SINDUSFARMA

LaBioTec
Laboratório de Biotecnologia Farmacêutica FCF/USP

28/03/18 - Conferência: BIOFÁRMACOS

Da bioprospecção de moléculas produzidas por micro-organismos extremófilos às estratégias para o desenvolvimento de *biobetters*.

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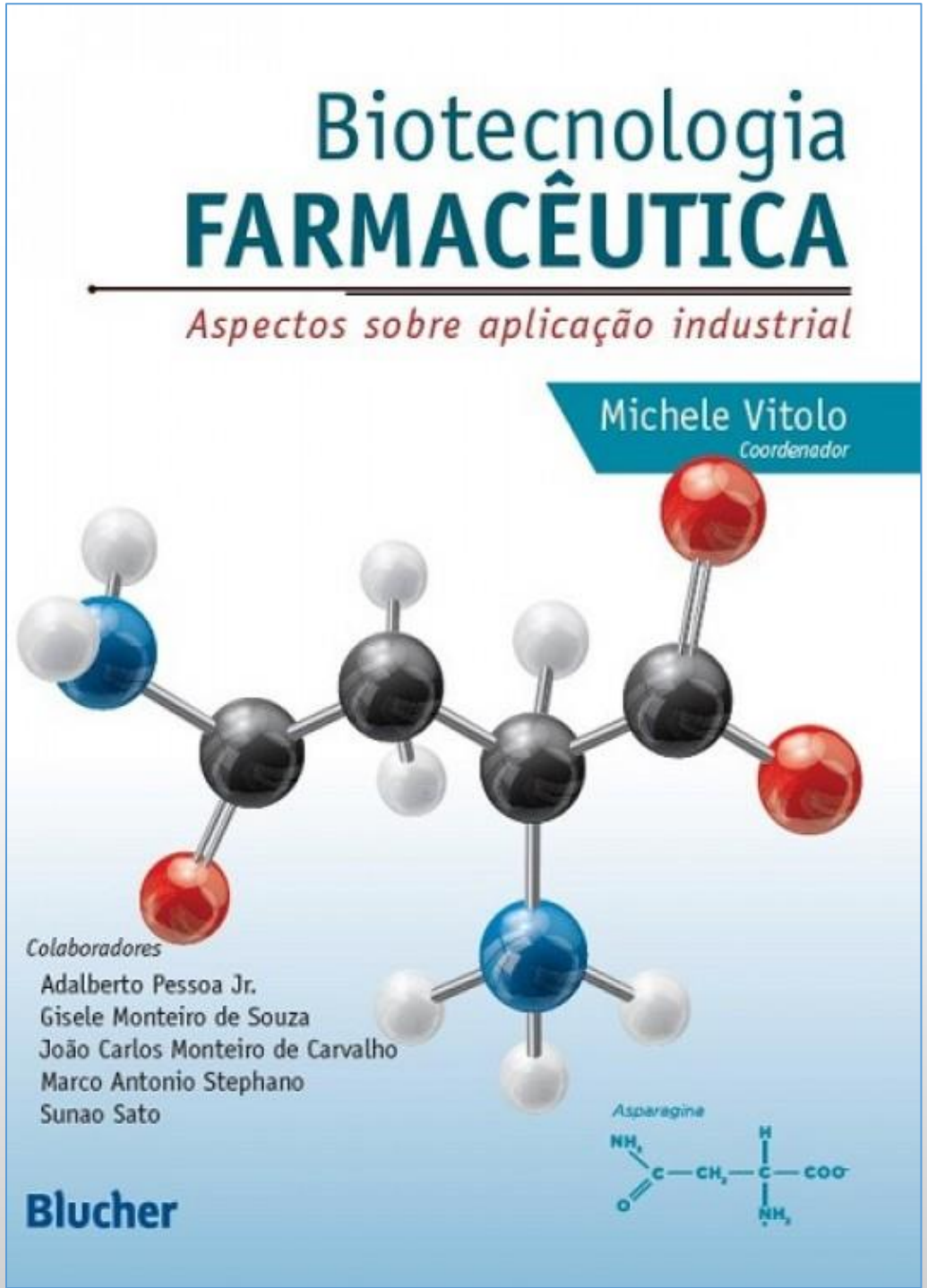
FCF USP 120 1898 • 2018



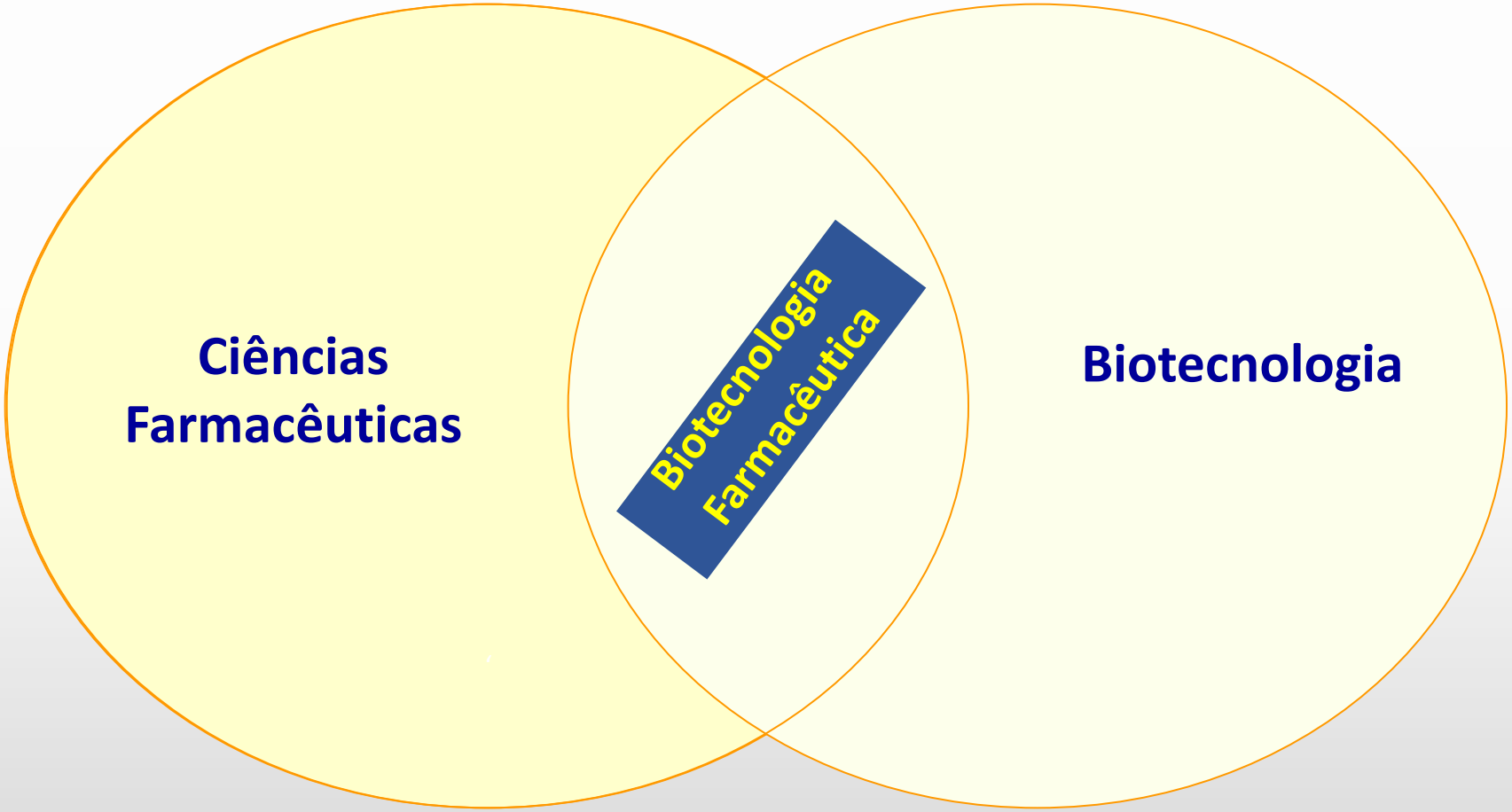
Biotecnologia Farmacêutica Aspectos sobre Aplicação Industrial

