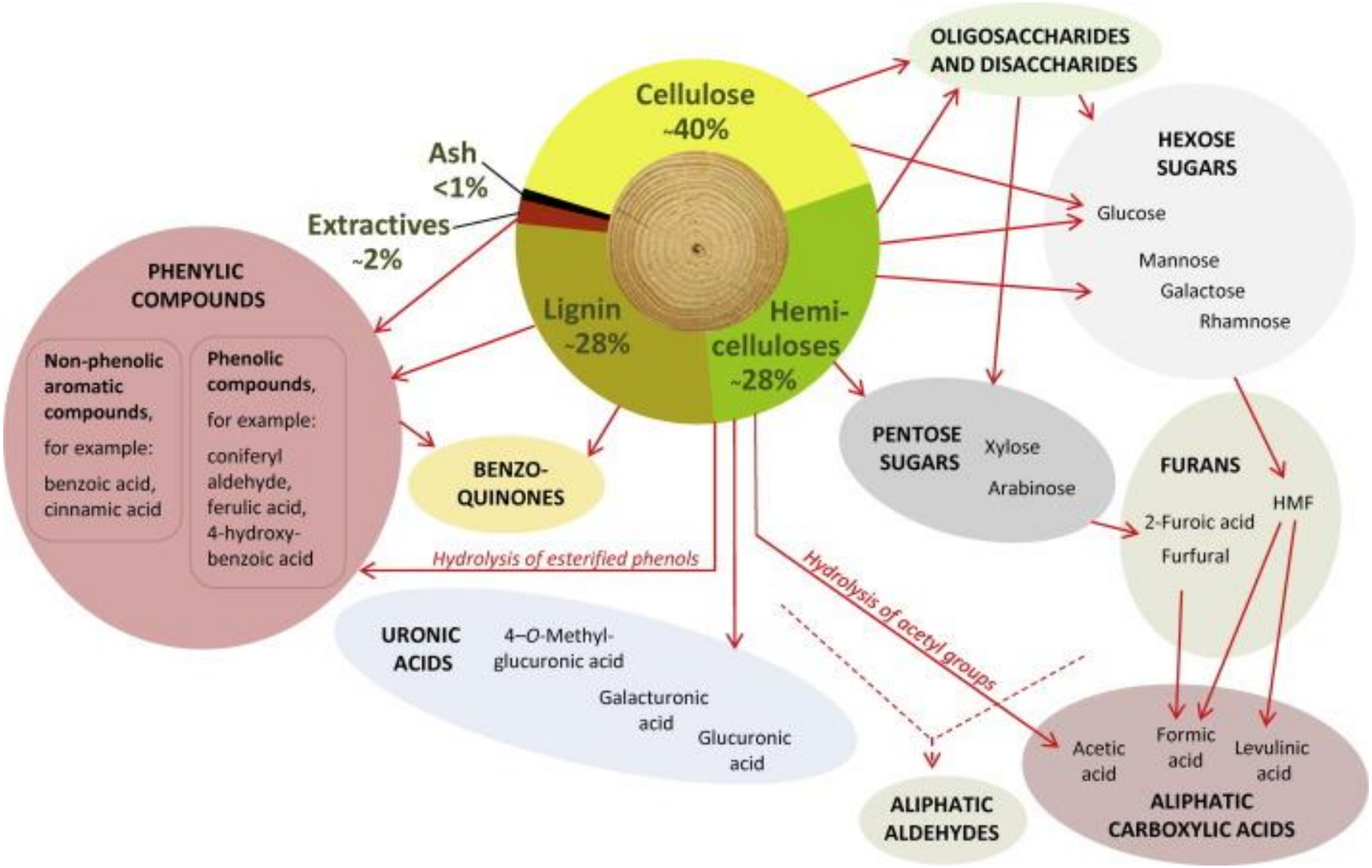


A biorefinery is a concept borrowed from the petroleum oil refinery, which goes beyond the exhaustion of **biomass** into a spectrum of products.

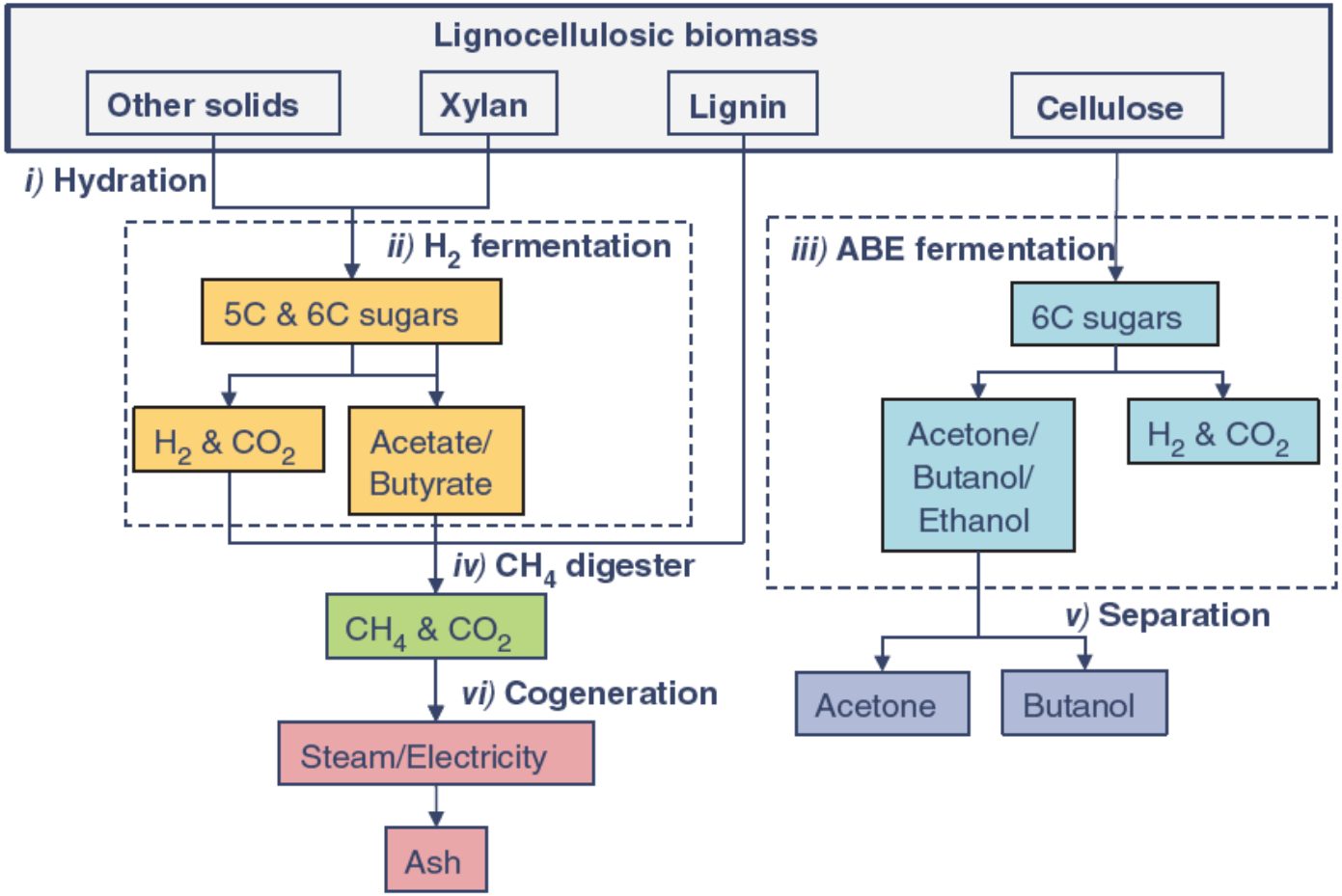
Principles of a biorefinery

Biorefineries are based on four principles, namely principles of sustainability, cascading, non-conflict with food, and neutral carbon footprint. It may be considered that the latter three are branches of the main principle of sustainability.



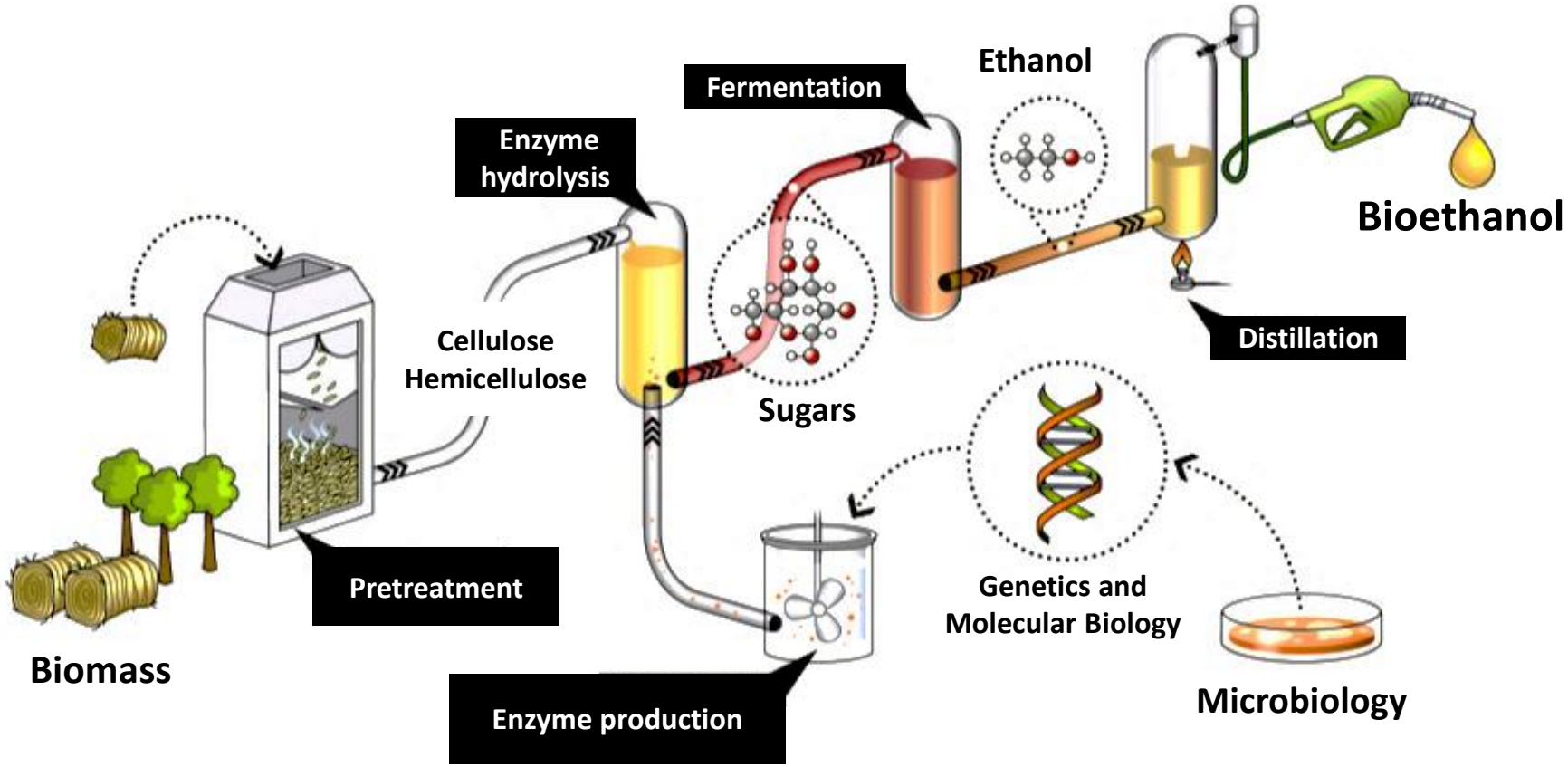


(Jönsson & Martín, 2015)



Valdés-Vázquez et al. (2017)

