

# Optimization of enzymatic cartilage hydrolysis from *Prionace glauca* wastes for the production of chondroitin sulphate

José A. Vázquez<sup>1\*</sup>, María Blanco<sup>2</sup>, **Diana Noriega**<sup>2,3</sup>, Javier Fraguas<sup>1</sup>, Lorenzo Pastrana<sup>3</sup>, Carmen G. Sotelo<sup>2</sup> & Ricardo Pérez-Martín<sup>2</sup>

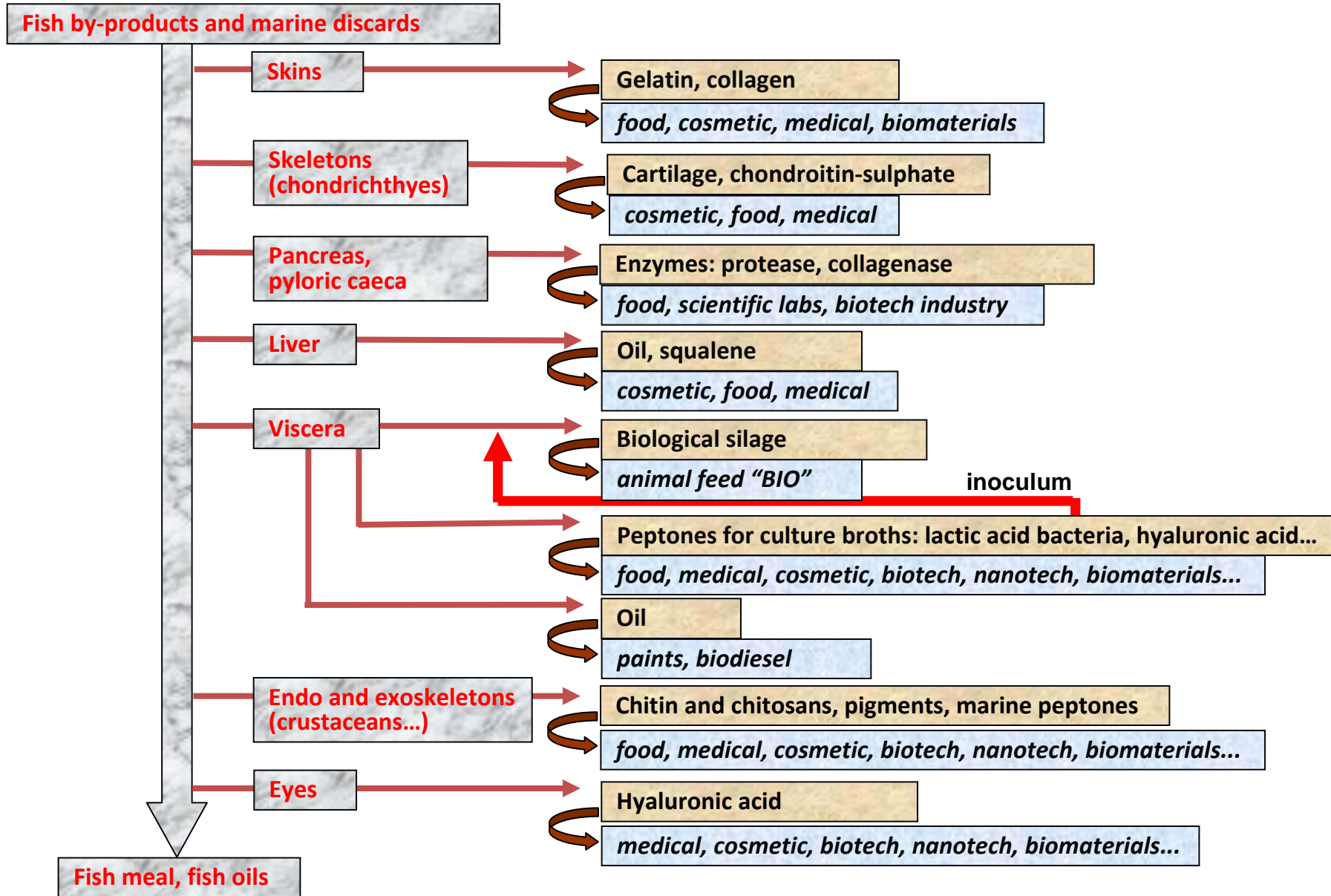
<sup>1</sup>Grupo de Reciclado y Valorización de Materiales Residuales (REVAL), (IIM-CSIC), Vigo (Spain)

