UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
PROGRAMA DE PÓS GRADUAÇÃO EM CIÊNCIA E TECNOLOGIA DE ALIMENTOS
INSTITUTO DE CIÊNCIA E TECNOLOGIA DE ALIMENTOS

OBTENÇÃO E AVALIAÇÃO DE LINHAGENS HÍBRIDAS E DESENVOLVIMENTO DOS PROCESSOS DE INÓCULOS LÍQUIDOS PARA CULTIVO AXÊNICO DE *Lentinula edodes* (BERK.) Pegler

Diego Melo Pereira

Porto Alegre

2015

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
PROGRAMA DE PÓS GRADUAÇÃO EM CIÊNCIA E TECNOLOGIA DE ALIMENTOS
INSTITUTO DE CIÊNCIA E TECNOLOGIA DE ALIMENTOS

OBTENÇÃO E AVALIAÇÃO DE LINHAGENS HÍBRIDAS E DESENVOLVIMENTO DOS PROCESSOS DE INÓCULOS LÍQUIDOS PARA CULTIVO AXÊNICO DE *Lentinula edodes* (BERK.) Pegler

Diego Melo Pereira

Dissertação apresentada ao Programa de Pós Graduação em Ciência e Tecnologia de Alimentos como requisito parcial para obtenção do grau de Mestre em Ciência e Tecnologia de Alimentos

Orientador: Prof. PhD Marco Antônio Záchia Ayub

Porto Alegre

2015

Ficha Catalográfica

Pereira, Diego Melo
Obtenção e avaliação de linhagens híbridas e desenvolvimento dos processos de inóculos líquidos para cultivo axênico de lentinula edodes (berk.) pegler / Diego Melo Pereira. -- 2015.
82 f.

Orientador: Marco Antônio Záchia Ayub.

Dissertação (Mestrado) -- Universidade Federal do Rio Grande do Sul, Instituto de Ciência e Tecnologia de Alimentos, Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos, Porto Alegre, BR-RS, 2015.

1. Lentinula edodes. 2. shiitake. 3. biorreator airlift. 4. enzima extracelular. 5. basidiomiceto. I. Ayub, Marco Antônio Záchia, orient. II. Título.

DISSERTAÇÃO
Submetida como parte dos requisitos para obtenção do grau de
MESTRE EM CIÊNCIA E TECNOLOGIA DE ALIMENTOS
Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos (PPGCTA)
Universidade Federal do Rio Grande do Sul (UFRGS)

Aprovada em:___/____/____

Pela banca examinadora:

Homologada em: ___/____/____

Por:

Prof. PhD Marco Antônio Záchia Ayub

Orientador – PPGCTA/UFRGS

Profª. Drª. Rosane Rech

Coordenadora – PPGCTA/UFRGS

Prof. Dr. Plinho Francisco Hertz

Docente - PPGCTA/UFRGS

Prof. Dr. Vitor Manfroi

Diretor - ICTA/UFRGS

Profa. Dra. Rosa Mara Borges da Silveira

Docente – PPG Botânica/UFRGS

Prof. Dr. Enilson Luiz Saccol de Sá

Docente – PPGMAA/UFRGS

Diego Melo Pereira
Engenheiro Agrônomo

Porto Alegre

2015

OBTENÇÃO E AVALIAÇÃO DE LINHAGENS HÍBRIDAS E DESENVOLVIMENTO DOS PROCESSOS DE INÓCULOS LÍQUIDOS PARA CULTIVO AXÊNICO DE *Lentinula edodes* (BERK.) Pegler[1]

Resumo

A utilização de inóculos líquidos na produção de Shiitake (*Lentinula edodes* (Berk.) Pegler) é uma tecnologia promissora para a industrialização do cultivo em sistema axênico, uma vez que permite a inoculação do micélio de forma rápida e bem distribuída, reduzindo riscos de contaminação e o período de incubação dos substratos. Neste trabalho, o cultivo submerso para a produção de micélio de Shiitake foi avaliado em um biorreator airlift de circulação externa, idealizado e projetado no grupo, utilizando o meio de cultura Mushroom Complete Medium adicionado de micronutrientes, nas condições de operação: taxas de aeração: 0.16, 0.20, e 0.24 vvm e tamanhos de inóculo (i.s.): 1.0 $g.L^{-1}$ e 2.0 $g.L^{-1}$. Na melhor condição de operação (0.16 vvm e 1.0 $g.L^{-1}$ i.s.), um modelo matemático foi desenvolvido usando o software EMSO para descrever a cinética da cultura em ajuste aos dados experimentais. Foram atingidos 15,47 $g.L^{-1}$ de biomassa total, após 10 dias de cultivo, 0,516 d^{-1} de taxa máxima de crescimento específico, fatores de rendimento $Y_{x/s}$ 1,06 $g.g^{-1}$, $Y_{P/X}$ 0,485 $(g.L^{-1}).g^{-1}$, $Y_{P/S}$ 0,140 $(g.L^{-1}).g^{-1}$ e produtividade P_X 0,061 $(g.L^{-1}).h^{-1}$. A predição do modelo demonstrou ser um parâmetro confiável para estudos de aumento de escala já que descreveu bons resultados preditivos para biomassa em relação aos produtos do metabolismo (CO_2 e síntese de H^+ pela acidificação do meio) e ao consumo de nutrientes (O_2 e glicose). A seleção e a avaliação de linhagens no cultivo também são fatores a serem considerados para obtenção de boas produtividades e, deste modo, o desempenho produtivo de cinco linhagens de Shiitake foi avaliado em cultivo axênico, utilizando um planejamento composto central (CCD) para avaliar as variáveis independentes: tempo de incubação $I_{(t)}$, e percentual de farelo de trigo em relação a serragem *Eucalypitus saligna* (representada pela relação carbono/nitrogênio ($R_{C/N}$)), tendo a eficiência biológica (BE) e o peso unitário dos cogumelos como resultados de destino . Os substratos utilizados tiveram sua composição físico-química determinada e as taxas de suplementação do substrato foram otimizadas, não só para o rendimento em cultivo, mas também para o crescimento vegetativo do micélio. Os melhores resultados de BE em todas as linhagens foram obtidos em cultivos com $R_{C/N}$ 57,62/1, com indução dos primórdios em 130 dias de $I_{(t)}$. Os cogumelos colhidos nesta condição de produção tiveram sua composição centesimal determinada. Atividades enzimáticas amilolíticas e celulolíticas, β-glicosidase e proteases específicas foram realizadas em ensaios de colonização do substrato. Três linhagens foram selecionadas para o isolamento de esporos e hibridização intraespecífica. As linhagens híbridas foram comparadas com as parentais em termos de rendimento e síntese das enzimas extracelulares. Os resultados deste trabalho demonstraram que a linhagem cultivada tem um efeito considerável sobre o rendimento, sobre o peso unitário e sobre a síntese de enzimas extracelulares produzidas pelo fungo, tanto pelas linhagens híbridas, como pelas parentais.