Michele Vitolo

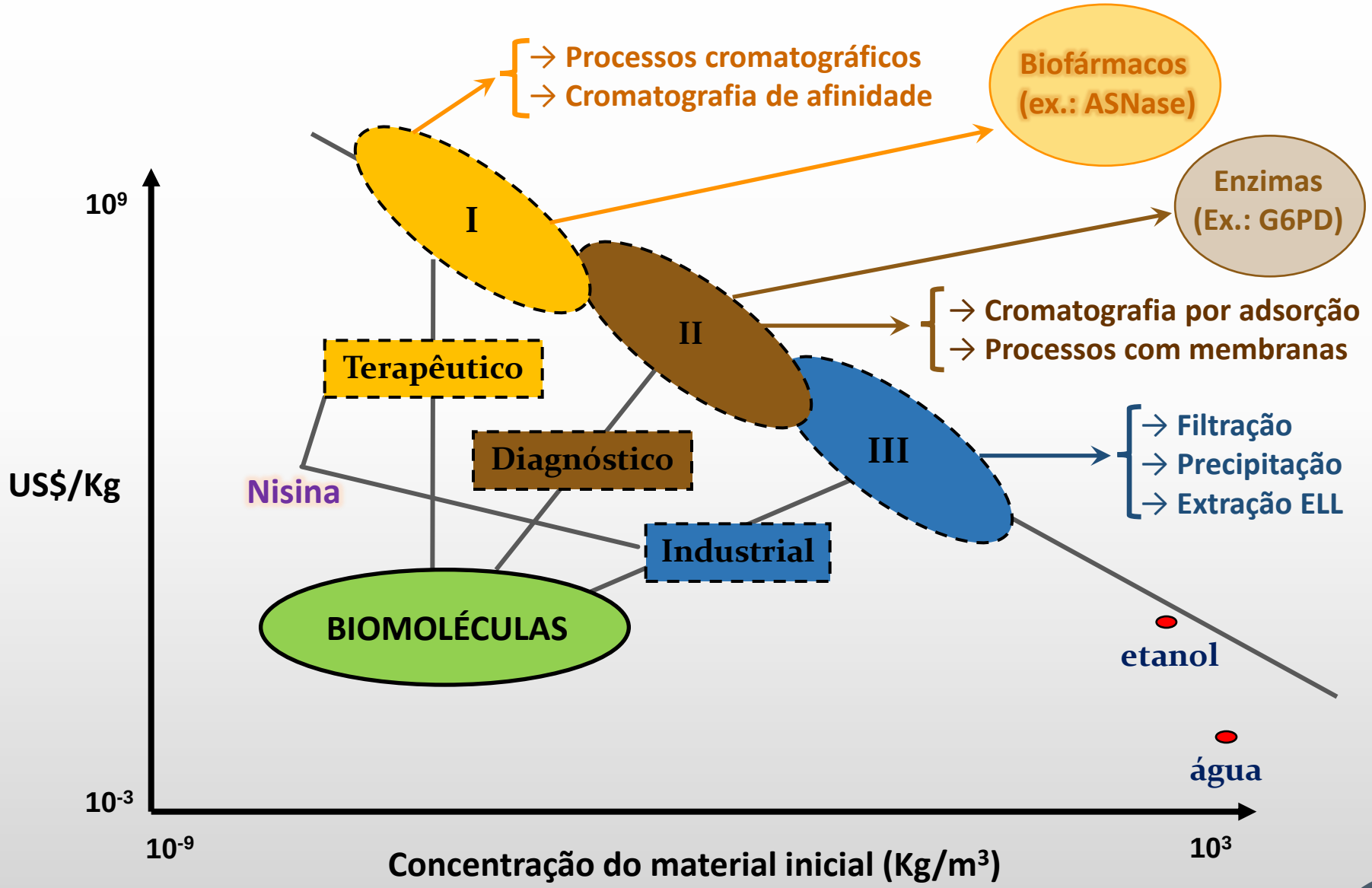
2014 — 1ª edição

http://www.blucher.com.br/livro/detalhes/biotecnologia-farmaceutica-175

CIÊNCIAS FARMACÊUTICAS E BIOTECNOLOGIA



Preços de bioprodutos em função de sua concentração no meio inicial



The global market for bioproducts should reach $714.6 billion by 2021.

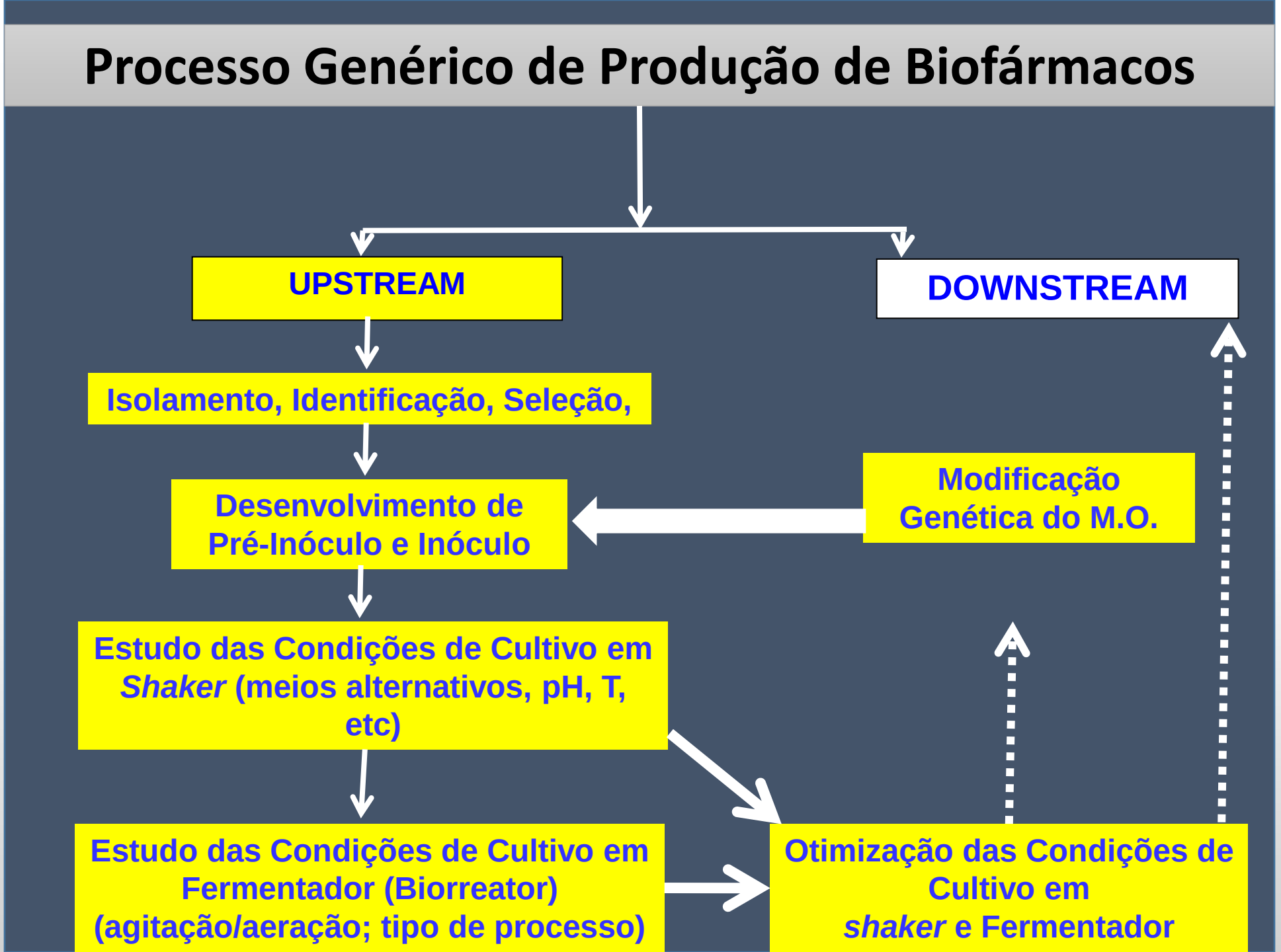
SUMMARY FIGURE GLOBAL MARKET FOR BIOPRODUCTS BY CATEGORY, 2013-2021 ($ BILLIONS *Estimated values*)

Bioproduct	Value (US$)
Antibiotics	10 billions/year
Monoclonal antibodies	10 billions
DNA Probes	1 billion
Insulin	700 millions
Somatostatine	100 millions/year
Transgenic hemoglobin	10 billions

DESENVOLVIMENTO DE BIOPROCESSOS MICROBIANOS

- Possibilidade de otimização (em até milhares de vezes) da produção.
- Obtenção de biomoléculas “extremofílicas”.
- Cultivos microbianos são mais rápidos que “plantas” e “animais”.
- Processo simples de seleção de microrganismos.
- Capaz de crescer e originar o produto em culturas de larga escala
- Possibilita uso de inúmeras fontes de carbono e nitrogênio
- Relação área/volume favorável ⇒ rápida absorção de nutrientes, alta velocidade de síntese e metabolismo
- Diversidade metabólica
- Adaptabilidade a distintos ambientes e condições de crescimento
- Capacidade de sintetizar enantiômeros específicos
- Tecnologias *“limpas”*

Processo Genérico de Produção de Biofármacos



Antártica – Ambiente Extremo
Pode ser fonte de Biofármacos?

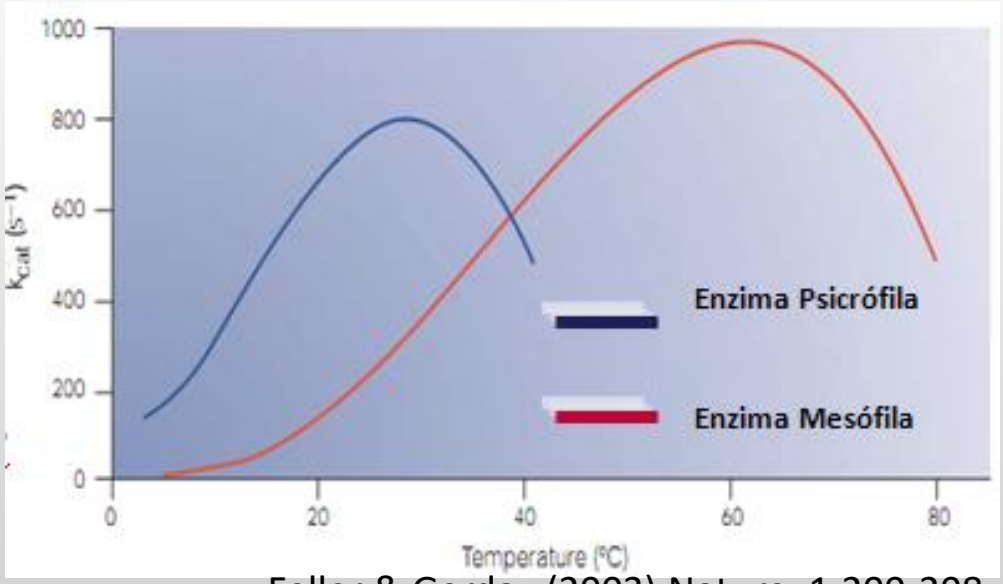
PROTEASES

ASPARAGINASES

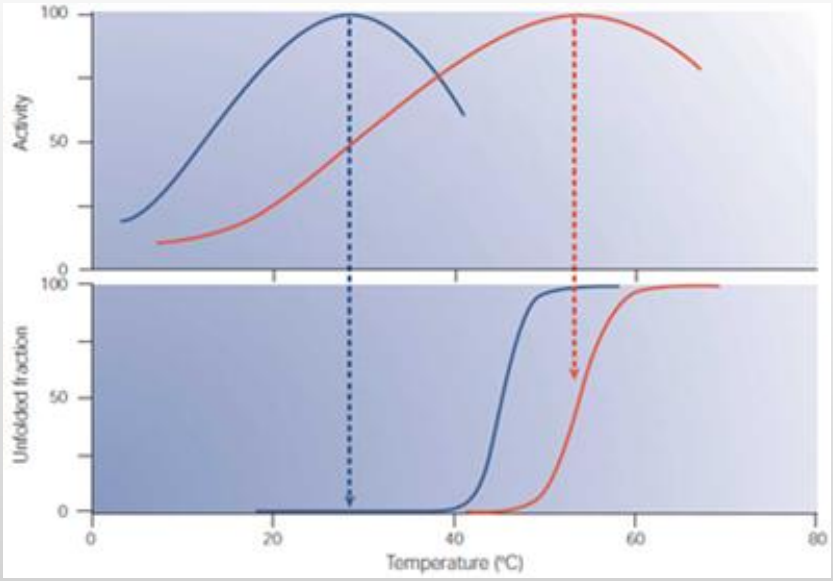
Enzimas adaptadas ao frio

- **Podem catalisar reações a temperaturas baixas e moderadas (< 40ºC) de forma mais eficiente com poucas reações químicas indesejáveis que ocorrem a altas temperaturas.**
- **Maior Resistência a solventes orgânicos e detergentes.**
- **Redução do gasto energético associados às etapas de aquecimento no processo produtivo.**
- **Maior estabilidade a estresses ambientais.**