<sup>2</sup>Grupo de Bioquímica de Alimentos (BA), (IIM-CSIC), Vigo (Spain)

<sup>3</sup>Departamento de Química Analítica y Alimentaria, Universidad de Vigo, Ourense (Spain)

\* [jvazquez@iim.csic.es](mailto:jvazquez@iim.csic.es)

# Recycling and marine waste valorisation from IIM-CSIC



# STATE OF THE PROBLEM and MOTIVATION



Fishing Port of Vigo

- Largest fishing port in Europe (fresh and frozen fish).
- Largest fishing market in Europe (litoral, big fishes, coastal auctions, etc.).
- ~1 MTm of fish/year landed, commercialized and moved from the Port of Vigo.
- 35% are by-products (350000 Tm/year).

## **Prionace glauca fishery:**

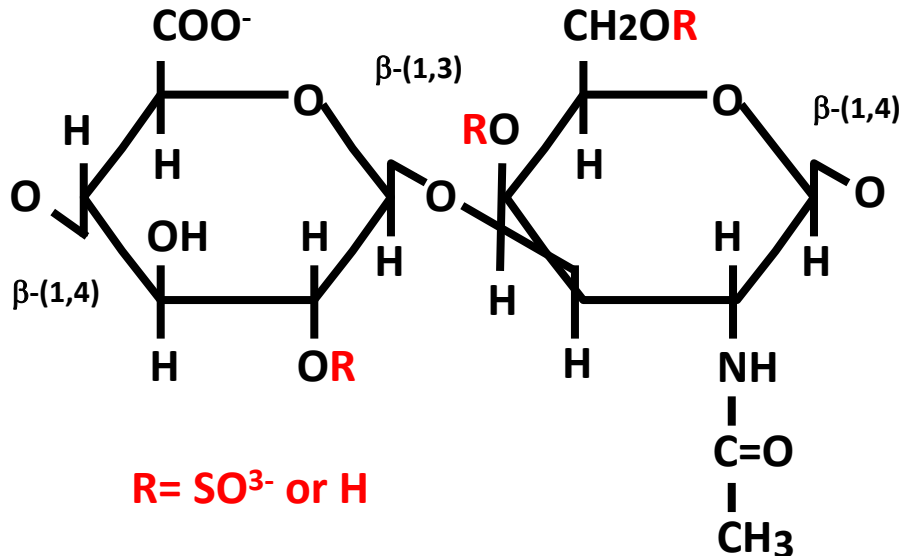
- 50% of sharks landed in Europe were *P. glauca* (>60% in Spain, 2009).
- 2500 Tm (fresh) were landed in Vigo (2013).
- It is the 6<sup>th</sup> most landed species in Vigo.
- 33% of life weight is a by-product (visceras, skin and head). 15% of this weight are heads.



# CHONDROITIN SULPHATE from CARTILAGE

**Cartilage** is a connective tissue formed by chondrocytes cells and an extracellular matrix including proteoglycan and protein fibers (elastin, collagen). Proteoglycan is the covalent association of glycosaminoglycans chains (chondroitin sulphate, etc.) and proteins (aggrecan, etc.).

**Chondroitin sulphate** is a linear polysaccharide consisting of repeating disaccharide units of glucuronic acid and sulfated N-acetyl galactosamine, mainly in C4 or/and C6.



Increasing demand in tissue bioengineering, nanomaterials, hydrogels, repair of skin and bone lesions, dental material support, arthritis and osteoarthritis treatment, etc.