BIOETHANOL PRODUCTION



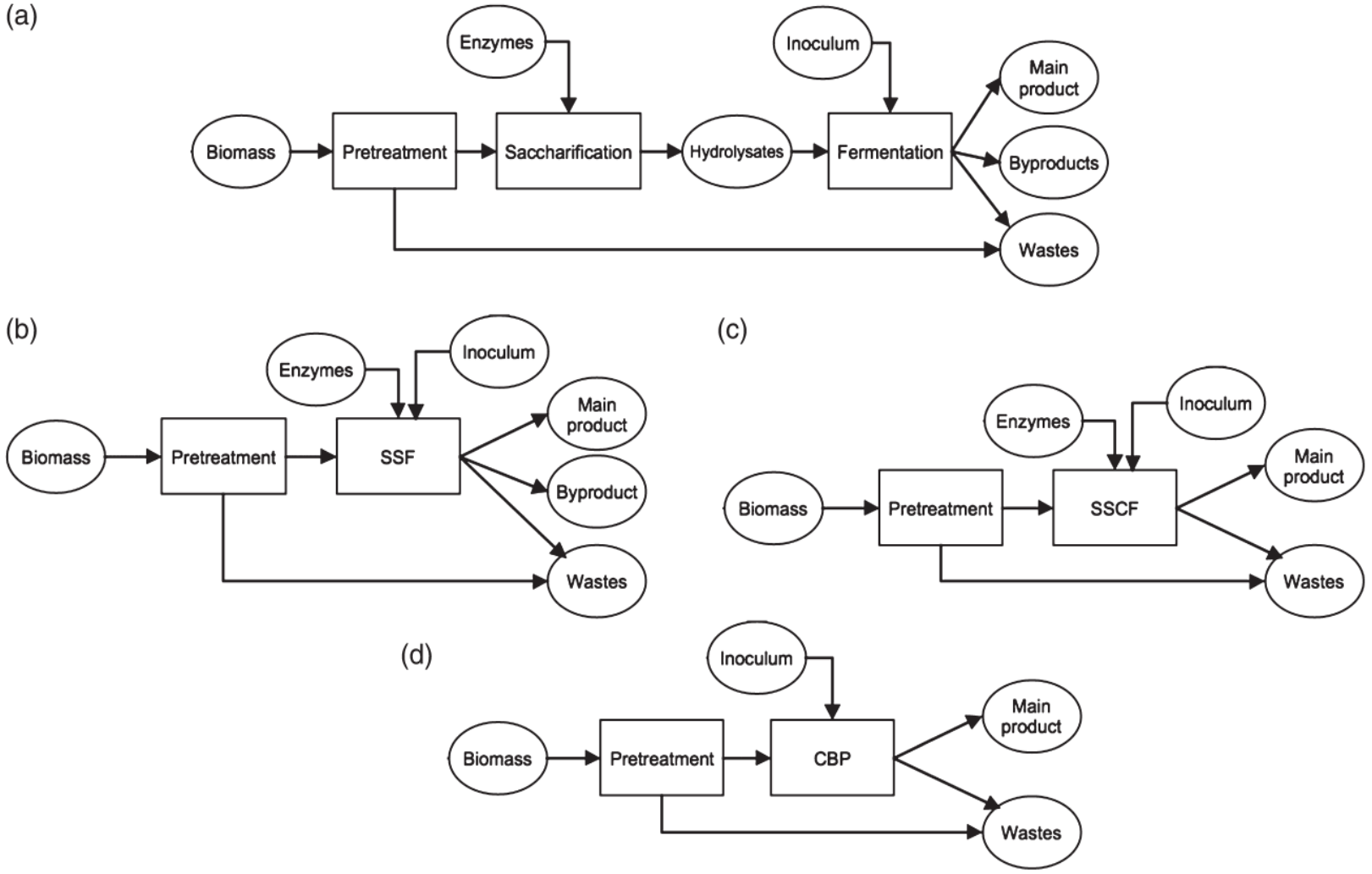


Figure 2. Saccharification and fermentation of biomass: (a) conventional; (b) SSF; and (c) SSCF and CBP.

Escamilla et al. (2016)

SACCHARIFICATION

It is a limiting factor for the production of bioethanol.

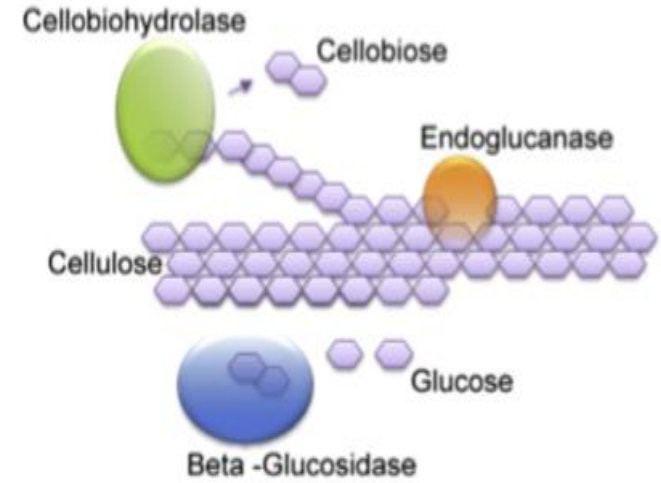
High cost of production and recovery of enzymes.

More efficient enzymes

Hyperproductive strains.

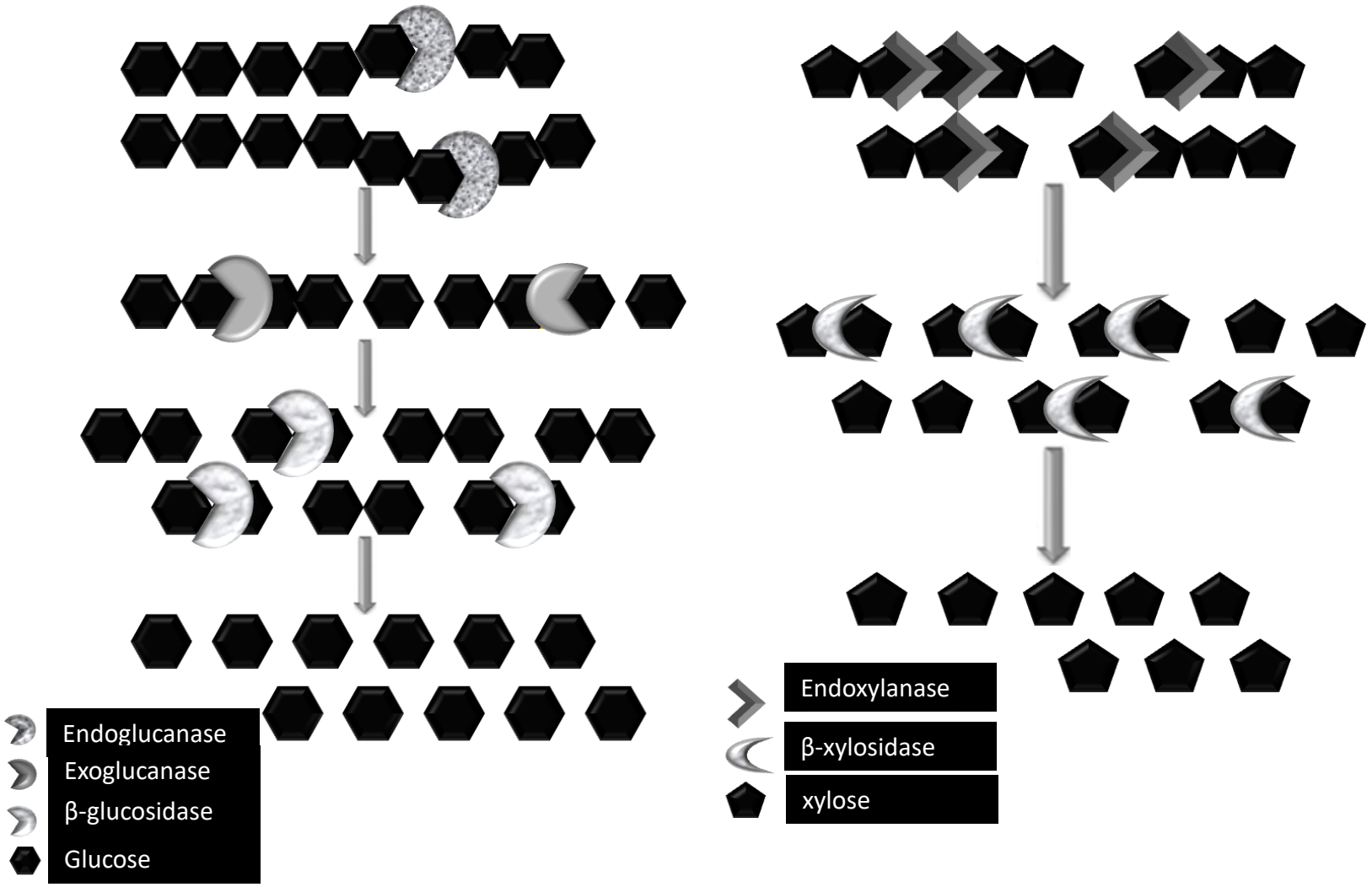
Optimal conditions.

Genetic modification



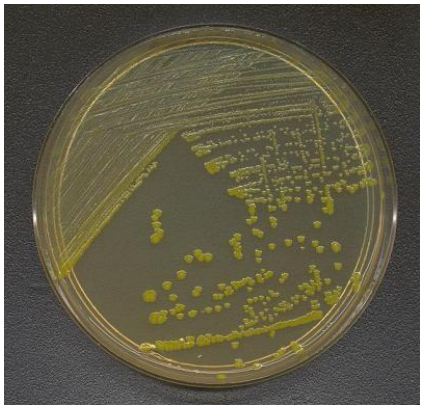
(Percival *& Himmel.*, 2017; Zhang *et al., 2006*)

Hydrolysis of cellulose and xylan



Cellulomonas flavigena

Gram (+) bacterium able to grow in several agricultural wastes and mineral medium formulated with industrial salts. Produces multiple cellulases and xylanases



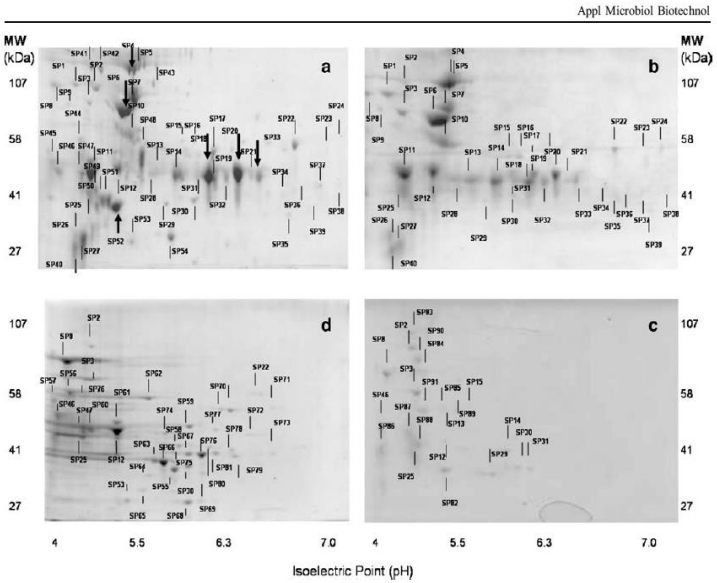


Fig. 1 Secreted proteome of *C. flavigena* grown on: **a** sugar cane bagasse, **b** Solka-floc, **c** xylan, and **d** glucose

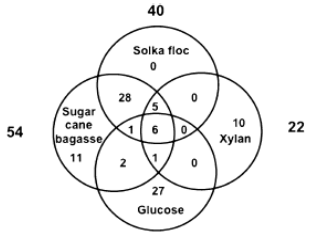
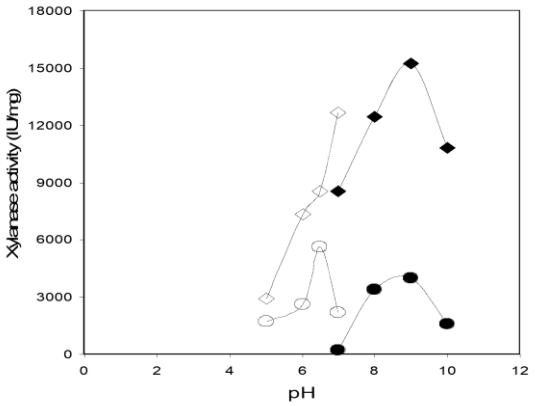


Fig. 2 Venn diagram of the proteins secreted by *C. flavigena* grown on sugar cane bagasse, Solka-floc, xylan, or glucose. *Numbers* indicate the amount of proteins in the specific or coinciding pattern

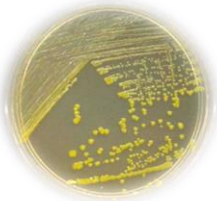


De la Torre, (1981); Martínez-Trujillo et al. (2003); Sánchez-Herrera et at. (2007)

C. flavigena mutants

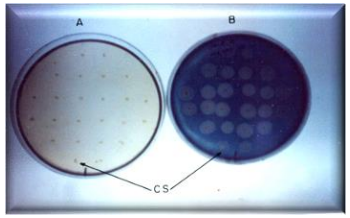
C. flavigena wt

$\mu_{max} = 0.08\ h^{-1}$



UV

PN-10



NTG

PN-120

$\mu_{max} = 0.25 h^{-1}$

NTG

PR-22

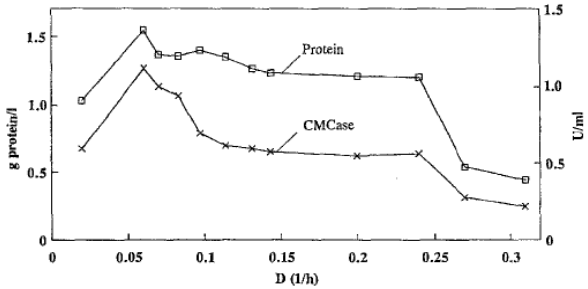


Fig. 3 Growth and CMCase production by mutant PN-10 (after mutagenesis) at increasing dilution rates. This method was used to isolate improved mutant PN-120

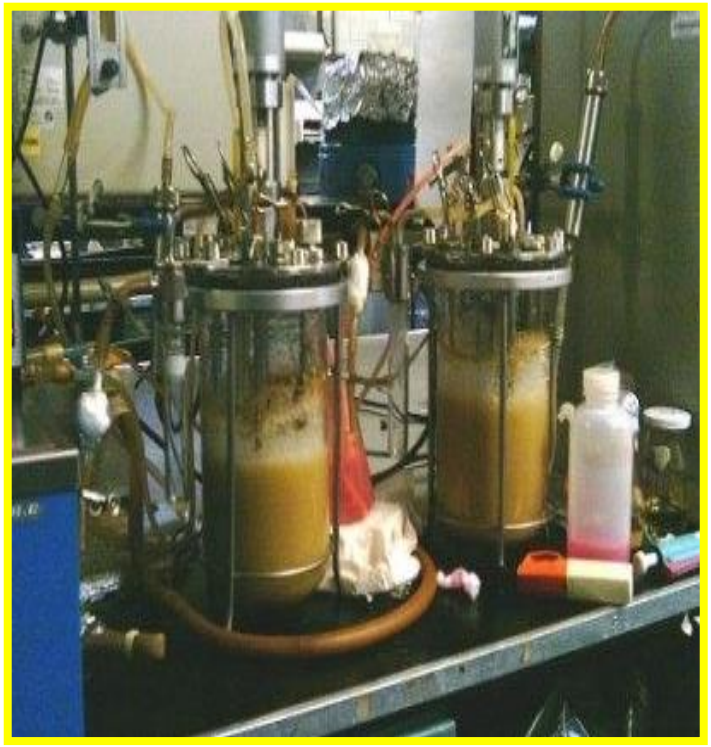
Ponce-Noyola & de la Torre (1995); Rojas –Rejón *et al.* (2011).