São até 10 vezes mais ativas a temperaturas baixas/moderadas do que as homólogas mesofílicas



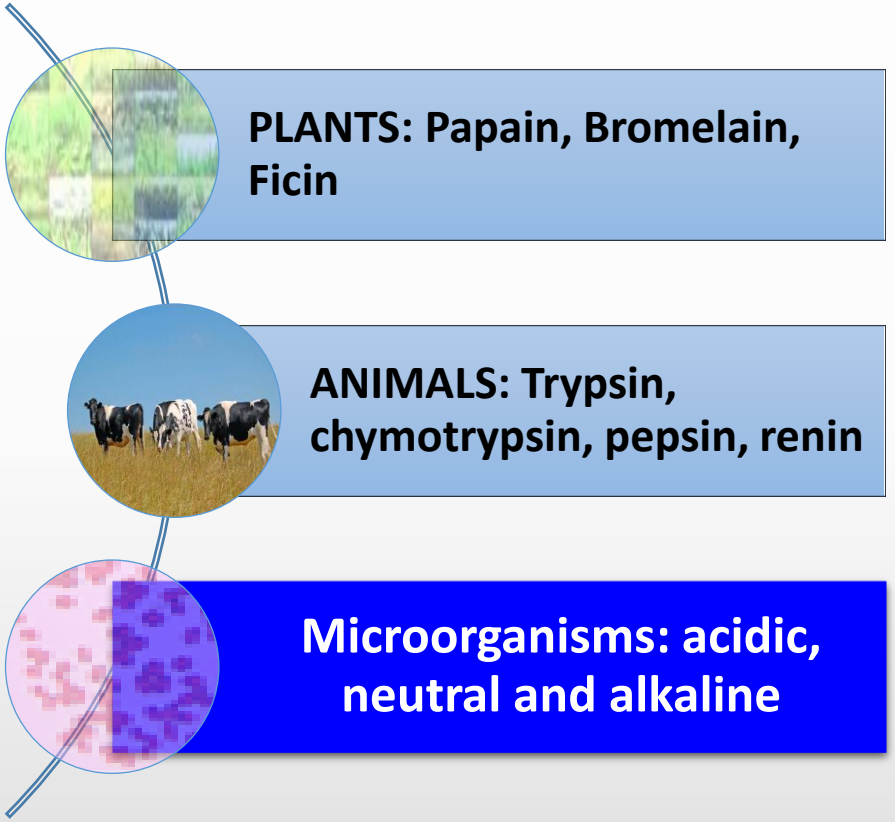
São inativadas a temperaturas maiores do que as ótimas para catálise



Feller & Gerday (2003) Nature, 1:200-208

Extremophile Producers of Biopharmaceuticals

PROTEASES - PROPERTIES E APPLICATIONS

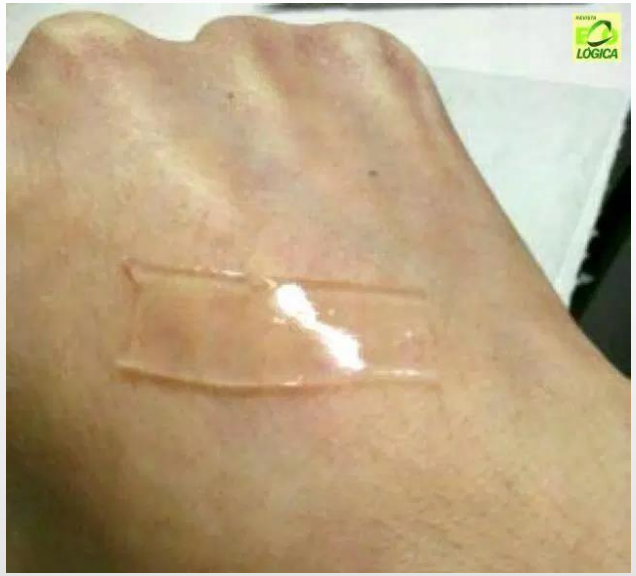


- INDUSTRY OF DETERGENTS
- FOOD INDUSTRY
- **PHARMACEUTICAL INDUSTRY**
- FINE CHEMISTRY
- WASTE TREATMENT
- LEATHER PROCESSING
- **COSMETICS**

PROTEASES

digestive aid

PAPAIN gel (2%)
Healing - debriding - antibacterial and anti-inflammatory



ISOLATION OF FUNGI FROM ANTARCTIC CONTINENT

SEA SPONGE

STAR SEA

SEAWEED

ASCIDIA

SNAIL

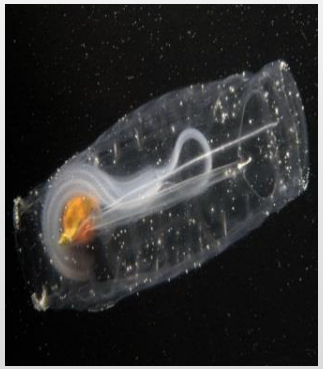
SOIL
PENGUINS

LICHEN

SEA URCHIN

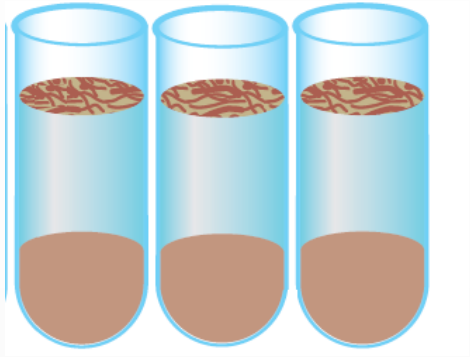
NACELLA

SALPA

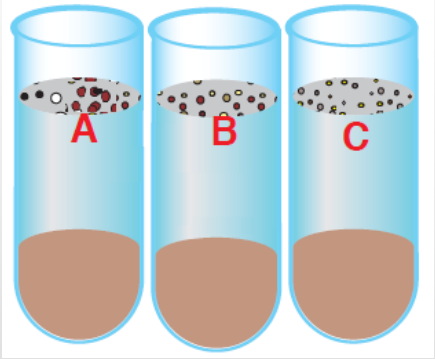


ISOPODE

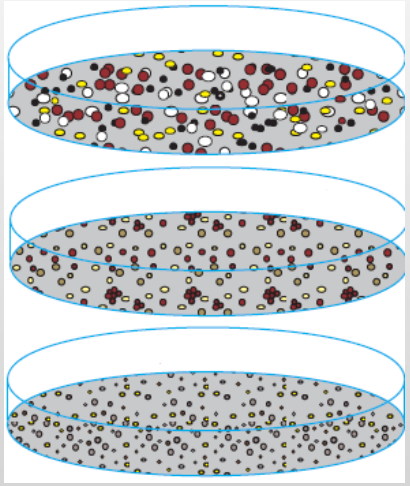
Fungi Isolation - Methodology



Samples collected in sterile saline



Serial dilution in steril saline



Isolation by direct plating and serial dilution.

Antibiotic + medium

Isolation of producers of Proteases: yeasts

Sterilized Agar

Skimmed milk 10%

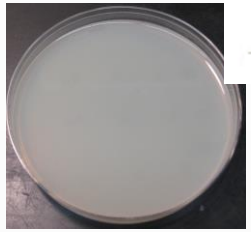
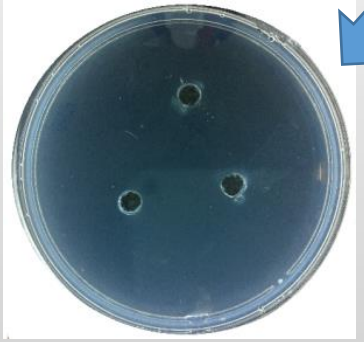


Plate of agar-milk

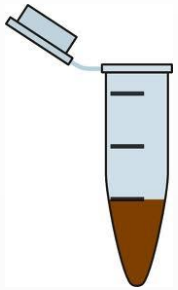
100 µL

inoculum



Diameters of the halos on the plates after 24, 48 and 72 hours of cultivation

YEAST SUBMERGED CULTIVATION

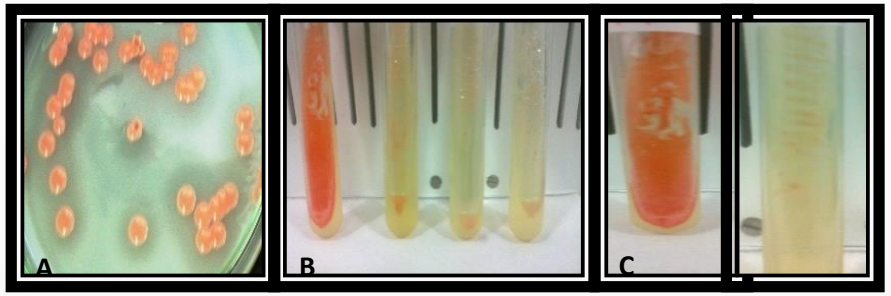


Isolated Yeast:
Stock culture -
Glycerol at -80°C

Medium PDA
(activation)