# OBJECTIVES and WORKING PLAN

- 1: To study** the first step (enzymatic hydrolysis of cartilage) to extract chondroitin sulphate from shark head wastes.
- 2: To assess** the joint effect of  $pH$  and  $T$  in the proteolytic hydrolysis run by alcalase in cartilage of shark head.
- 3: To optimize** the best conditions for the alcalase catalysis by combination of kinetics and response surface methodology approaches.

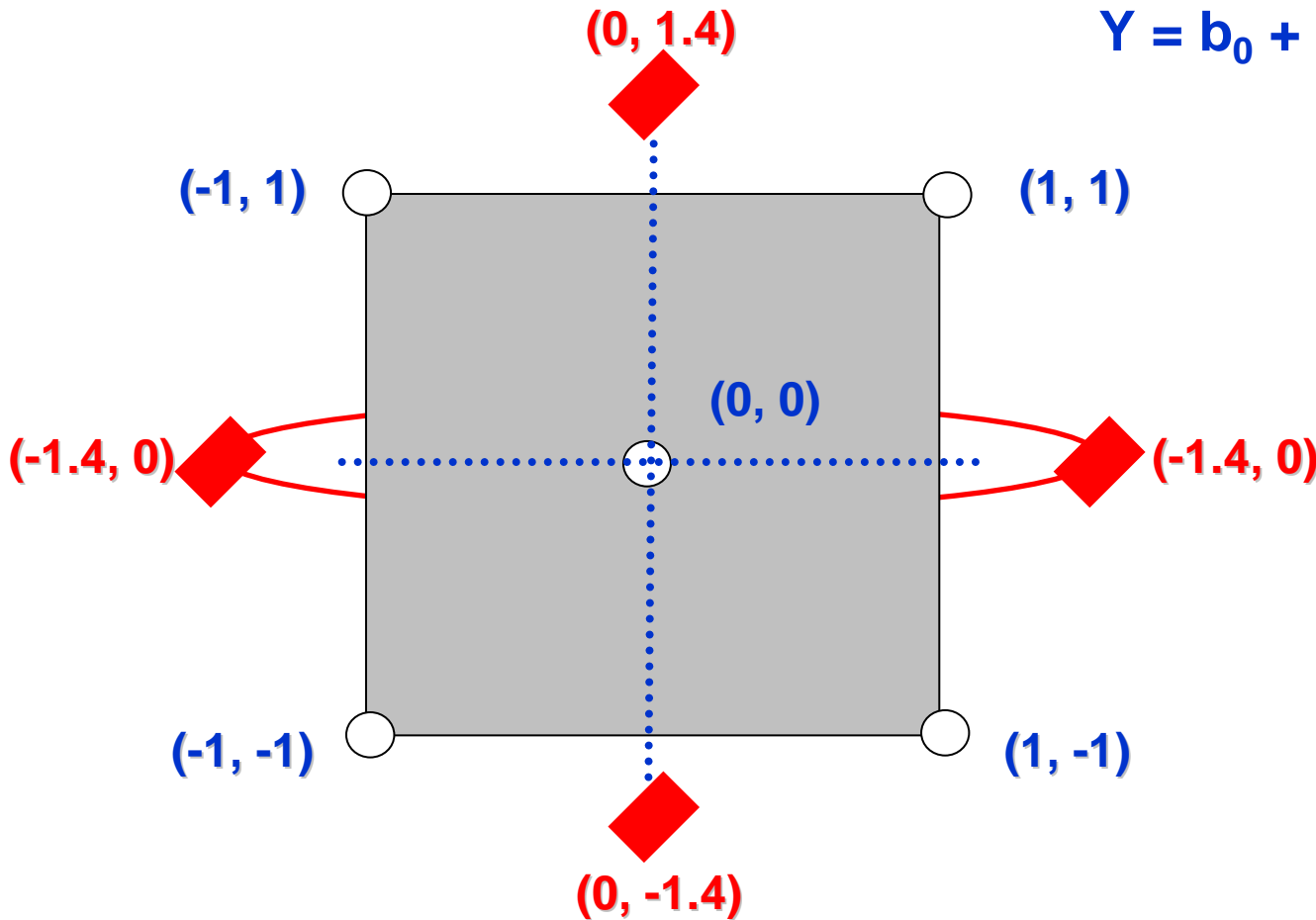
**Cartilaginous material** from *P. glauca* head wastes was initially prepared following the next steps:

- Hot water cooking (80°C/1 h).
- Manual or mechanical cleaning of the muscle remains.
- Homogenization of sample by grinding.