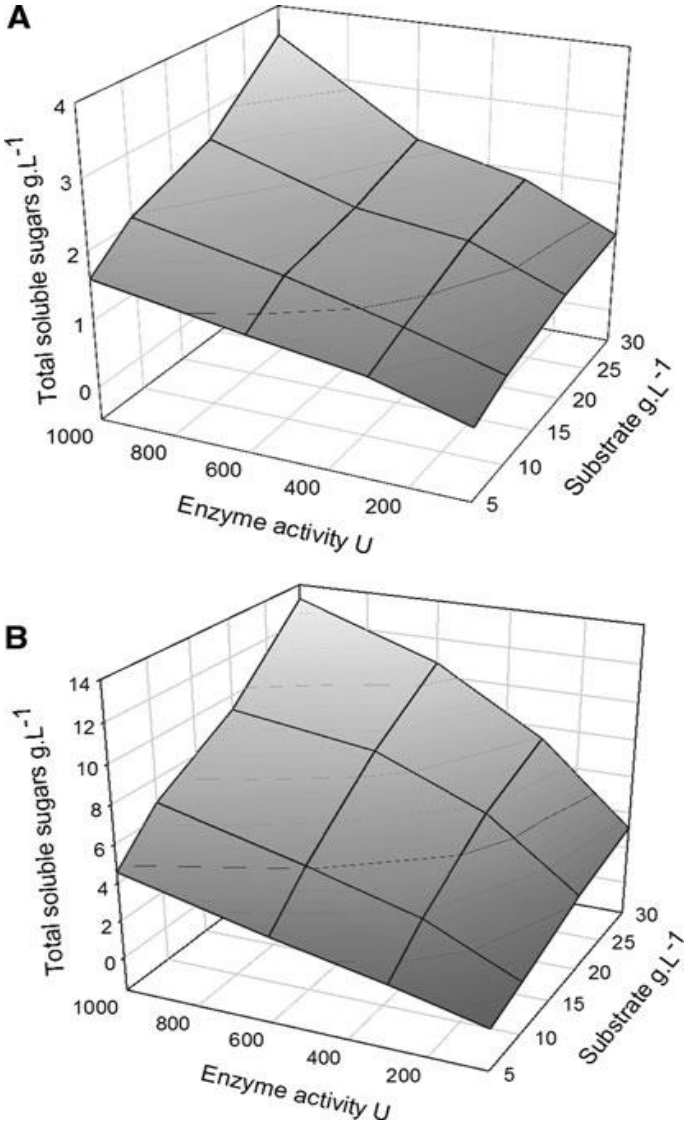
Table 1 Enzyme production by *Cellulomonas flavigena* wild type and mutants PN-10 and PN-120 grown on sugar cane bagasse (*CMCase* carboxymethylcellulase, *FPase* filter paper activity)

C. flavigena	Enzyme activity (u/ml)			
	CMCase	FPase	Xylanase	β-Glucosidase
Wild type	0.98	0.041	2.3	0.078
PN-10	1.4	0.050	4.4	0.243
PN-120	0.87	0.045	10.25	0.73
PR-22	1.6	ND	20.5	0.98

Ponce-Noyola and de la Torre, (1995); Rojas-Rejón, (2007); Ríos-Fránquez, (2012).

Enzyme production and saccharification





Rojas-Rejón *et al*., (2011)

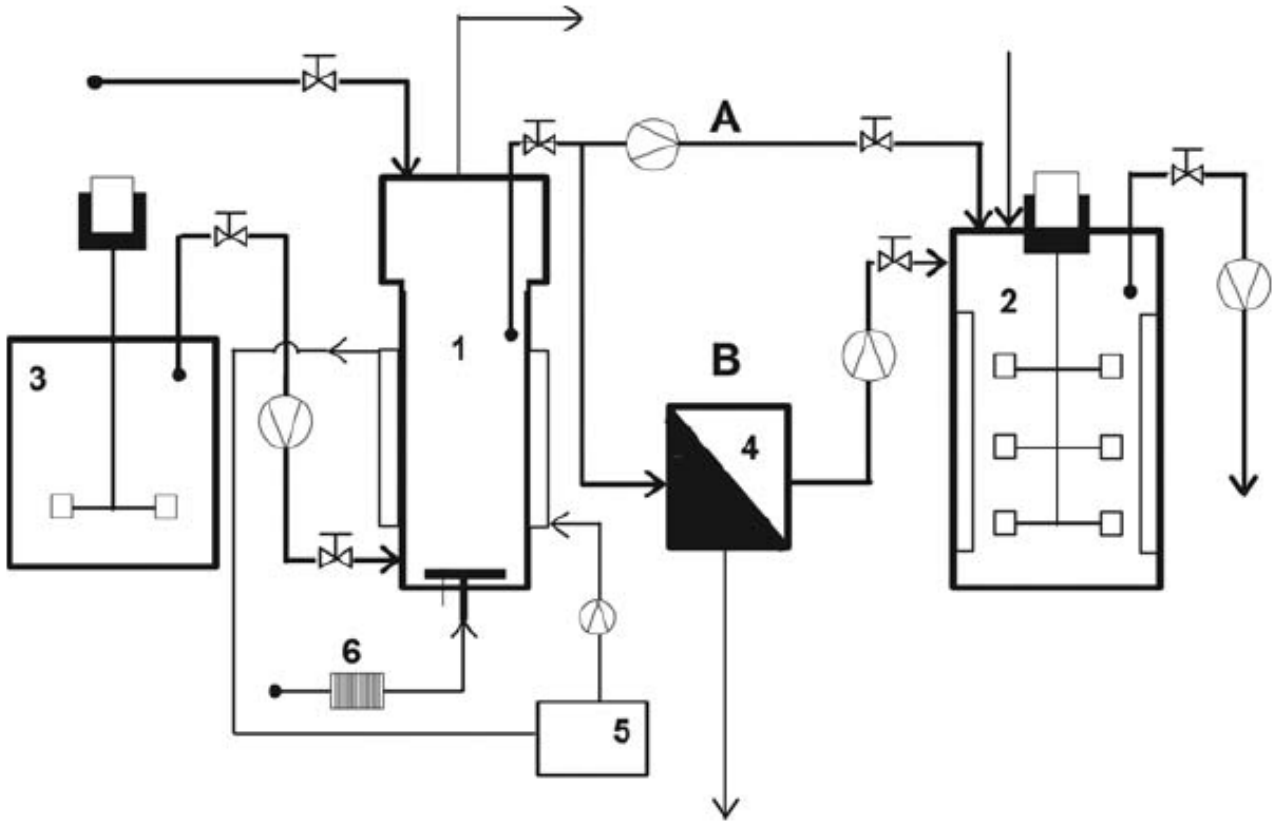


Figure 1. Continuous coupled enzyme production and saccharification system. (1) Bubble column (CMCase and xylanase producing reactor); (2) stirred tank (RENZ); (3) sterile reservoir; (4) centrifuge; (5) warm bath, and (6) air filter.

Rojas-Rejón, et al. (2016)

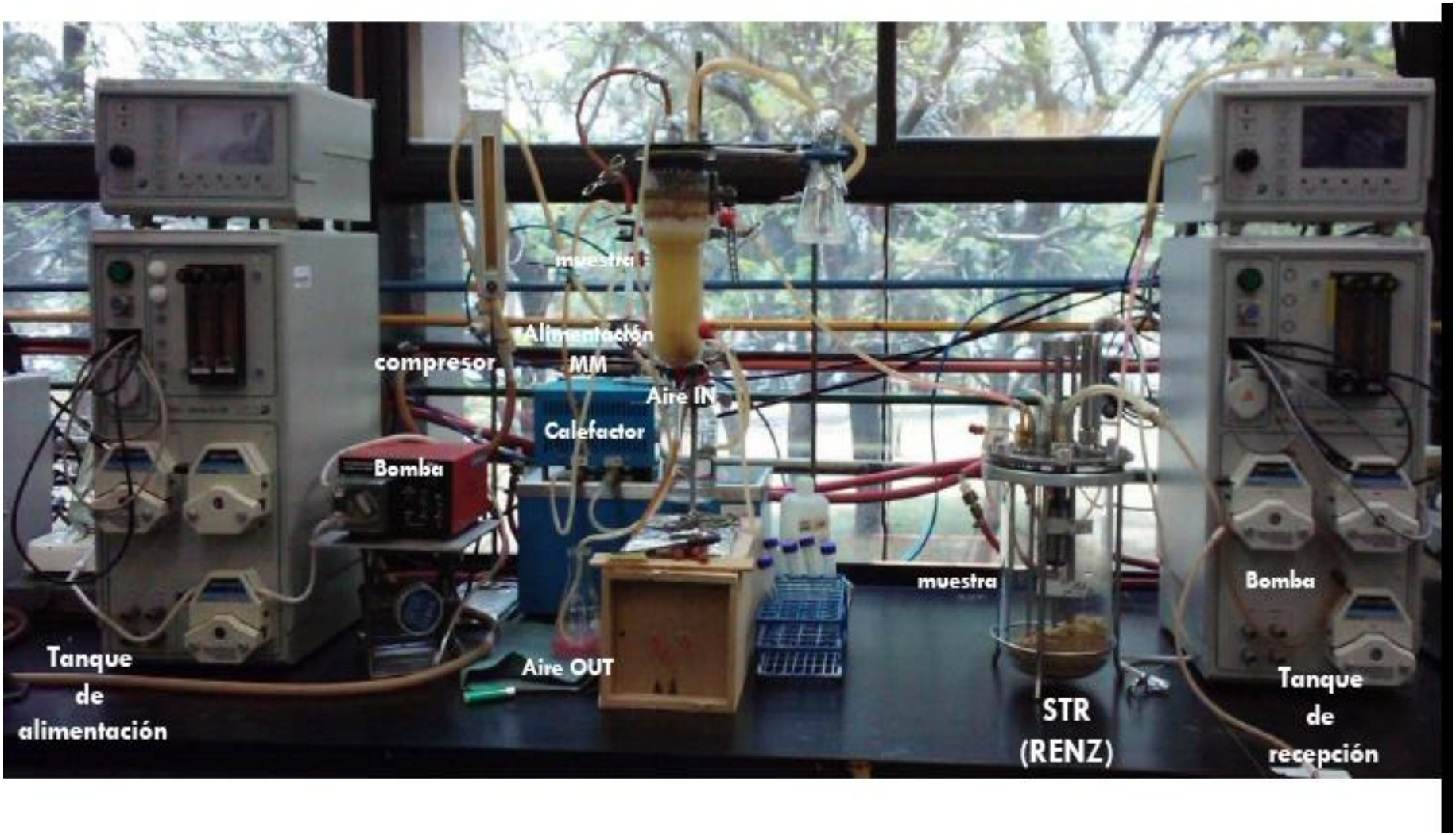

muestra
Alimentación
MM
compresor
Aire IN
Calefactor
Bomba
muestra
Bomba
Tanque
de
alimentación
Aire OUT
STR
(RENZ)
Tanque
de
recepción

Table 1. Comparison of Kinetic Parameters of *Cellulomonas flavigena* PR-22 Cultured in BCR and STR Configurations

*RC	Growth Rate (1/h) μ	Protein (g/L)		Enzyme Activities (U/mL)		‡Q_{VOL} (U/L h)	
		Cellular	Soluble	Xylanase	CMCase	Xylanase	CMCase
BCR	0.13 ± 0.01	1.22 ± 0.09	1.08 ± 0.03	17.69 ± 0.21	1.94 ± 0.19	368.54 ± 4.38	40.3 ± 3.96
STR	0.09 ± 0.02	1.73 ± 0.12	0.89 ± 0.06	14.70 ± 0.25	1.18 ± 0.25	306.25 ± 5.21	24.58 ± 5.51
(ξ)†	44	−6	22	33	63	65	98

*RC stands for reactor configuration.
†The performance index (ξ) represents improvement when the value is positive and repression, inhibition or decrease when it is negative.
‡Q_{VOL}: Enzymatic volumetric productivity.