7 days
28°C

Yeast growth



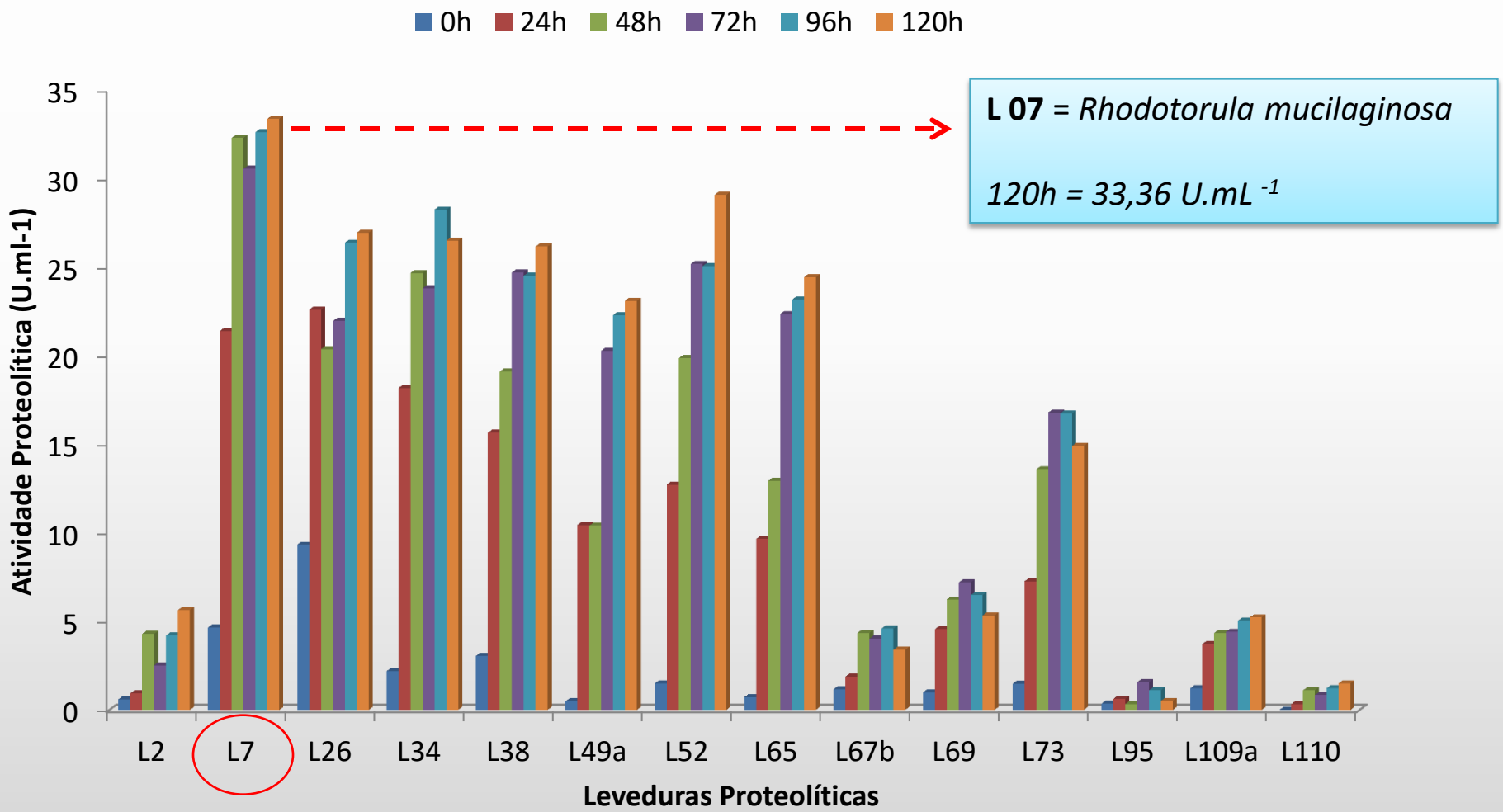
Cell suspension
in 0,9% NaCl

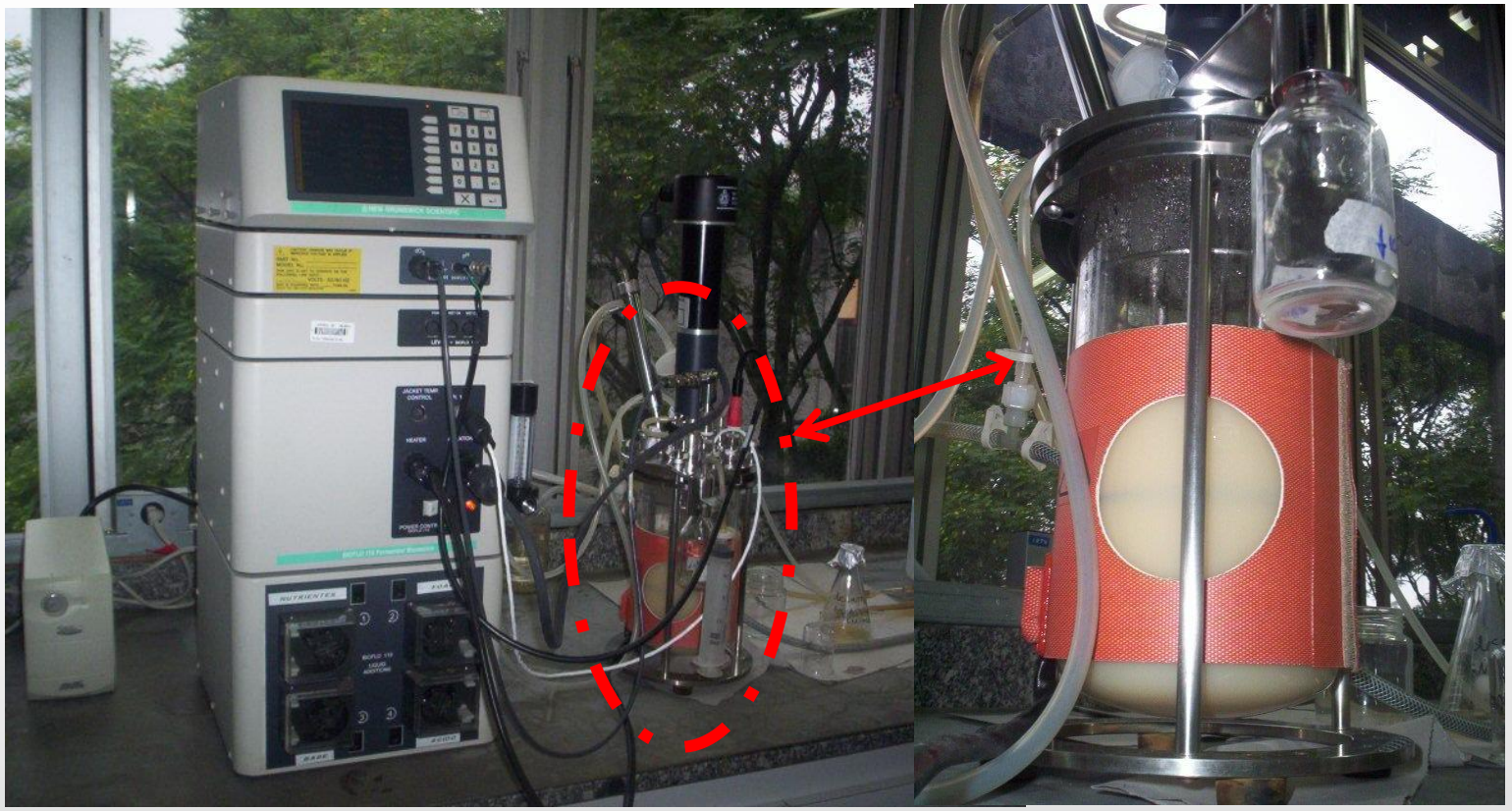
Cultivation:
3% Sabouraud medium
2% pepton
1% skimmed milk

120 h
28°C

Protease
Activity
Measurement

CULTIVATION OF THE ISOLATED YEASTS – THE POSITIVE-PROTEASES





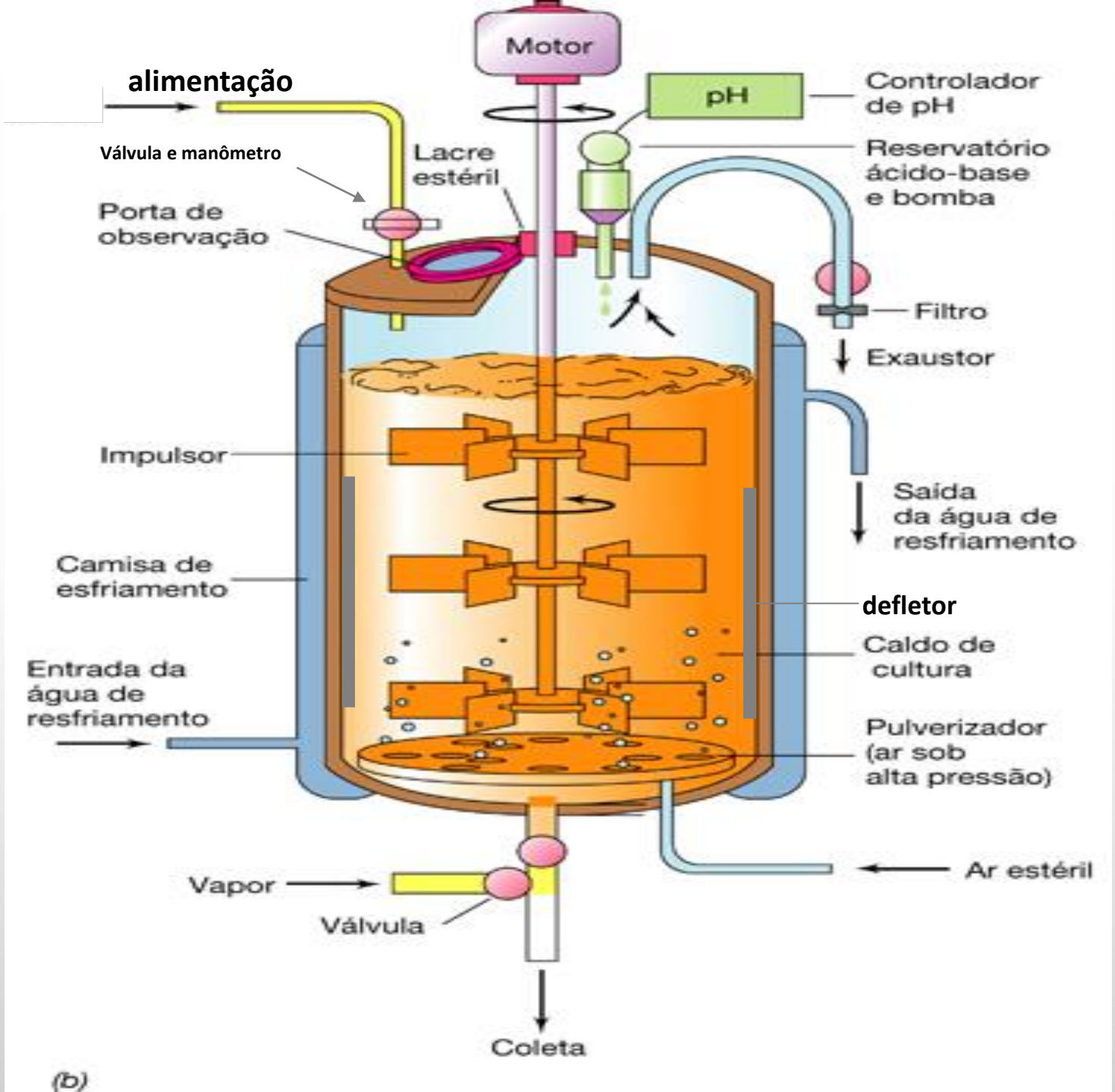


Diagrama de um fermentador, ilustrando a construção e os dispositivos para a aeração e controle do processo.