# EXPERIMENTAL CONDITIONS (rotatable second order design)

$$Y = b_0 + b_1pH + b_2T + b_{12}TpH$$



RESPONSE	SURFACE METHODOLOGY (RSM):
1) Parameters significance:	t-Student test ( $\alpha=0.05$ ).
2) Equation consistency:	F-Fisher test ( $\alpha=0.05$ ).
3) Equation-data correlation:	$R^2_{adj}$

$$Y = b_0 + b_1pH + b_2T + b_{12}TpH + b_{11}pH^2 + b_{22}T^2$$

# EXPERIMENTAL CONDITIONS (rotatable second order design)

Nº Exp	T (°C)	pH	Tcod	pHcod
1	37.3	6.9	-1	-1
2	72.7	6.9	1	-1
3	37.3	11.1	-1	1
4	72.7	11.1	1	1
5	30.0	9.0	-1.41	0
6	80.0	9.0	1.41	0
7	55.0	6.0	0	-1.41
8	55.0	12.0	0	1.41
9	55.0	9.0	0	0
10	55.0	9.0	0	0
11	55.0	9.0	0	0
12	55.0	9.0	0	0
13	55.0	9.0	0	0

## Constant conditions:

[alcalase]= 0.3% (v/w), 7.2 AU/kg

R (S:L)= 1:3 (25 g:75 mL H<sub>2</sub>O)

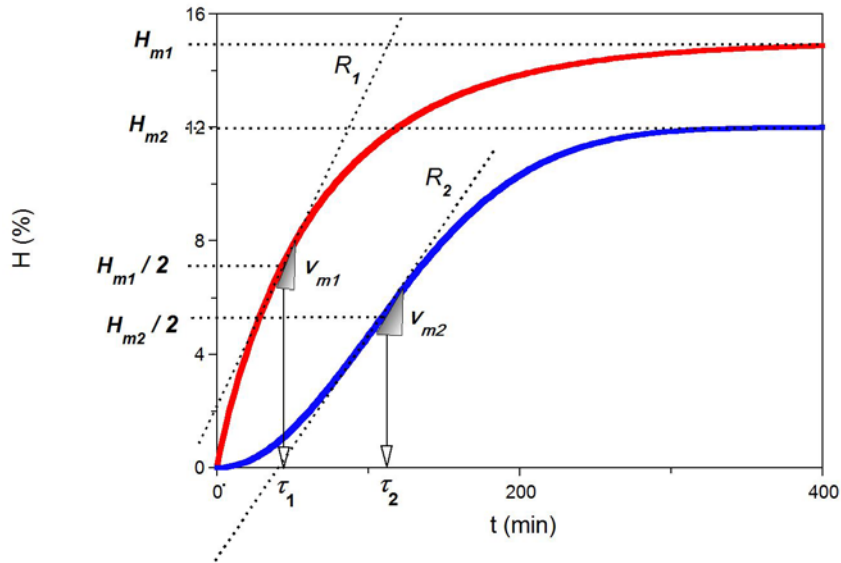
t= 4 h

Agitation ~ 250 rpm

pH-stat Reactor

CODIFICATION	DECODIFICATION
$V_c = (V_n - V_o) / \Delta V_n$	$V_n = V_o + (\Delta V_n \times V_c)$
$V_n$ : natural value of the variable to codify.	$V_o$ : natural value in the centre of the domain.
$V_c$ : codified value of the variable.	$\Delta V_c$ : increment of $V_n$ per unit of $V_c$