Table 3. Final Mass of Soluble Sugars and Total Enzyme Units in RENZ

Condition	Protein (g)		Enzyme activities (U)		Soluble sugars (g)	
	Cellular	Soluble	Xylanase	CMCase	Total	Reducing
RENZ A	2.19 ± 0.21	3.80 ± 0.01	35.66 ± 2.34	16.73 ± 0.63	14.81 ± 0.54	12.75 ± 2.04
RENZ B	0.95 ± 0.05	3.45 ± 0.17	10.06 ± 3.26	2.10 ± 0.18	15.79 ± 1.33	11.78 ± 1.16

Rojas-Rejón, et al. (2016)

Purification of β-glucosidase (PN-120)

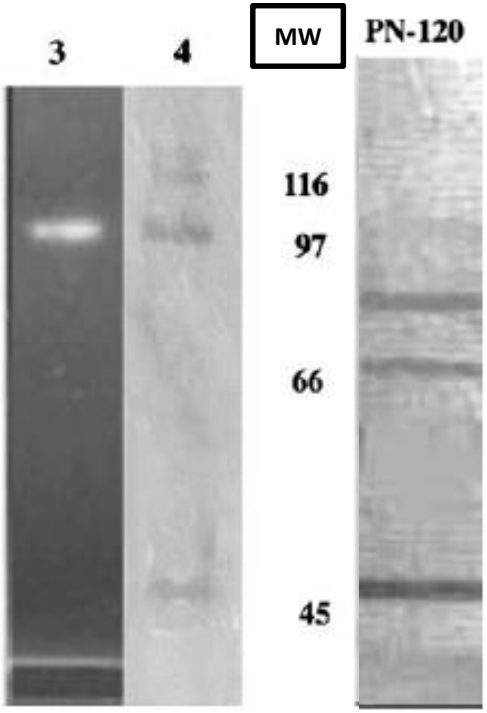


Fig. 1. PAGE of the protein eluted from the P100 column. (A) Protein separated under native conditions from wild-type (Lanes 1 and 2) and PN-120 (Lanes 3 and 4) strains of *C. flavigena* stained with Coomassie (Lanes 2 and 4) and zymogram of β-glucosidase using MUG as substrate (Lanes 1 and 3). (B) Protein separated under denaturizing conditions from wild-type and PN-120 strains stained with Coomassie. MW = molecular weight standards.

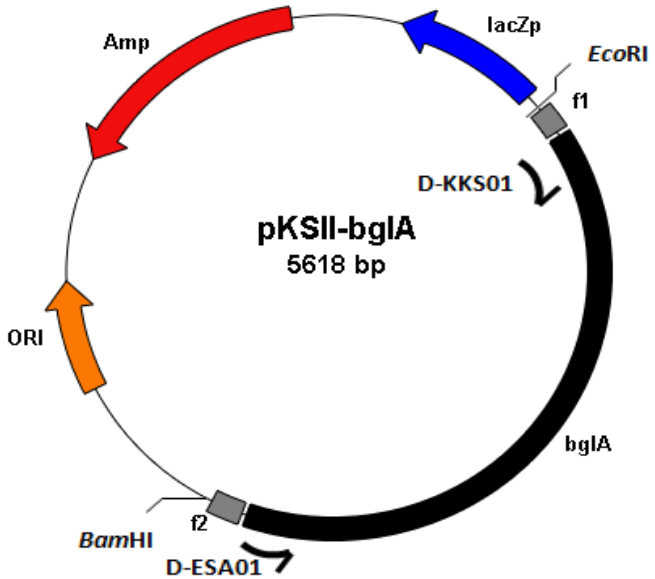
Barrera et al. (2005)

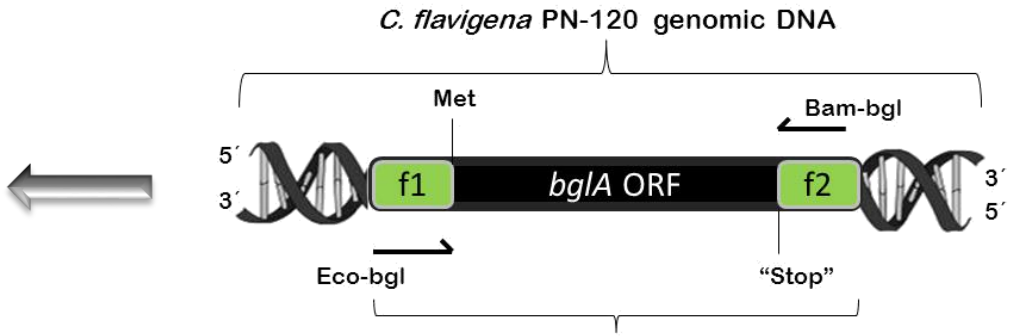
Fermentative microorganisms

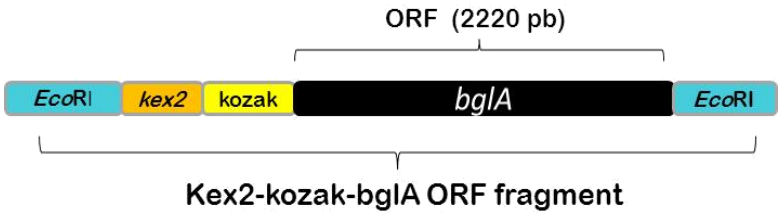
- ***Saccharomyces cerevisiae***

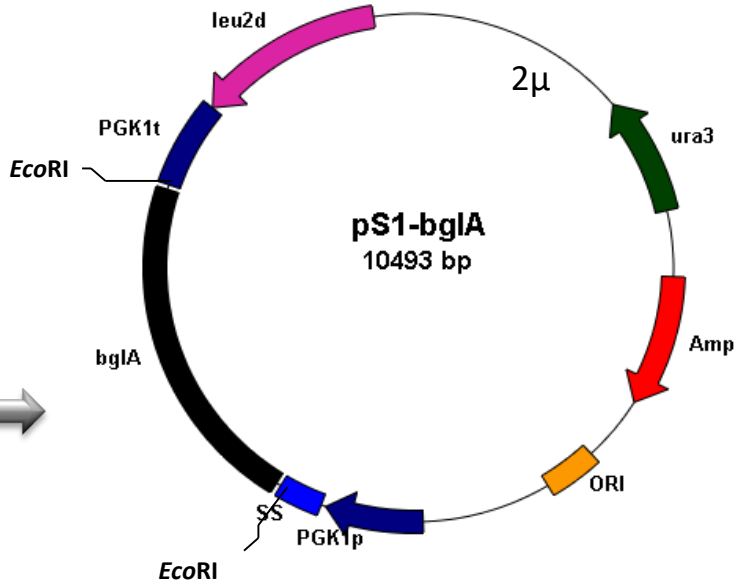
- **Via Embden-Meyerhoff**

Plasmid construction



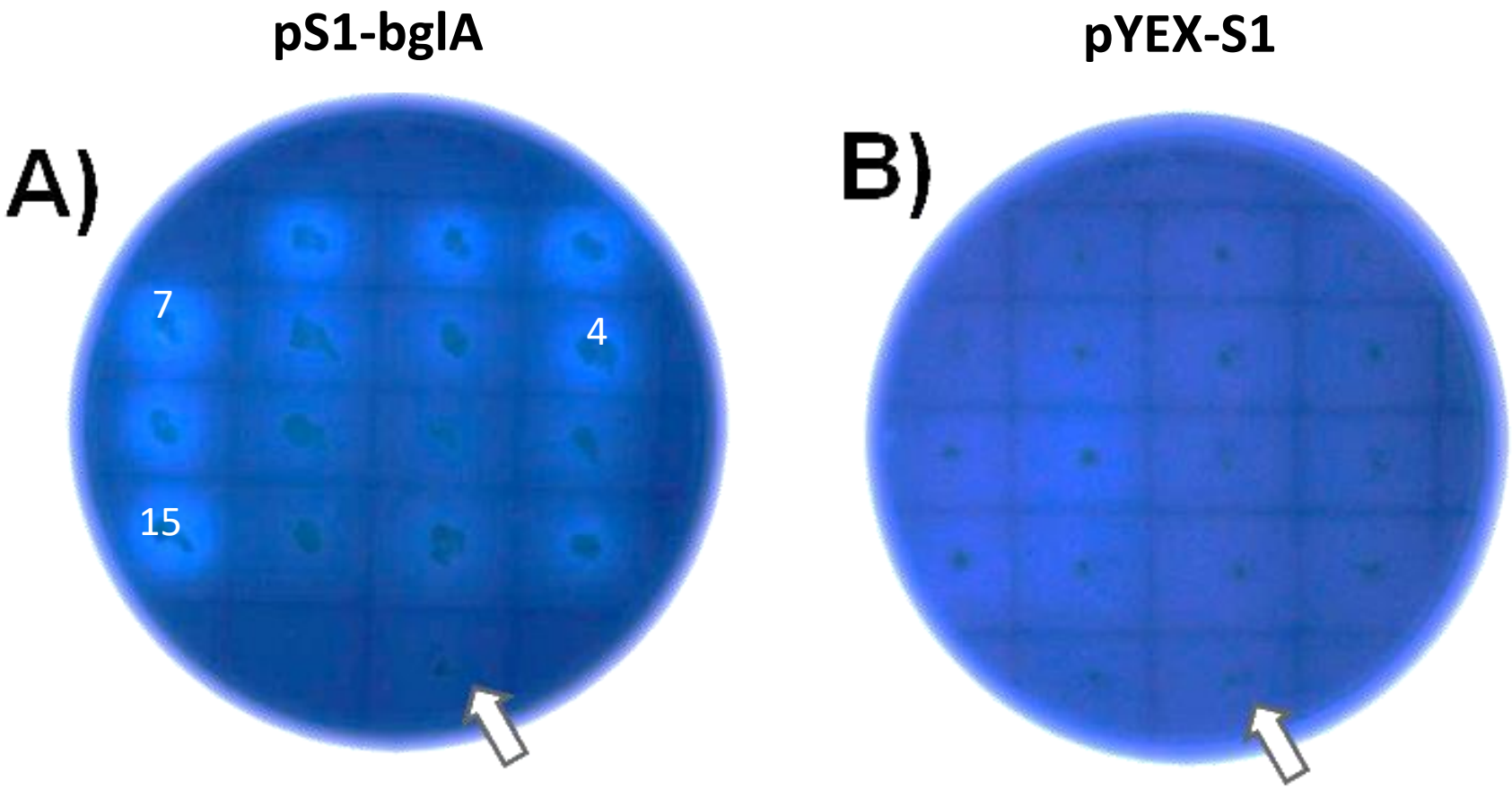






Mendoza-Aguayo et al. (2014)

Selection of transformed cells



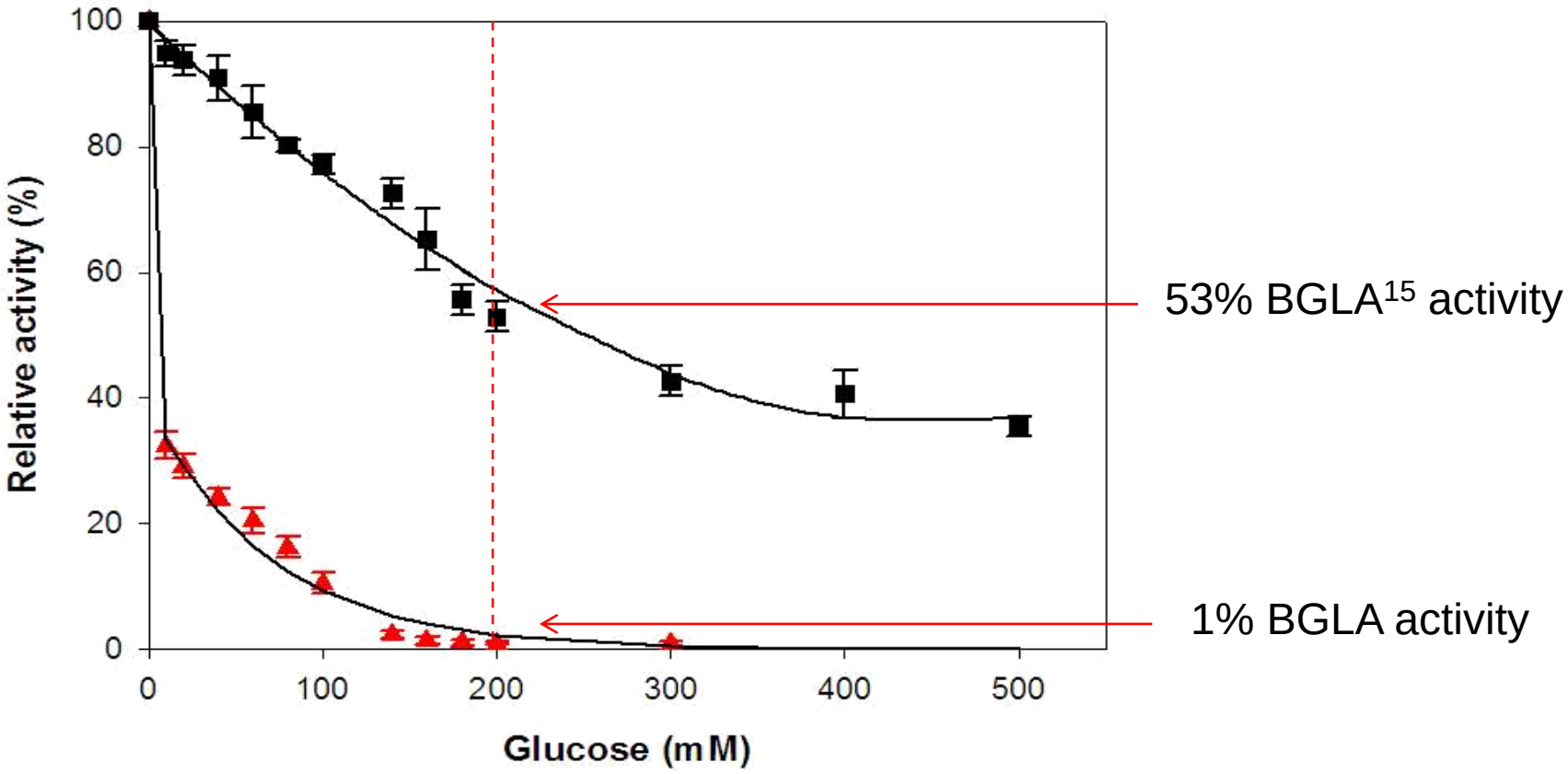
Extracellular β-glucosidase activity of recombinant strains of *S. cerevisiae*. A) Cells transformed with pS1-bglA. B) Cells transformed with pYEX-S1. Arrows show the wild strain. Strains were grown on SC plates with a MUG overlay.