PROTEASE PRODUCTION BY RHODOTORULA MUCILAGINOSA

Inoculum

Supernatant: proteases

Pellet: cells

New possibilities

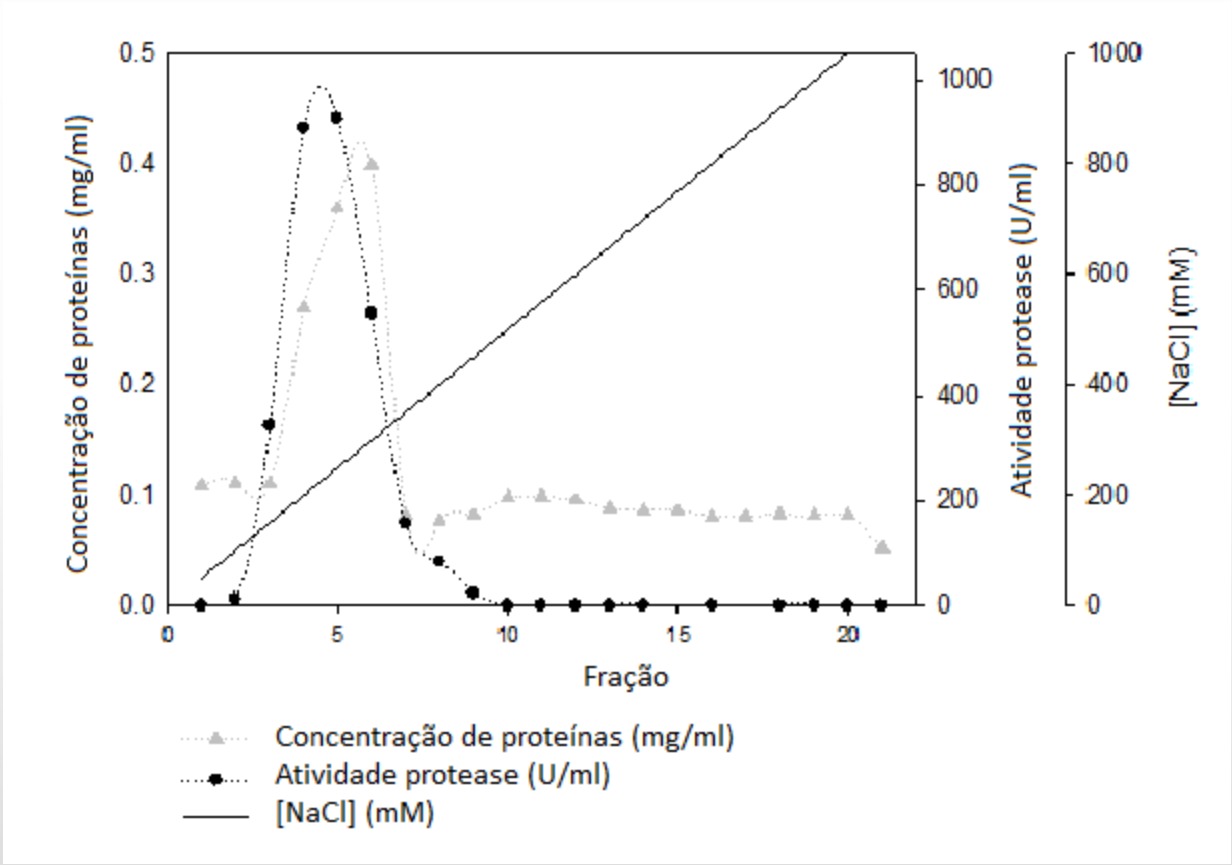
carotenoids

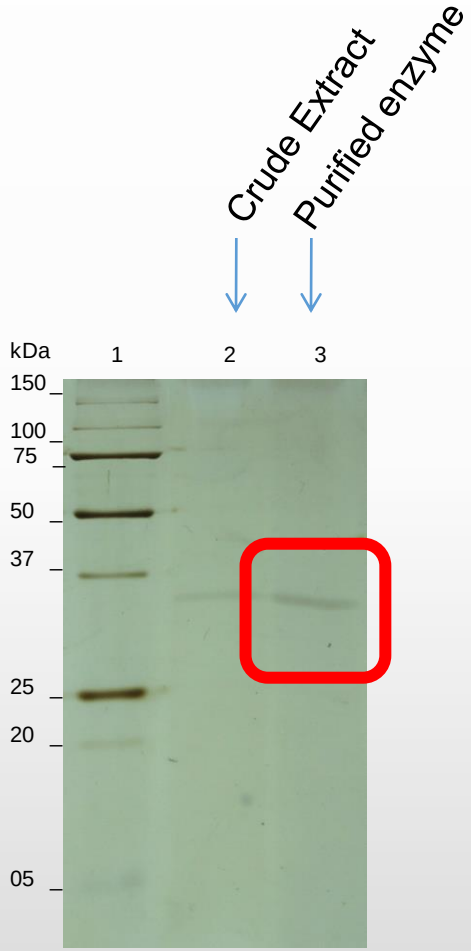
lipids

***Rhodotorula* can store more than 50% in lipids**

PROTEASE PURIFICATION - *Rhodotorula mucilaginosa L07*

Ionic Exchange: CM-Sepharose





Purification Yield: 48.5%
Purification Factor: 12.9 times

RELEVANT COMMENTS

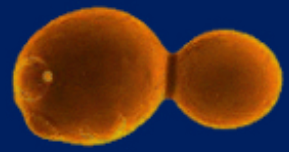
- **14,14% of the isolated yeasts from Antarctica were protease-positive**
- Protease from *R. mucilaginosa* L07 – acidic characteristic – **medical potential (as debriding, antibacterial and anti-inflammatory actions) and cosmetic potential (skin lighter and peeling)**.
- Highly stable**.**

L-Asparaginase

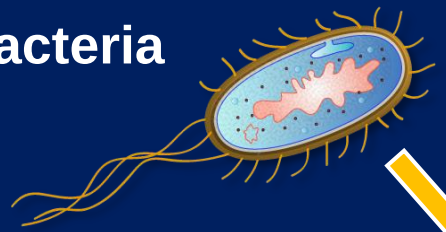
Biofármaco empregado no tratamento de Leucemia Linfoide Aguda - LLA

Source of L-asparaginase

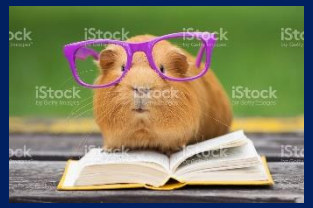
Yeasts



Bacteria

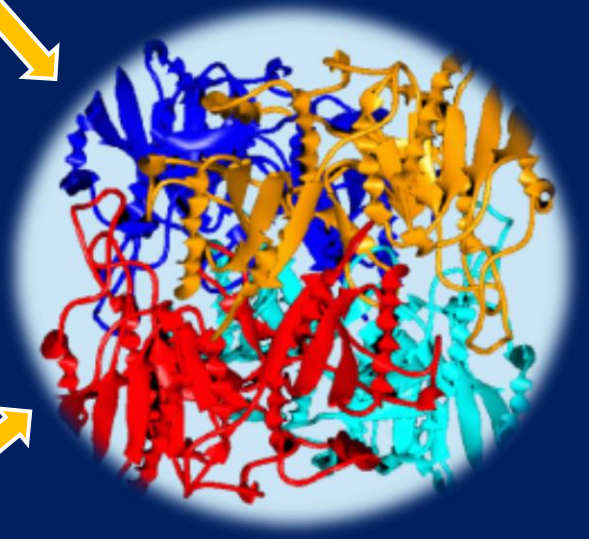


Animals



Plants

Filamentous fungi



L-ASPase

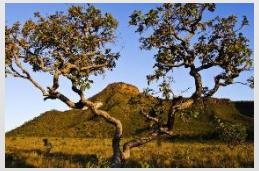
Where are we looking for new Asparaginases?