# RESULTS OF HYDROLYSIS KINETICS



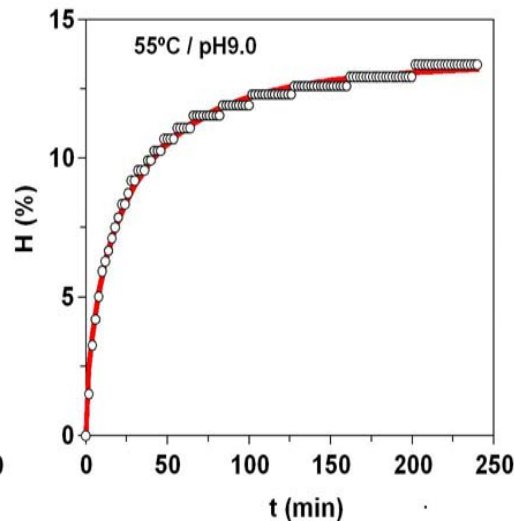
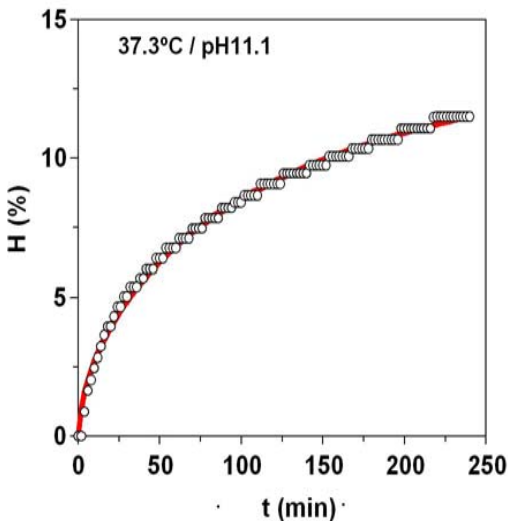
## Modelling of kinetics by Weibull equation

$$H = H_m \left\{ 1 - \exp \left[ -\ln 2 \left( \frac{t}{\tau} \right)^\alpha \right] \right\}$$

$$v_m = \frac{\alpha H_m \ln 2}{2\tau}$$

**$H$** : degree of hydrolysis (%).  
 **$t$** : time of hydrolysis (min).  
 **$H_m$** : maximum hydrolysis (%).  
 **$\alpha$** : parameter of form (dimensionless).  
 **$\tau$** : time to achieve  $H_m/2$  (min).  
 **$v_m$** : maximum hydrolysis rate (% min<sup>-1</sup>).

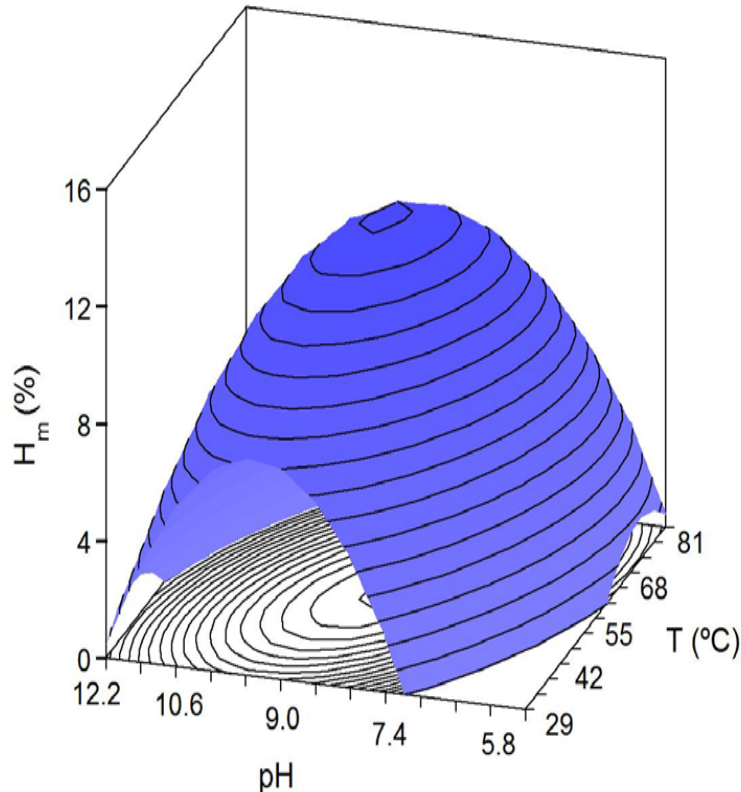
- 1) It is a flexible equation to model different experimental profiles: sigmoid, hyperbolic, etc.
- 2) It can be formulated with parameters of enzyme hydrolysis relevance.
- 3) Perfect agreement among the experimental data and the predicted fittings by equation ( $R^2 > 0.991$ ).