β-glucosidase activity localization

Strain	YPD medium					SC medium				
	Supernatant		Cell pellet		Total activity	Supernatant		Cell pellet		Total activity
	IU/mL	%	IU/mL	%	IU/mL	IU/mL	%	IU/mL	%	IU/mL
4	0.63 ± 0.13	48.1	0.68 ± 0.06	51.9	1.31 ± 0.15	0.10 ± 0.13	16.4	0.51 ± 0.08	83.6	0.61 ± 0.08
7	0.62 ± 0.06	45.3	0.75 ± 0.16	54.7	1.37 ± 0.12	0.09 ± 0.01	14.5	0.53 ± 0.05	85.5	0.62 ± 0.07
15	0.58 ± 0.03	42.6	0.78 ± 0.11	57.4	1.36 ± 0.09	0.08 ± 0.004	13.0	0.54 ± 0.06	87.0	0.62 ± 0.06

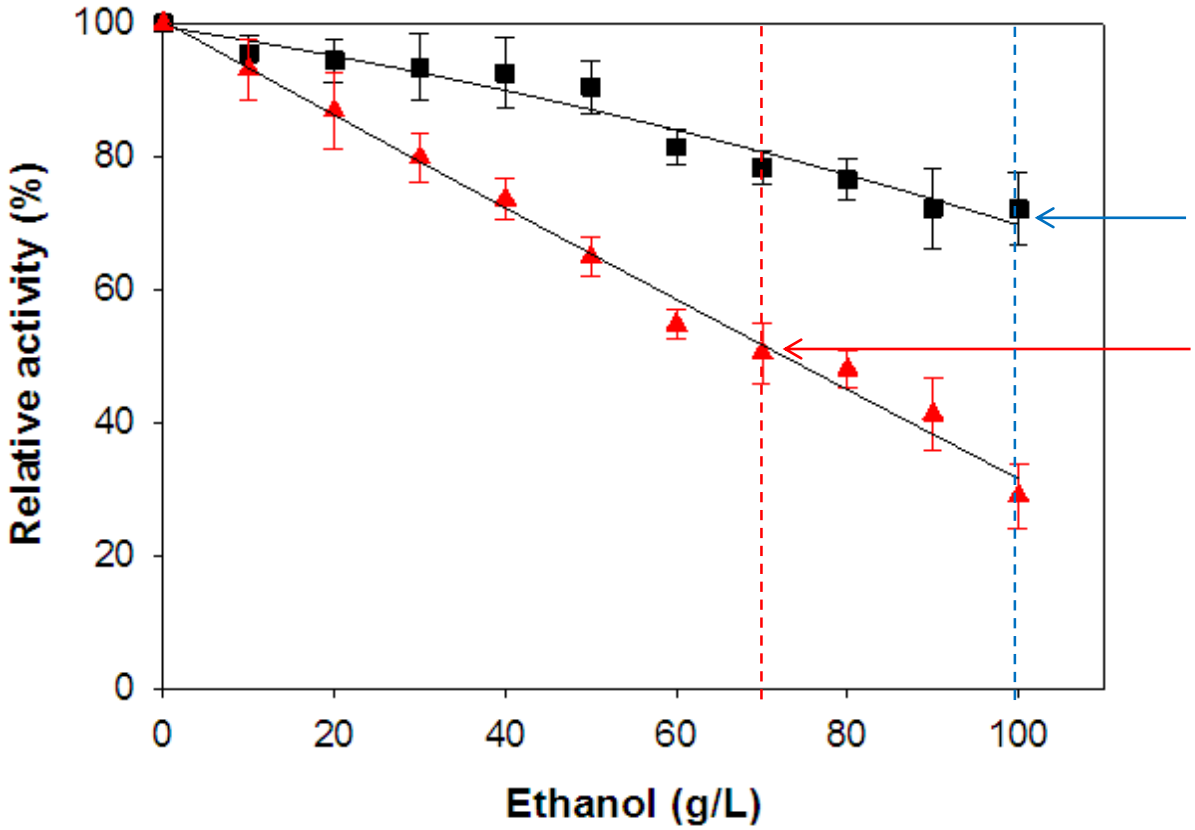
Mendoza-Aguayo et al. (2014)

Effect of glucose over β-glucosidase activity



Effect of glucose over activity of partially purified β-glucosidases. (■) BGLA[15], (▲) BGLA.

Effect of ethanol over β-glucosidase activity



72 % BGLA^{15} activity

50% BGLA activity

β-glucosidase activity. (■) BGLA^{15}, (▲) BGLA.

Codon usage in *S. cerevisiae.*

> bglA ### translated in organism: Saccharomyces cerevisiae

```
   1  mtlaekvgqm mqldsrgsvr glieethvgs ilhtspalvr eahevtagtr
  51  lriplliaed cihghsffeg atifptqlgm aaswdpglve raarvtavev
 101  aatgihwtfs pvlciardlr wgRvsetfge dpfligelas amvkgyqgdg
 151  ltdptailat akhfagyset qggRdatead isrrkltawf lppfervare
 201  gcatfmlgyq smdgtpitvn dwlltdvlrg ewgytgtlvt dwdnvgrmvw
 251  eqqiqpdhah aaaaavkagn dvimttpqff dgaqeavaag mldeaaidaa
 301  vsriltlkfe lglfedprrp ddariaqvlg tpehealnle ltrrsivllr
 351  negvlplagg ctagdderav apagaerRrv lvvgpnaddt daqlgdwagr
 401  sgqvdwlpdg hprhmietvl dglrrrspeg wevtyargad iltltpdpeg
 451  eyfpdgqprp qvvapcepdp eliaeavaaa Rdadyvvavv gdrielvgeg
 501  rstatlelig gqvalldala atgtplvivv vaskphvlpd saldaaavvw
 551  aanpgmrggq avaeillglv epsgRlpitf aRhvgqqptf ynqlrgqhgt
 601  Ryadltqrpa fafgaglsyt tleyadlevl tpsvtaadev raRvtlrntg
 651  trpaletvqv yvsdlvtttm waeselkayr qvevapgqsv vveltlpasa
 701  csivdaqarr vvepgafelr vgpssvreqq lvagftita*
```

> celB ### translated in organism: Saccharomyces cerevisiae

```
   1  vsapvsvtrr rtlrarvvag iaavaapaap lavaaaspva aaptsdwlht
  51  qgnkivdesg kevwltganw fgfnatervf hglwavnled vtrsmaehgi
 101  nivrvpistq pllewkagka avssgvntyv npelvgktsl evfdrflals
 151  ekyglkvmld vhsaeannag hfhpvwwkgt vtvddfysaw ewvtaryknn
 201  dtlvamdikn ephgtanssp rakwdsstdq dnfknlcqvt grkilainpn
 251  vlilcegiev ypkpgvswts tnkefygtww gcnlrgvtdh pvdlcanqdq
 301  lgdsphdygp mmfgsRtgst srsprltlea dvwdpnwlyi hkknisqpli
 351  rewggqvaqd eRqdrwmtal rdlmierrml htfwslnpns gdtgglldd
 401  wktwdsakyq llkpglwhdg Rqvptgfehq vglgRceqRt tprrttddpt
 451  qdptddrrri rrrpddgahr lvhgdlpgqr vehRdvgRgq gqehgtsals
 501  gwslqftlpa gqkleqgwsg twsqsgqtvt vtnaawngtl aaggtvevgf
 551  ngthtgstta psaftlnaad ctta*
```

Optimization of the *bgl*A and *cel*B genes.

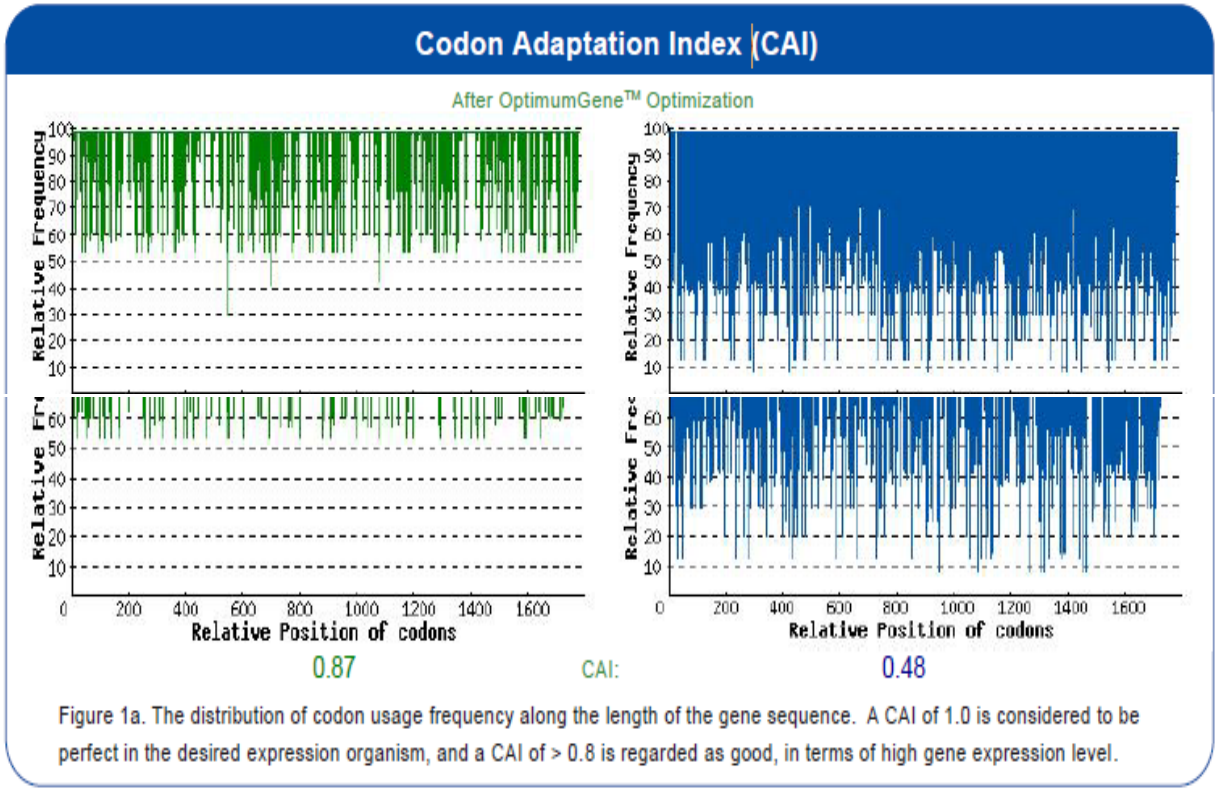
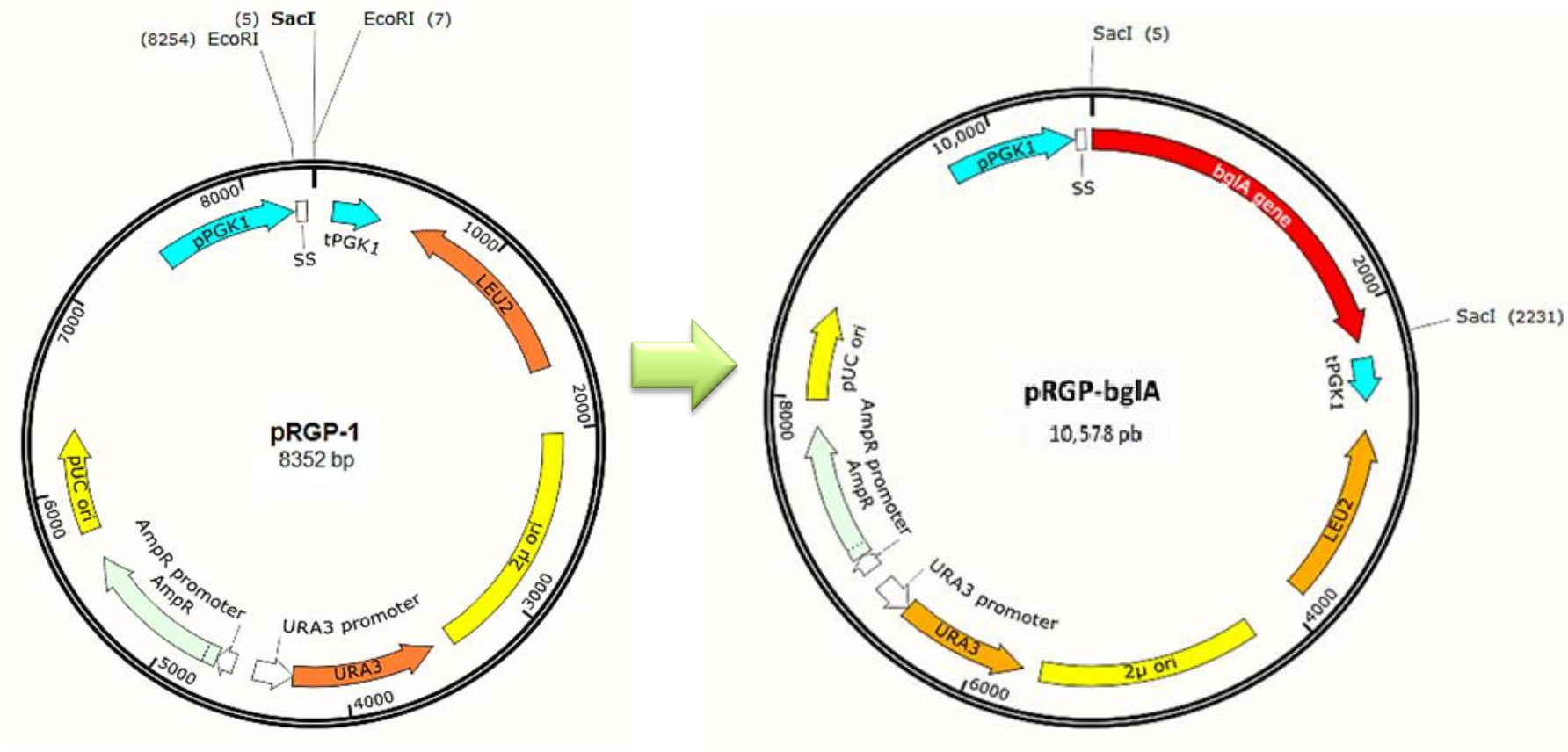