Antarctic Continent (Extremophile Environment - cold and dry)

Atlantic Ocean - North coast of the State of São Paulo (Extremophile environment - high pressure and saline concentration)

Coconut plantation - State of Paraíba - (dry environment - Biome little explored)

Caatinga - State of Pernambuco - (dry environment - Bioma little explored)

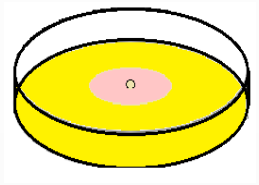


“Cerrado” - (dry environment - little explored biome)

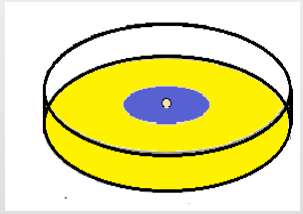
Results: we found hundreds of fungi producing L-asparaginase, but to date none of them presented characteristics suitable for a biopharmaceutical

Initial identification of fungi producers of L-asparaginase

Initial Screening – two diferente methodologies



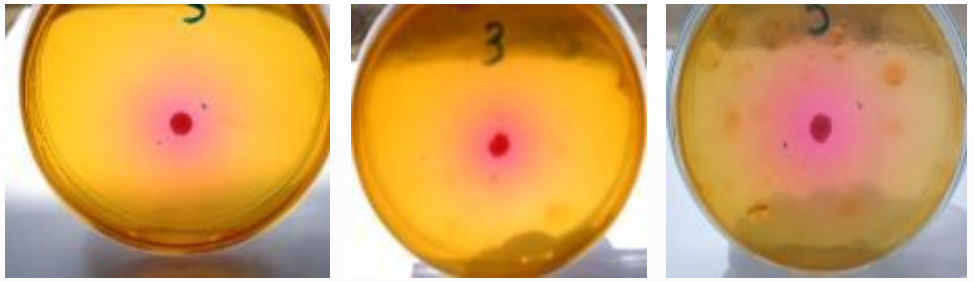
Red phenol		
pH below 6.6		*pH above 8,0*
Yellow	⇄	**red**



Bromothymol blue		
pH below 6.6		*pH above 7,6*
Yellow	⇄	**blue**

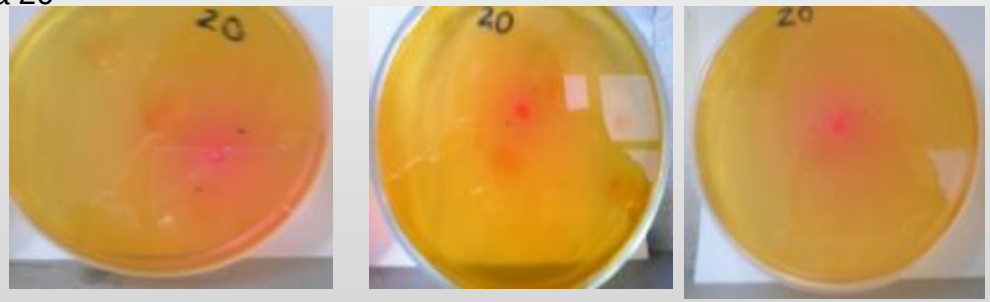
Examples: Plates with phenol red

Cepa 3

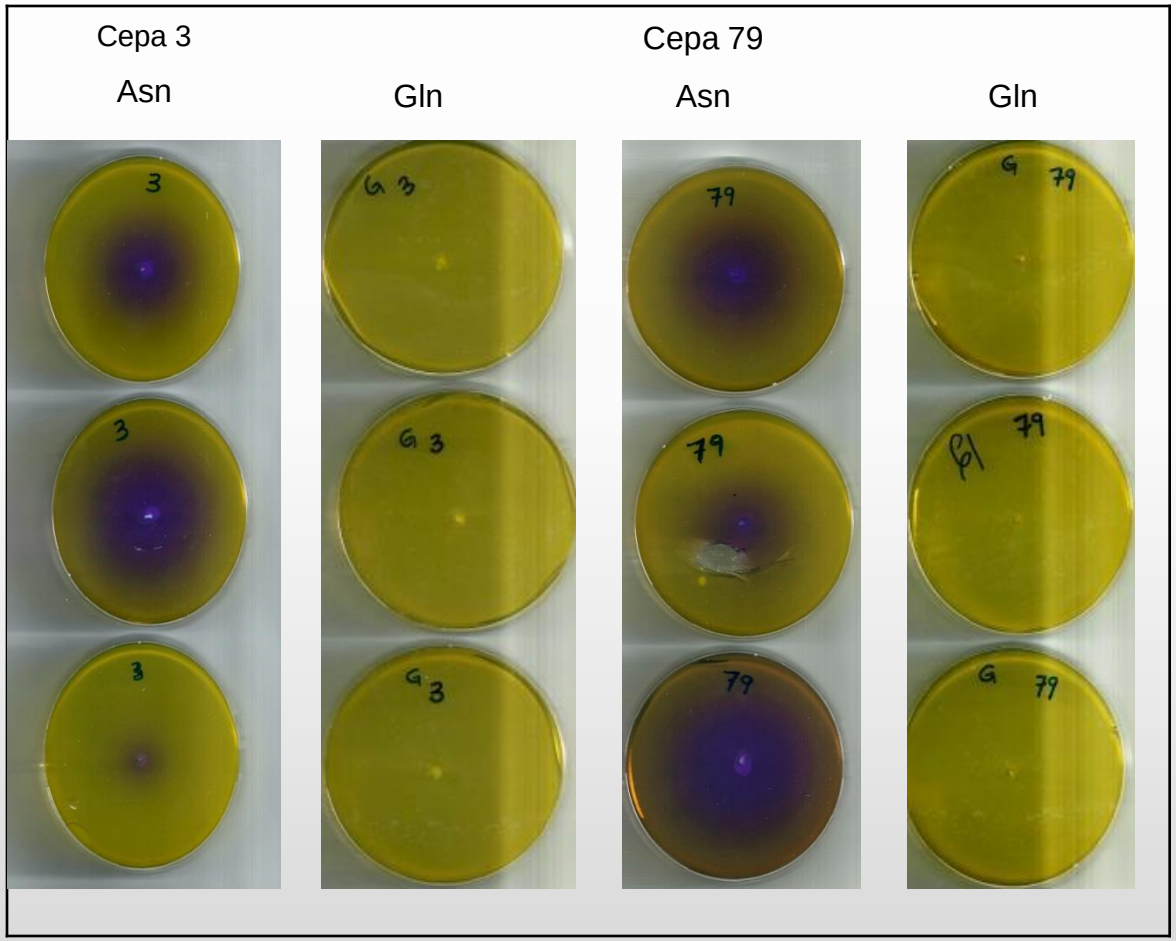


Cepa 79

Cepa 20



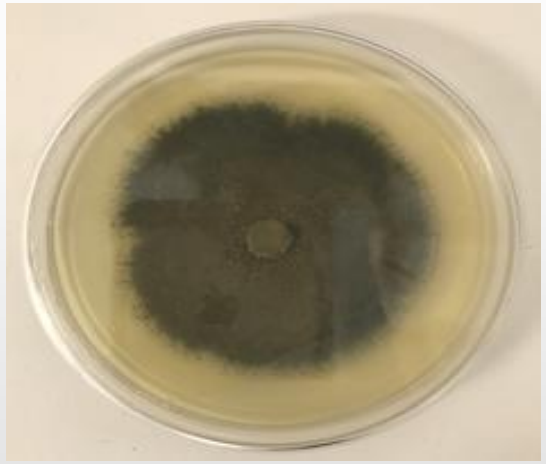
Examples: Plates with bromothymol blue



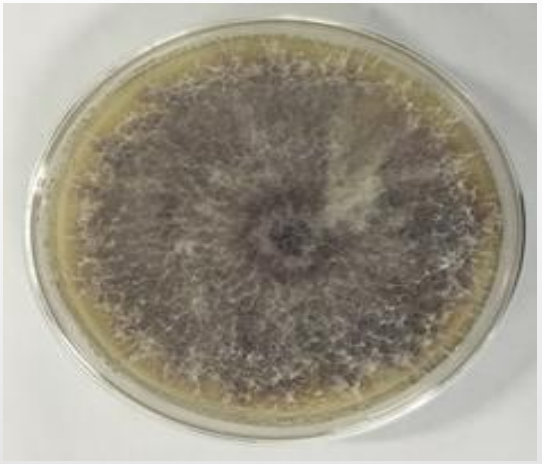
L-ASPARAGINASE PRODUCTION BY FILAMENTOUS FUNGI ISOLATED FROM THE BRAZILIAN BIOME (CERRADO)