# ALCALASE HYDROLYSIS OPTIMIZATION

Maximum hydrolysis ( $H_m$ ) as response (dependent variable)



$$R_{adj}^2 = 0.720$$

$$H_m = 13.0 - 3.72T\text{pH} - 3.01T^2 - 6.28\text{pH}^2$$

$$\frac{\partial H_m}{\partial T} = -3.72\text{pH} - 6.02T$$

$$-3.72\text{pH}_{\max} - 6.02T_{\max} = 0$$

$$\frac{\partial H_m}{\partial \text{pH}} = -3.72T - 12.56\text{pH}$$

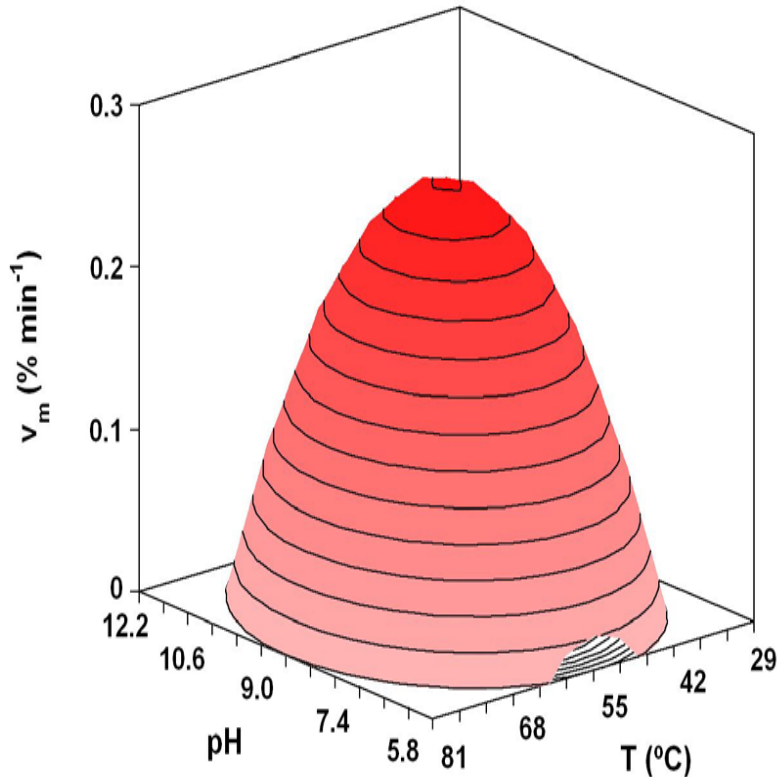
$$-3.72T_{\max} - 12.56\text{pH}_{\max} = 0$$

$$T_{opt} = 55^\circ\text{C}$$

$$\text{pH}_{opt} = 9.0$$

# ALCALASE HYDROLYSIS OPTIMIZATION

Maximum hydrolysis rate ( $v_m$ ) as response (dependent variable)