Figure 1a. The distribution of codon usage frequency along the length of the gene sequence. A CAI of 1.0 is considered to be perfect in the desired expression organism, and a CAI of > 0.8 is regarded as good, in terms of high gene expression level.

Codon adaptation index

a) *bgl*A, **b)** *cel*B.

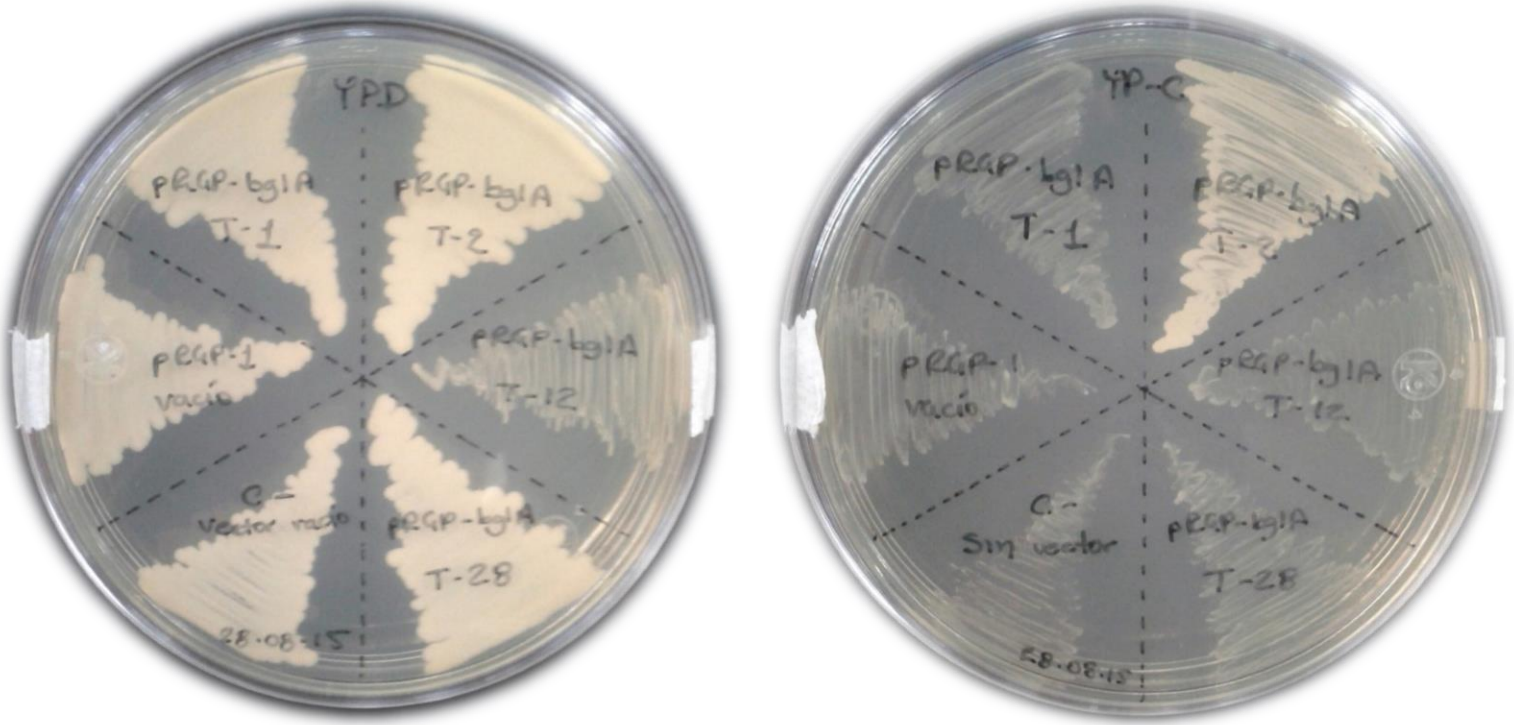
Plasmid construction



Cloning of the *bglA* optimized gen into the vector pRGP-1

Ríos-Fránquez, (2017)

S. cerevisiae transformants carried pRGP-bglA opt



a **b**

YP-Dextrose (a) YP-Cellobiose (b)

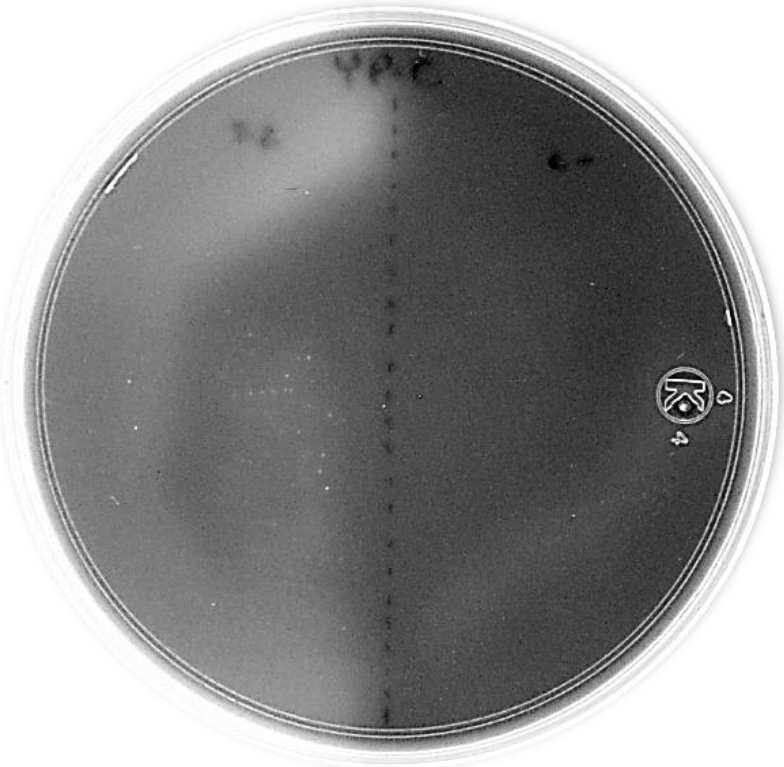
Ríos-Fránquez (2015)

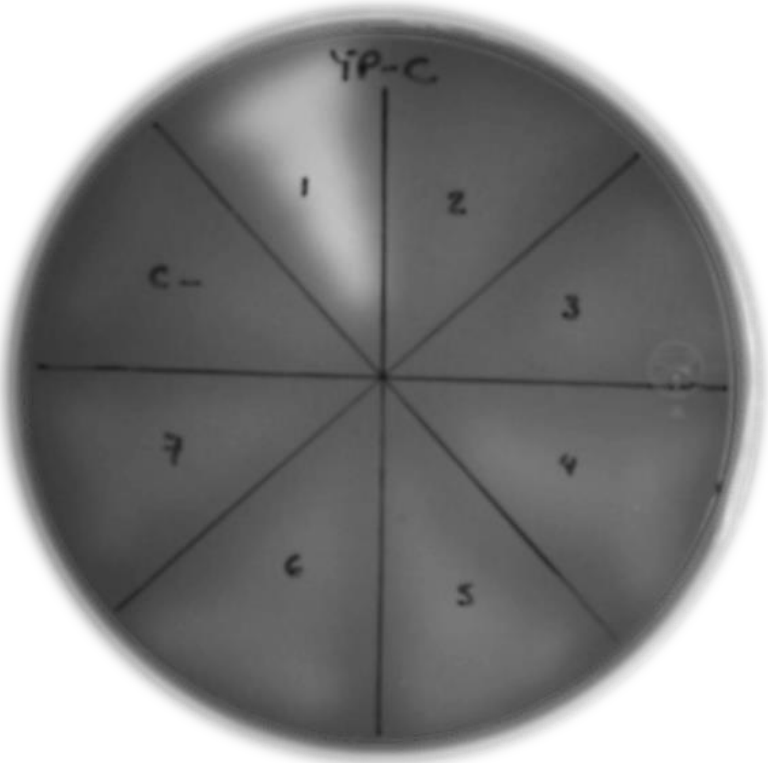
Zymograms using MUG in YPC (cellobiose)