FUNGI SELECTED AS L-ASPARAGINASE WITH LOW GLUTAMINASE ACTIVITY

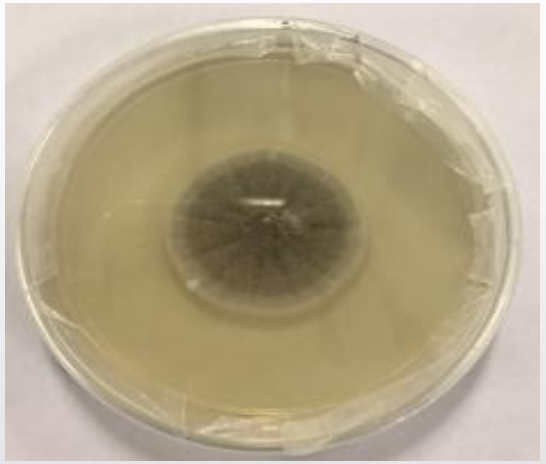
Penicillium sizovae **(2DSST1)**



Fusarium proliferatum **(DCFS10)**



Penicillium decumbens **(DCFS6)**



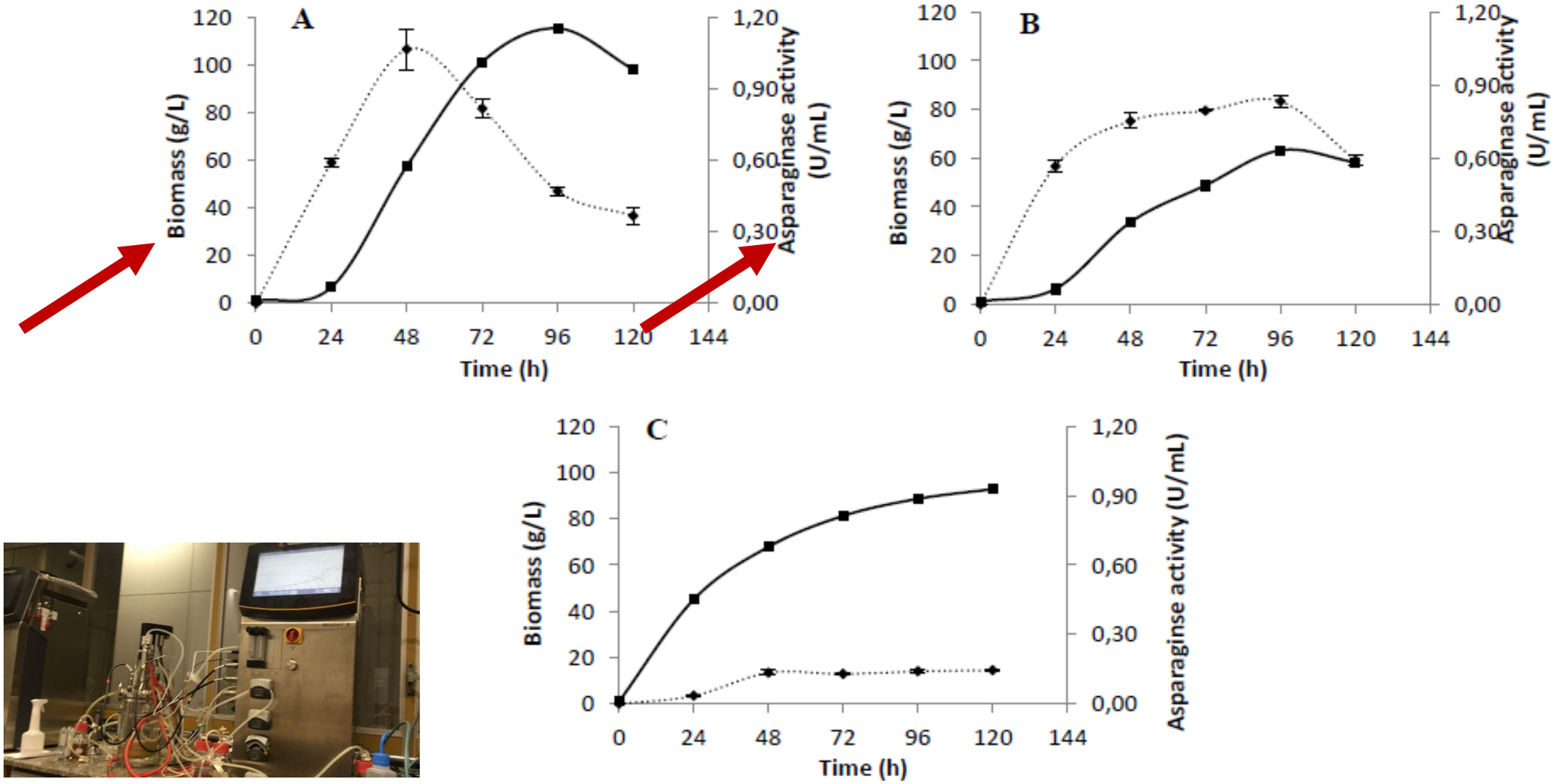
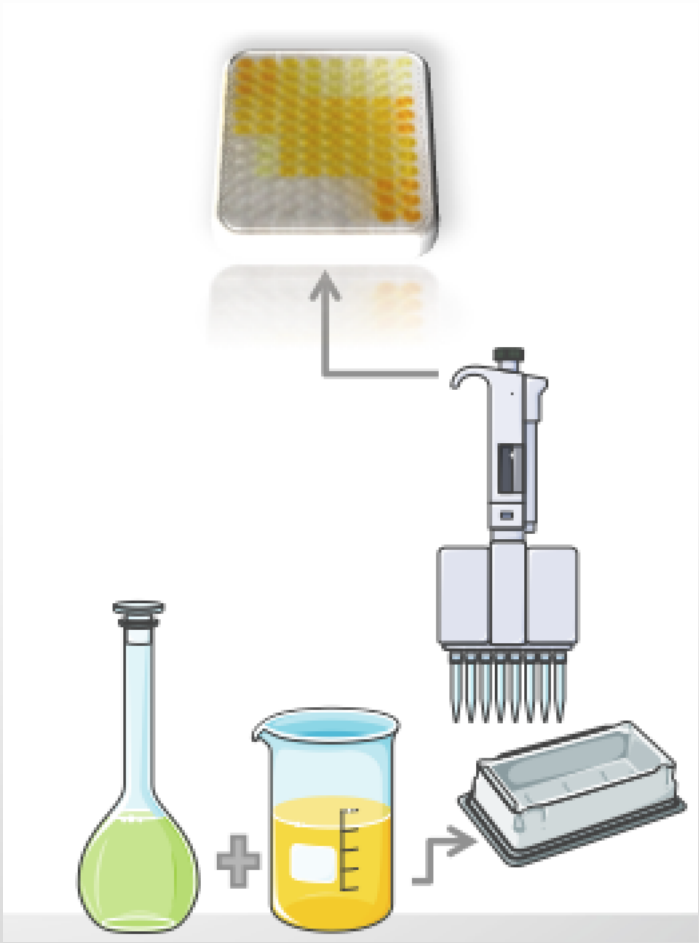


*Figure 1. Growth curves and L-asparaginase production of fungi 2DSST1 (**A**), DCFS10 (**B**) and DCFS6 (**C**).Line (—) biomass concentration and line (…) L-asparaginase activity.*

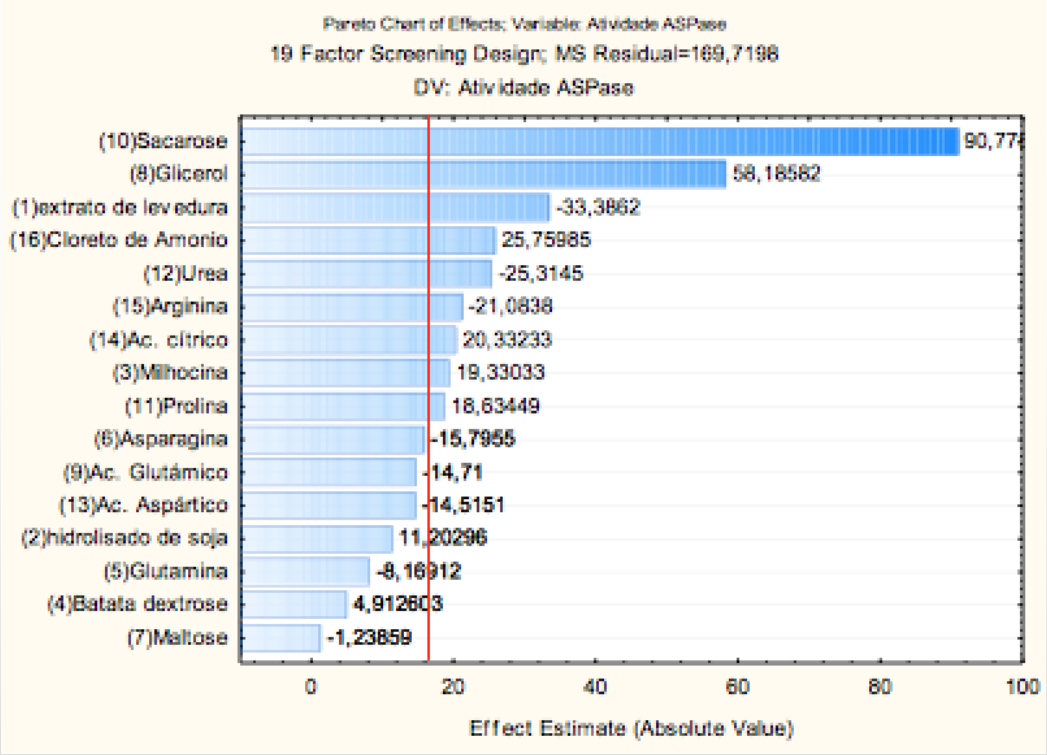
Caracterização de enzimas de interesse industrial

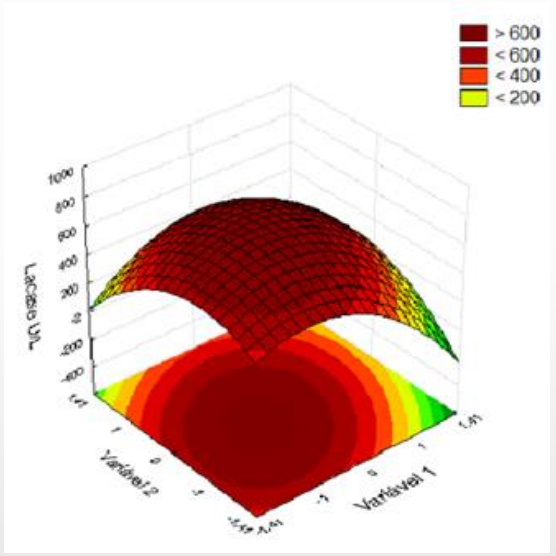
- Avaliação das Atividades enzimáticas
- Avaliação de Inibidores Enzimáticos
- Estabilidade ao pH e temperatura
- Temperatura e pH ótimos
- Efeito de Sais e de Surfactantes
- Massa Molecular e Ponto Isoelétrico



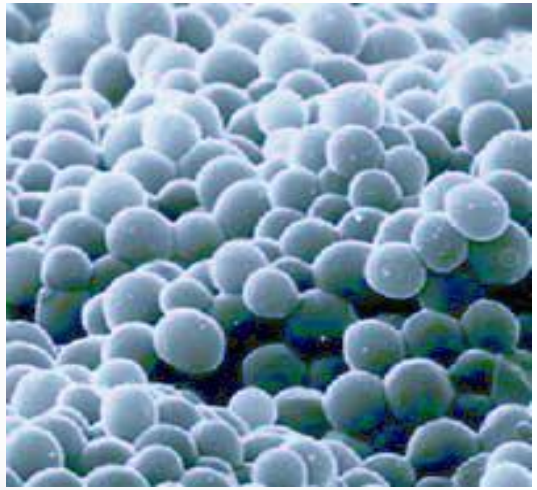
Avaliação de diferentes fatores na produção de enzimas de interesse industrial e/ou ambiental

Desenho experimental





L-Asparaginase from Antarctica



Search for extracellular L-ASNase with high specificity for L-asparagine and low activity of L-glutamine from Antarctic Continent can generate a new enzyme with higher stability for the production of a new biopharmaceutical.