$$R_{adj}^2 = 0.776$$

$$v_m = 0.264 - 0.095T^2 - 0.140pH^2$$

$$\frac{\partial v_m}{\partial T} = -0.19T$$

$$-0.19T_{max} = 0$$

$$\frac{\partial v_m}{\partial pH} = -0.28T$$

$$-0.28pH_{max} = 0$$

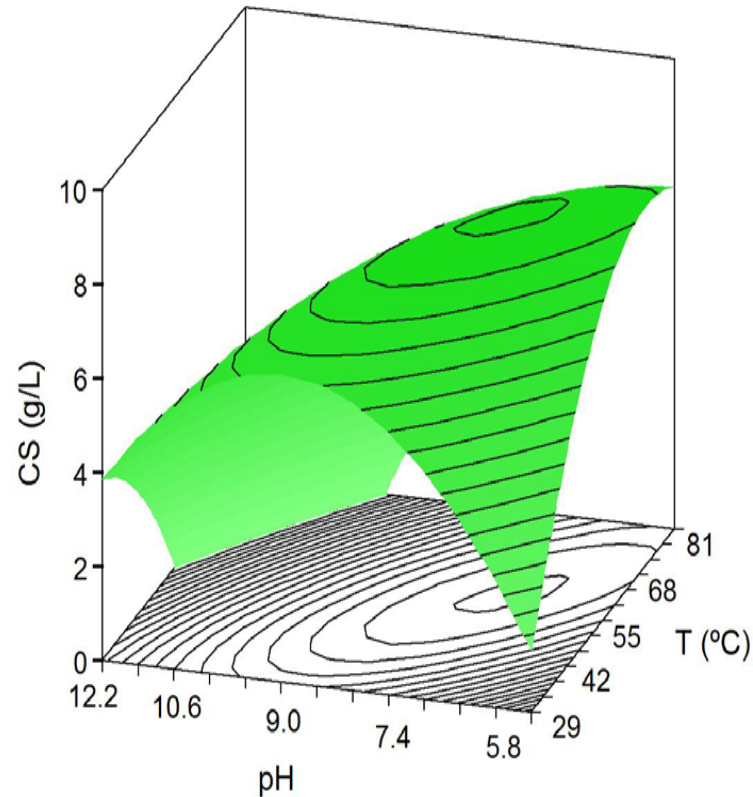
$$T_{opt} = 55^\circ\text{C}$$

$$pH_{opt} = 9.0$$

# ALCALASE HYDROLYSIS OPTIMIZATION

**Chondroitin sulphate concentration (CS)** as response: purified in pseudo-optimal conditions according to *Murado et al. (2009): Biochem. Eng. J. 49 (2010) 126–132*

$$CS = 7.34 - 1.07T - 2.21pH - 2.04TpH - 1.11T^2 - 1.72pH^2$$



$$R_{adj}^2 = 0.812$$

$$\frac{\partial CS}{\partial T} = -1.07 - 2.04pH - 2.22T \quad -1.07 - 2.04pH_m - 2.22T_m = 0$$

$$\frac{\partial CS}{\partial pH} = -2.21 - 2.04T - 3.44pH \quad -2.21 - 2.04T_m - 3.44pH_m = 0$$