Sc
RP2-BGL

Sc
pRGP-1

Sc
RP2-BGL





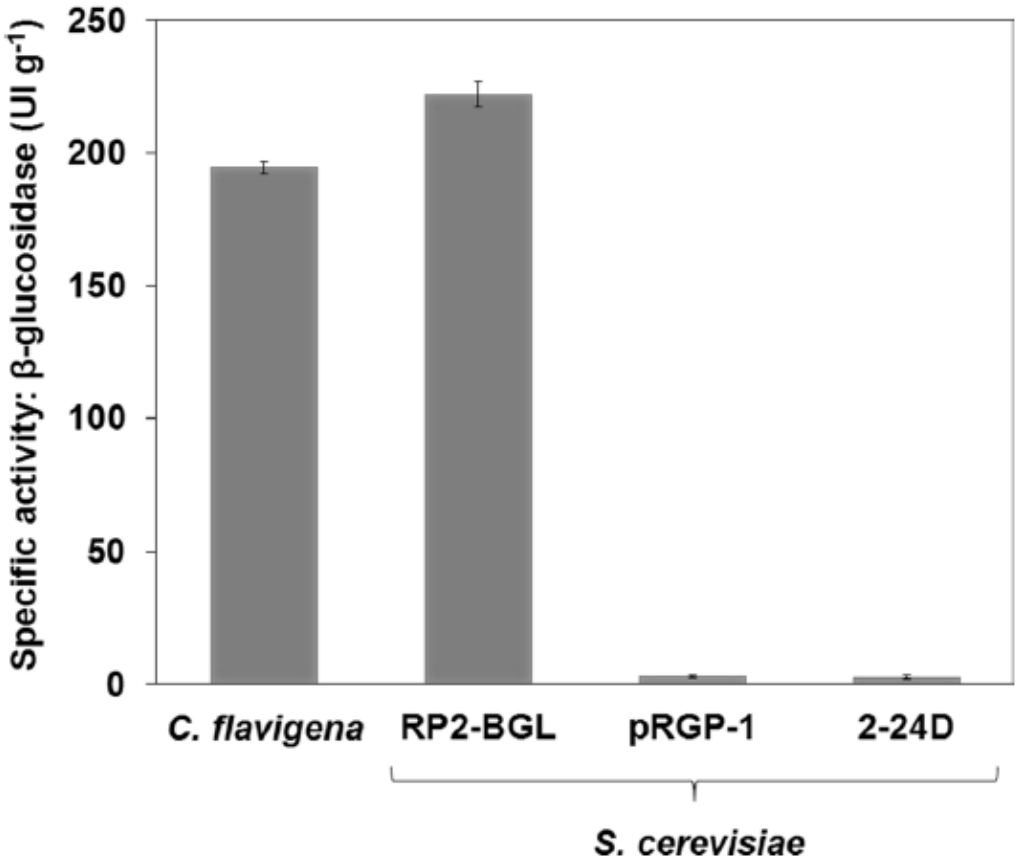
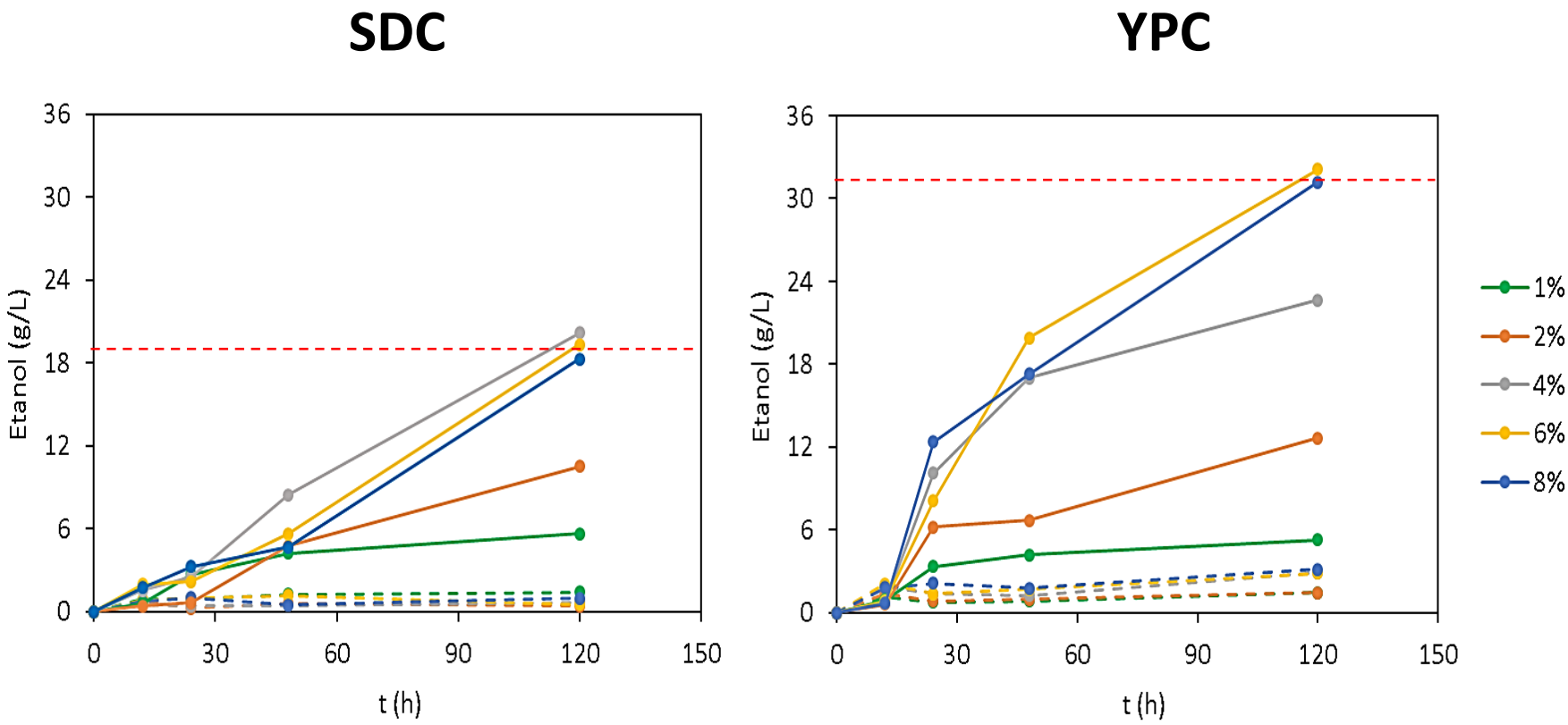


Fig. 2 Specific β-glucosidase activity in supernatants of *S. cerevisiae* growing in SD medium of transformants RP2-BGL (pRGP-bglA OPT), pRGP-1 and the 2-24D strain, and a concentrated crude cell-free extract from *C. flavigena* PR-22 as a control

Ríos-Fránquez et al. (2015)

Ethanol from cellobiose using *Sc* RP2-BGL



Ethanol production from cellobiose using *Sc* RP2-BGL (solid) and *Sc* pRGP-1 (dotted) in SDC and YPC media.

Yield of ethanol from cellobiose using engineered *S. cerevisiae* strains expressing β-glucosidase activities.

Microorganism	strain	Yield ($g_p/g_{s)}$	Reference
Saccharomyces cerevisiae	Y294 [SFI]	0.41	Van Rooyen et al., 2015
	RP2-BGL	0.41	Ríos-Fránquez, 2017
	RP2-BGL	0.50	Este trabajo
	NAN-227	0.532	Shen et al., 2008

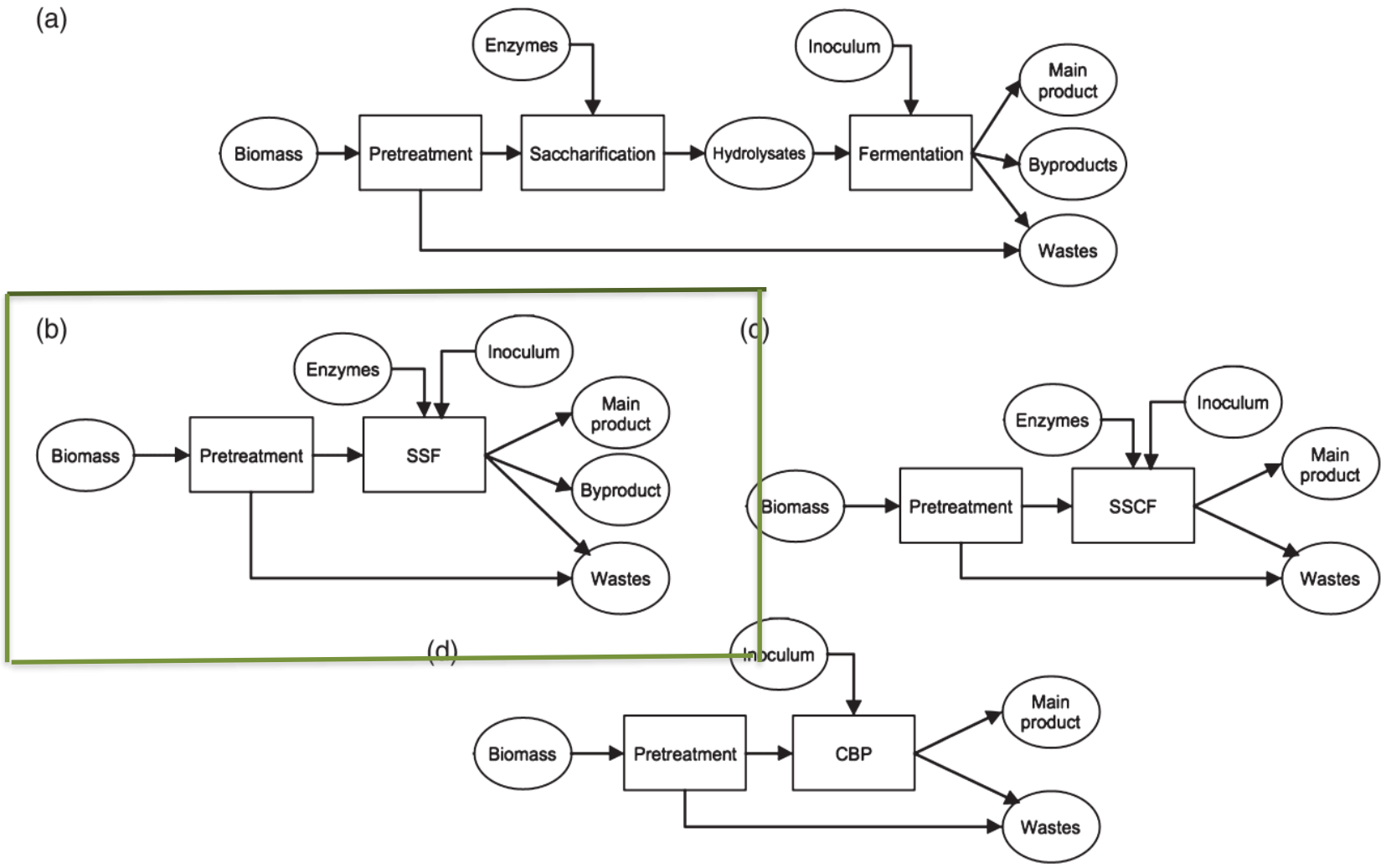


Figure 2. Saccharification and fermentation of biomass: (a) conventional; (b) SSF; and (c) SSCF and CBP.

Escamilla et al. (2016)

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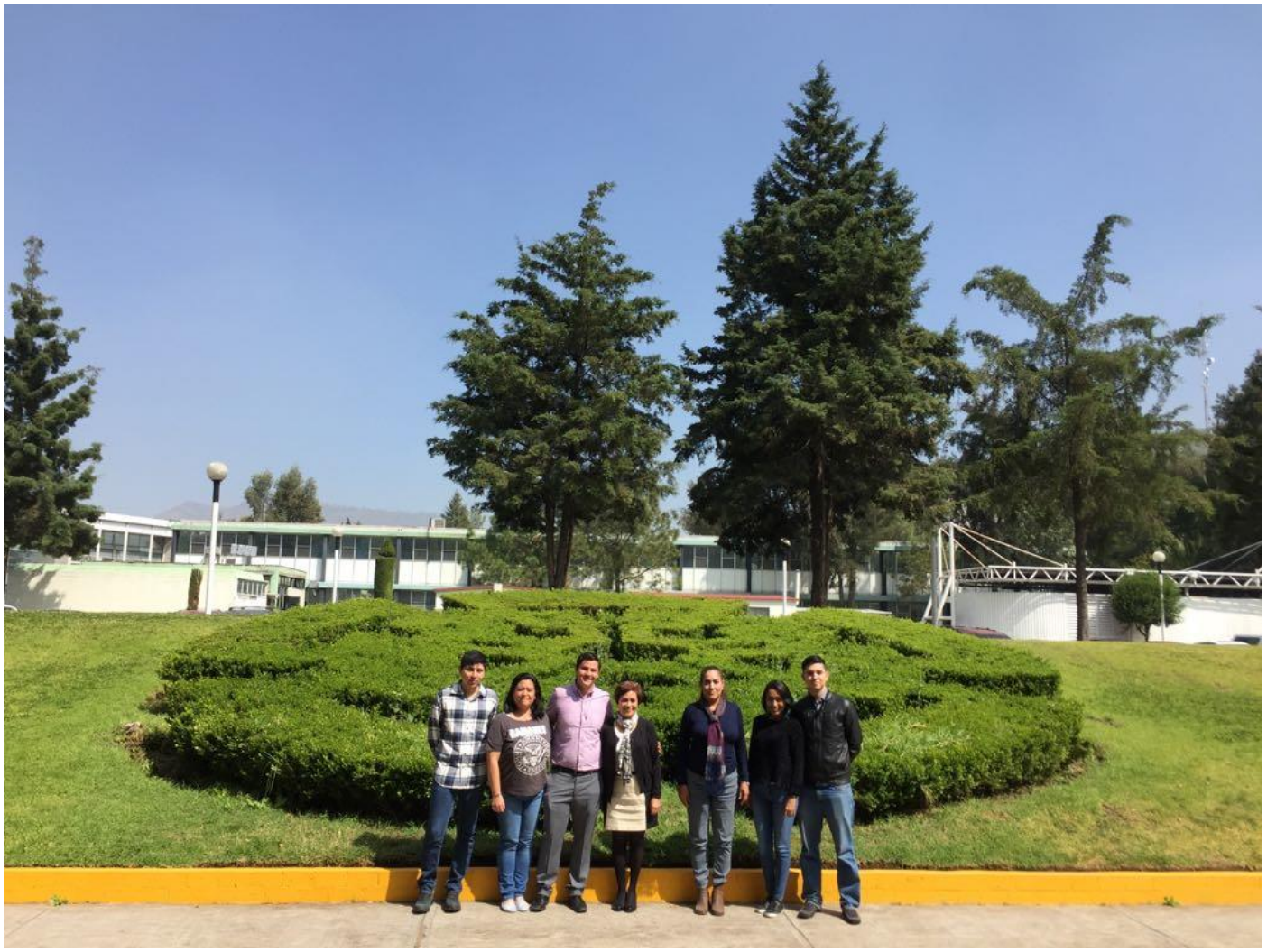

¡GRACIAS, LOS ESPERAMOS!