Asparaginase

Glutaminase

Rhodotorula mucilaginosa

Leucosporidium scottii

U/ mg of cells

0
0.2
0.4
0.6
0.8
1

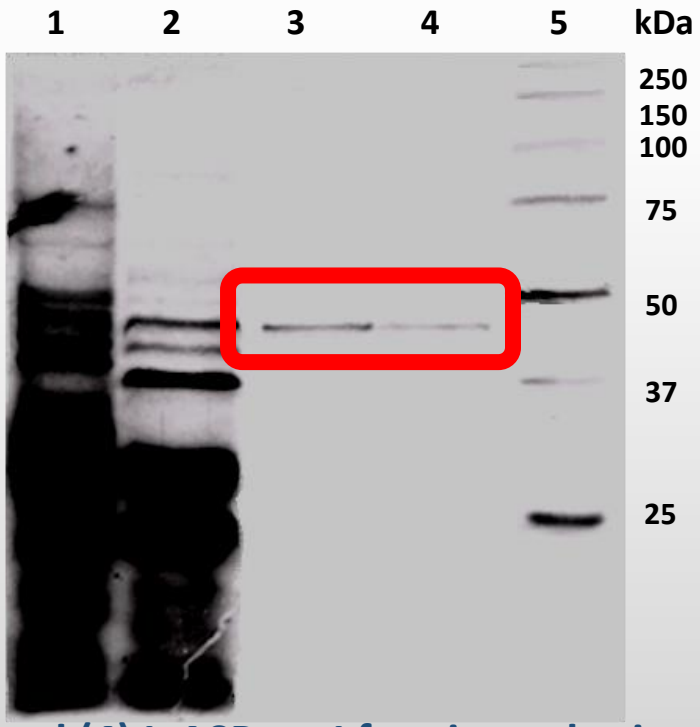
3 4b 5 6 7 9 11 13 13' 17 18 19 20 21 26 32 35 36 37 38 42 43 55 58 79 81 82 83 84 88 92 94 97 114 115 117 118 122 123 124

Numbers – represent the identification of the evaluated yeasts

Purification factor: 500 fold
Specific activity: 15.02 U/mg$_{prot}$
Decamer: 440 kDa
Allosteric regulation
k_M = 207 µM
k_{cat} = 1.53 s^{-1}

Antarctic

SDS-PAGE

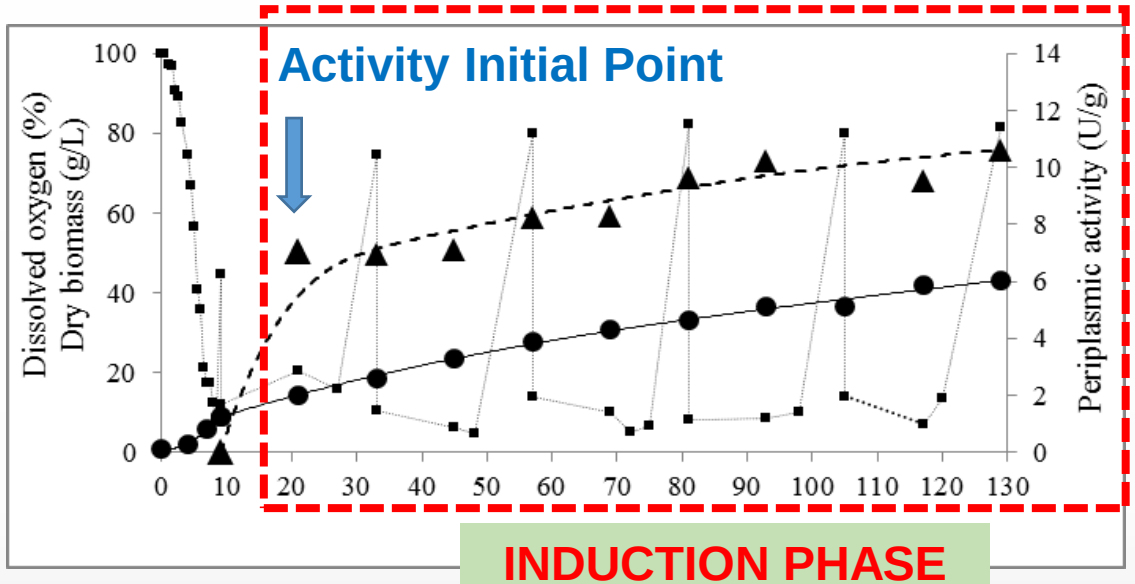


(3) and (4) L-ASPase I fractions obtained by Size exclusion chromatography.

CULTIVO EM BIORREATOR DE BANCADA

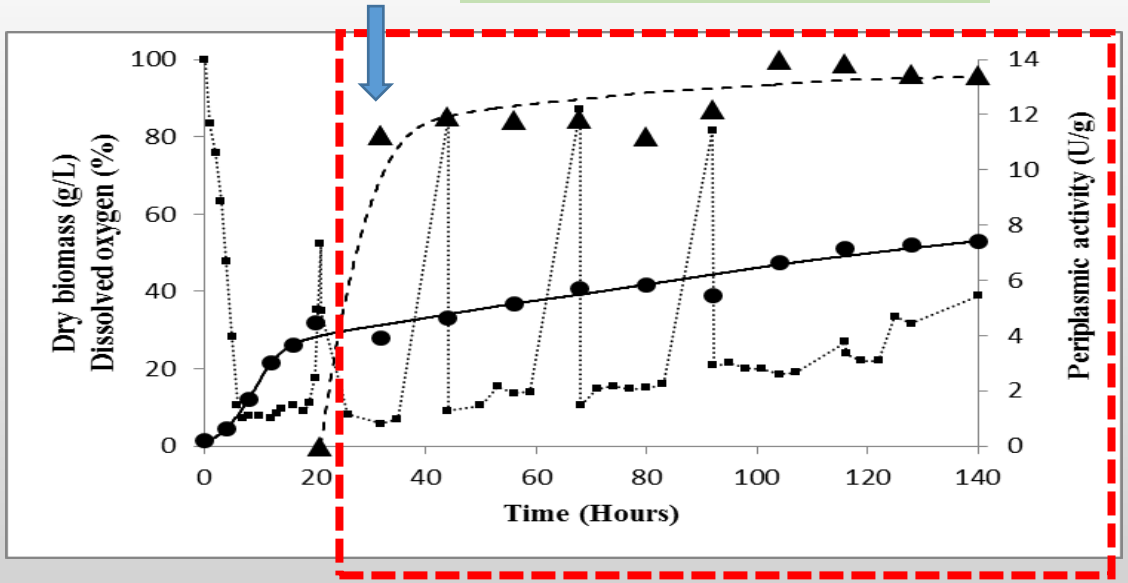
Avaliar parâmetros importantes para o escalonamento do processo

3L BIORREACTOR PRODUCTION



BATCH 1: Growth phase with Glycerol 10 g/L, 30 °C.

INDUCTION PHASE

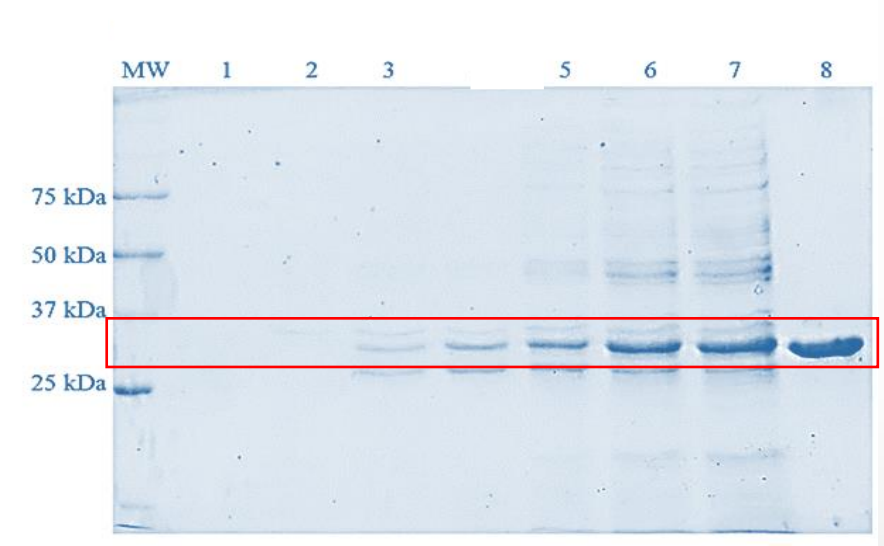


Variation of cell concentration (●), dissolved oxygen (■) and periplasmic ASNase activity (▲) against time for **recombinant *P. pastoris*** grown in 500 rpm and 1 vvm, inducing phase: 3% (v/v) of methanol, 20 °C.

BATCH 2: Growth phase with Glycerol 40 g/L, 30°C.

Asparaginase from recombinant *E. coli*

Extracellular ASNase secretion – new method



SDS-PAGE – extracellular extract from *E. coli* BL21 with addition of glycine and n-dodecane

Cuiltivation time: 0 h; (2) 4 h; (3) 8 h; (4) 12 h; (5) 16 h; (6) 20 h; (7) 24 h. (8) 1 mg/mL of commercial ASNase.

Patent Application

INPI INSTITUTO NACIONAL DA PROPRIEDADE INDUSTRIAL

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Anotação de transferência de titular

Número do Processo: BR 10 2016 024702 0

Dados do Interessado

Interessado 1 de 2

Nome ou Razão Social: UNIVERSIDADE DE SÃO PAULO - USP

Low Molecular Mass ASNase from *E. coli*

sob nº 63.025.530/0001-04, titular do pedido de patente sob o título: "**Método de obtenção de L-Asparaginase recombinante de baixa massa molar, L-Asparaginase recombinante e uso da mesma**", identificado pelo nº **BR 10 2016 024702-0**, depositado em **21/10/2016**, neste ato, com fulcro no inciso I do artigo 6º da Portaria GR nº 6.651, de

AUSPIN Agência USP de Inovação

Comunicação de Criação

ID:	CC-PI-2017-0034
Título Provisório:	NOVA ENZIMA L-ASPARAGINASE DE BACTERIA DICKEYA CHRYSANTHEMI, PRODUZIDA EM SISTEMA DE EXPRESSÃO EUCARIOTO
Modalidade de Proteção:	Patente
Houve divulgação do conteúdo da criação?	Não houve divulgação do conteúdo total ou parcial dessa criação. [ ]
Existe ex criação?	
A pesquisa foi desenvolvida em cooperação com instituições externas?	[Anexo 2 - Quadro Resumo]

ASNase from *Pichia pastoris* with human glycosylation pattern

OBRIGADO !!!

FAPESP – Thematic Project 2013/08617-7

CAPES – Scholarship of some students

Adalberto Pessoa Junior – pessoajr@usp.br

Perguntas???

Preguntas???

Questions???