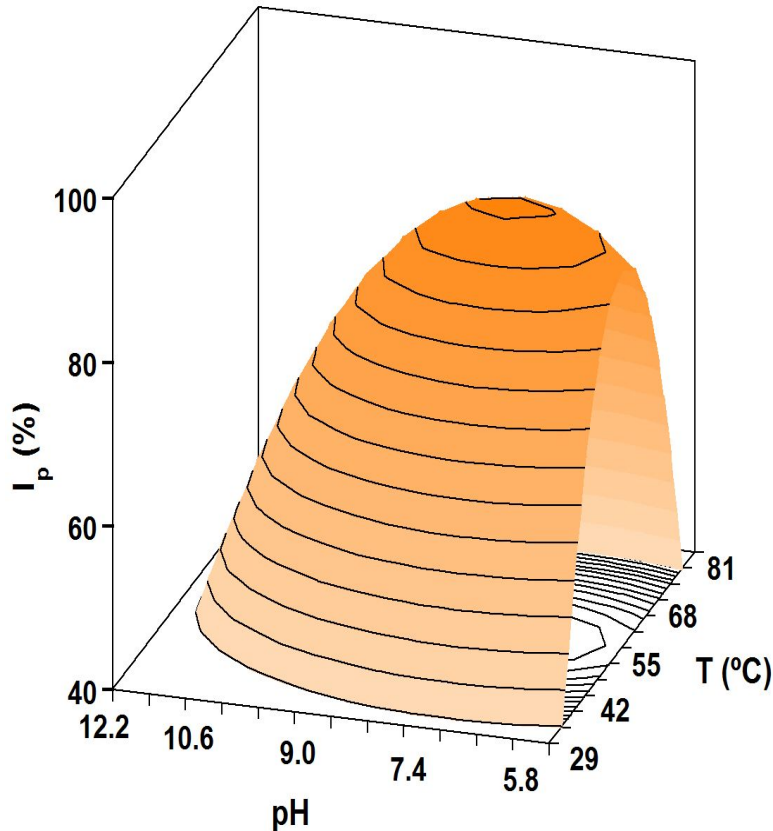


$$T_{opt} = 59.2^\circ\text{C}$$

$$pH_{opt} = 7.34$$

# ALCALASE HYDROLYSIS OPTIMIZATION

CS purity in relation to proteins ( $I_p$ ) as response



$$I_p = 86.87 - 17.38pH - 30.35T^2 - 11.84pH^2$$

$$\frac{\partial I_p}{\partial T} = -60.7T$$

$$-60.7T_m = 0$$

$$\frac{\partial I_p}{\partial pH} = -17.38 - 23.68pH$$

$$-17.38 - 23.68pH_m = 0$$

$$T_{opt} = 53.6^\circ\text{C}$$

$$pH_{opt} = 7.43$$

$$R_{adj}^2 = 0.939$$

# ALCALASE HYDROLYSIS OPTIMIZATION

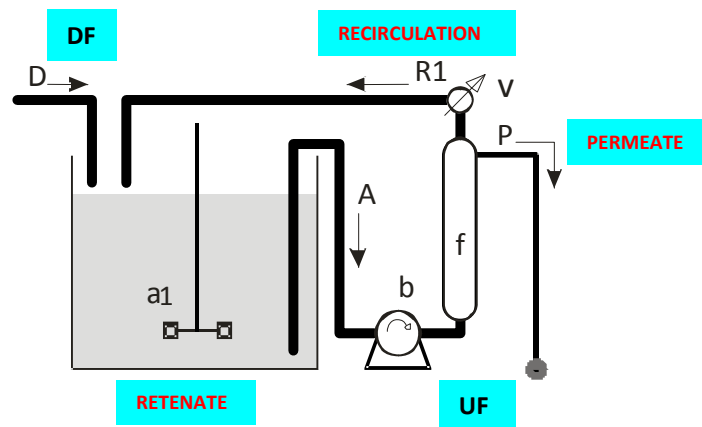
## OPTIMAL GLOBAL CONDITIONS (OGc)

RESPONSE (Y)	$T_{opt}$ (°C)	$pH_{opt}$	Maximum Response ( $Y_m$ )
$H_m$	55.0	9.0	13.0%
$v_m$	55.0	9.0	0.264 % min <sup>-1</sup>
CS	59.2	7.34	8.08 g/L
$I_p$	53.6	7.43	93.05%
<b>OGc</b>	<b>55.7</b>	<b>8.2</b>	<b>12.2% (<math>H_m</math>) 0.244 % min<sup>-1</sup> (<math>v_m</math>) 7.92 g/L (CS) 91.69% (<math>I_p</math>)</b>

# FUTURE DEVELOPMENTS

1: To optimize the best conditions for aqueous protein hydrolysis and chondroitin sulphate selective precipitation by alkaline-hydroalcoholic reaction.

2: To purify and maximize the chondroitin sulphate recovery by ultrafiltration and diafiltration performance at different cut-offs (30, 100 kDa...).



3: Physicochemical characterization: molecular weight, type of sulphation and pattern of sulphation.

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**THANK YOU  
FOR YOUR ATTENTION**

**chondroitin sulphate**