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ORIGINAL PAPER

Expression of a codon-optimized β-glucosidase from *Cellulomonas flavigena* PR-22 in *Saccharomyces cerevisiae* for bioethanol production from cellobiose

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Received: 15 July 2016 / Revised: 13 December 2016 / Accepted: 26 December 2016

Etanol de segunda generación

Una alternativa biotecnológica…

Bagazo de caña



(Himanish, 2004)

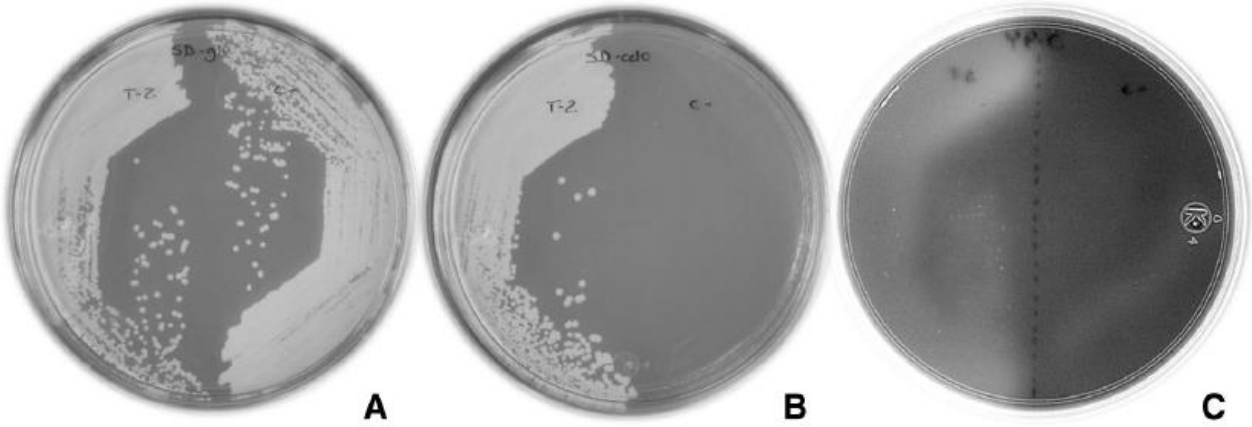
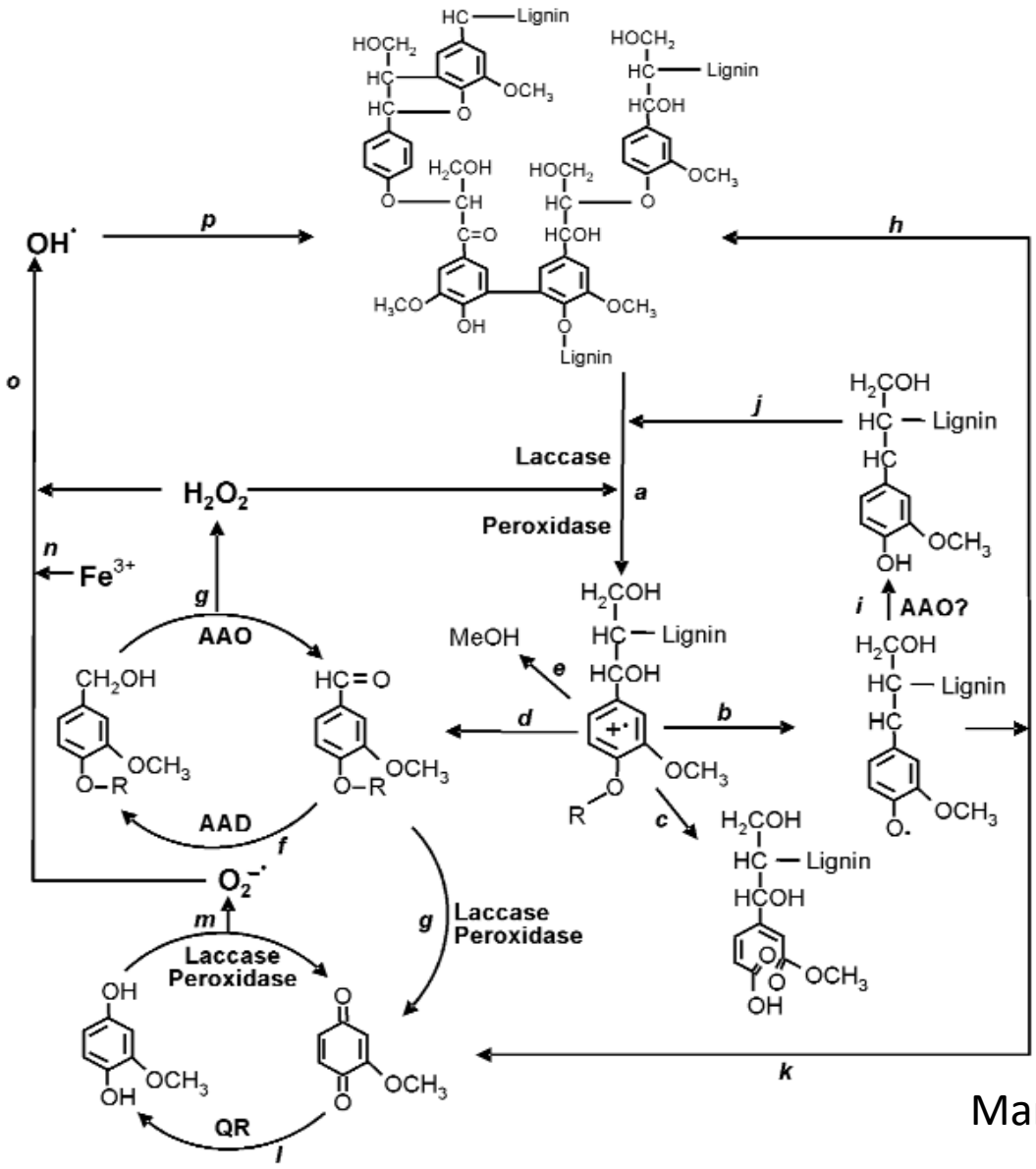


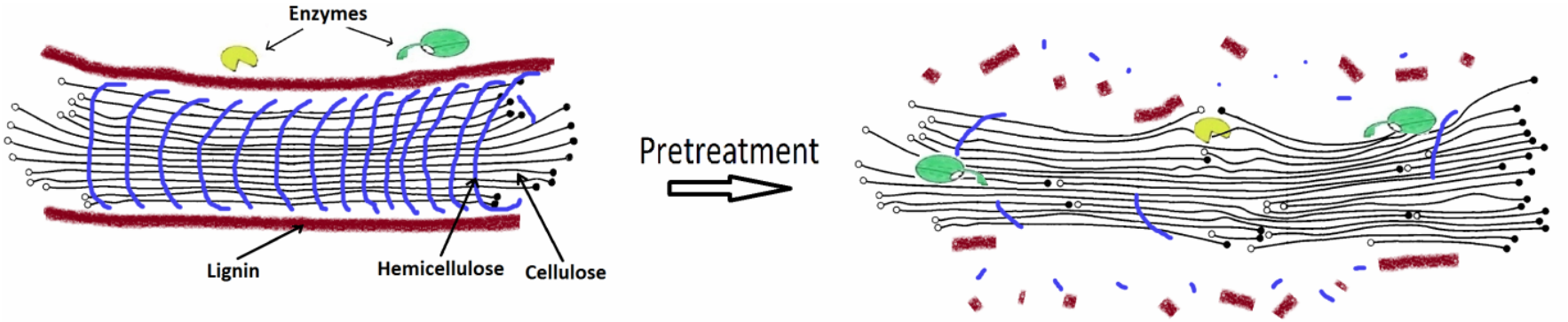
Fig. 1 Comparative growth in SD medium of *S. cerevisiae* RP2-BGL (*left*) and pRGP-1 (*right*) with **a** glucose and **b** cellobiose as the carbon source. **c** Zymogram overgrowth of the transformants on YPC medium using MUG as a substrate for β-glucosidase extracellular activity and revealed under UV light

Degradación de la lignina.



Martínez et al. 2005

Pretratamiento de la lignocelulosa



Físicos	Reducción mecánica Pirólisis
Químicos	Ozonólisis Tratamiento ácido Tratamiento alcalino

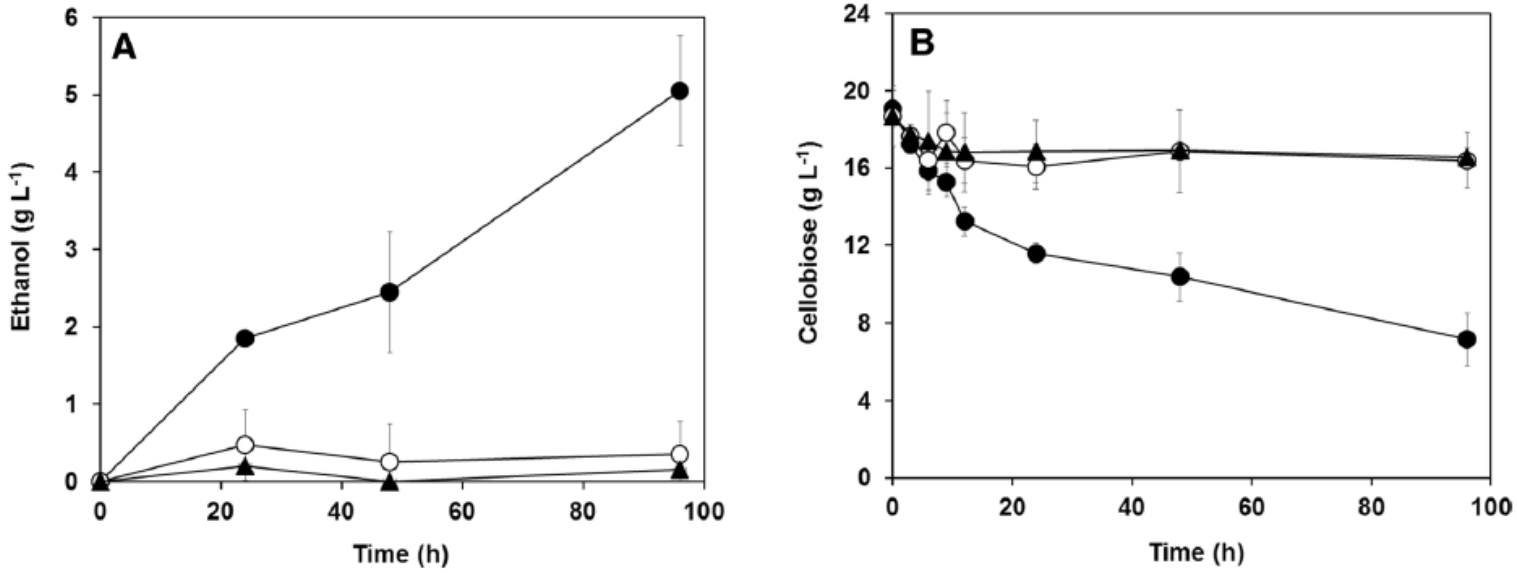


Fig. 3 Culture in anaerobic conditions on YPC medium of the *S. cerevisiae* transformants RP2-BGL (pRGP-bglA OPT), pRGP-1 and the 2-24D strain. **a** Production of ethanol and **b** cellobiose consumption. *Closed circle* RP2-BGL; *open circle* pRGP-1; *closed triangle* 2-24D

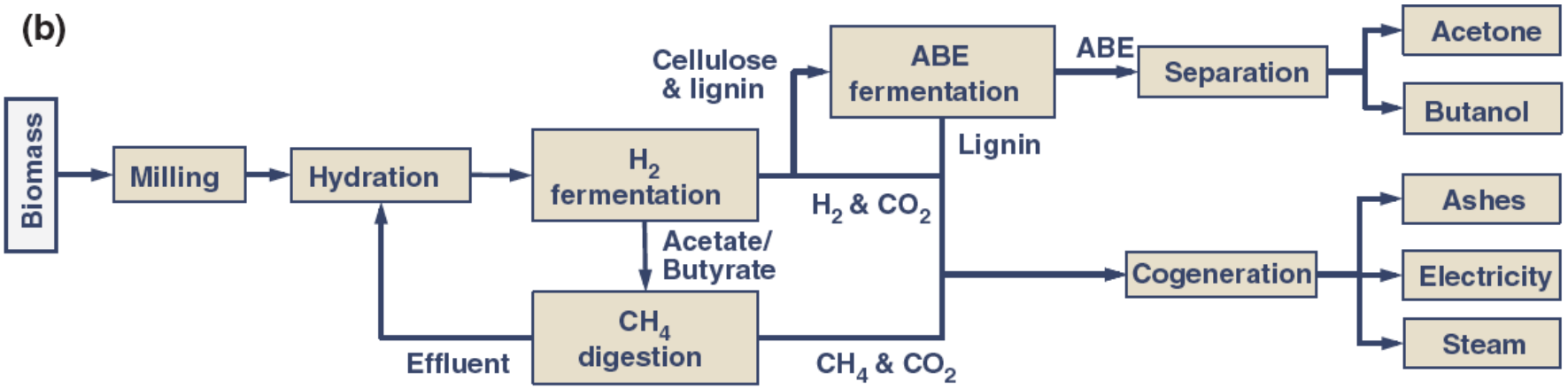


Figure 1. a) Reaction scheme for the biorefinery based on mixed cultures indicating the material flow. b) Process block diagram.

Estructura de la lignocelulosa