[1]/ Dissertação de mestrado em Ciência e Tecnologia de Alimentos, Instituto de Ciência e Tecnologia de Alimentos, Universidade Federal do Rio Grande do Sul (82 p.), outubro de 2015.

Palavras Chave: *Lentinula edodes*; shiitake; biorreator airlift; enzima extracelular; basidiomiceto.

OBTAINING AND EVALUATION OF HYBRID STRAINS AND DEVELOPMENT OF LIQUID INOCULANTS PROCESSES FOR AXENIC CULTIVATION OF *Lentinula edodes* (BERK.) Pegler[1]

Abstract

The use of liquid inoculant production of Shiitake (*Lentinula edodes* (Berk.) Pegler) is a promising technology for the industrialization farming of this fungus in axenic system because it allows the mycelium inoculation in a quick and well distributed way, thus reducing the risks of contamination and the incubation time of cultures. In this work, the submerged cultivation for the production of Shiitake mycelium was evaluated in an airlift bioreactor of external circulation, conceived and designed in the group, using the culture medium Mushroom Complete Medium added of micronutrients, under the operating conditions: rates of aeration: 0.16, 0.20, and 0.24 vvm and inoculum sizes (i.s.): 1.0 g. L^{-1} and 2.0 g. L^{-1}. Under the best operating condition (0.16 vvm and 1.0 g L^{-1} i.s.), a mathematical model was developed using the EMSO software to describe the kinetics of culture fittnes to experimental data. It was possible to obtain 15.47 g L^{-1} of total biomass, after 10 days of cultivation, a maximum specific growth rate of 0.516 d^{-1}, yields of $Y_{x/s}$ 1,06 $g.g^{-1}$, $Y_{P/X}$ 0,485 $(g.L^{-1}).g^{-1}$, $Y_{P/S}$ 0,140 $(g.L^{-1}).g^{-1}$ and P_X (productivity) 0.061 $(g.L^{-1}).h^{-1}$. The prediction of the model has proven to be a reliable parameter for studies for scaling up because it was predictive for biomass considering the products of metabolism (CO_2 and H^+ synthesis by acidification of the medium) and nutrient consumption (O_2 and glucose). The selection and evaluation of strains in cultivation are also factors to be considered to obtain good productivity and, therefore, the productive performance of five strains of Shiitake cultivated under axenic system, was carried out using a central composition design (CCD) to evaluate the independent variables: incubation time $I_{(t)}$, and percentage of wheat bran in relation to sawdust of *Eucalypitus saligna* (represented by the carbon/nitrogen ratio ($R_{C/N}$)), and having the biological efficiency (BE) and the unit weight of the mushrooms as target results. The best results for BE for all the strains were obtained in cultures with $R_{C/N}$ 57.62/1, with primordia induced at 130 days of $I_{(t)}$. The substrates used had their physico-chemical composition determined and the rates of substrate supplementation have been optimized, not only for the cultivation yields, but also for vegetative growth of the mycelium. The mushrooms harvested in this condition of production had their centesimal composition determined. Amilolytic, cellulolytic, β-glucosidase and specific proteases activities were performed in tests of colonization of the substrate. Three strains were selected for the isolation of spores and intraspecific hybridization was carried out. Hybrid strains were compared with the parental strains in terms of yields and synthesis of extracellular enzymes. The results of this study showed that strain has a considerable effect on both the yield and the unit weight of mushrooms.

[1]/ Master's thesis in Science and Technology of Food, Institute of Science and Technology of Food, Federal University of Rio Grande do Sul (82 p.), October, 2015.

Keywords: *Lentinula edodes*; shiitake; airlift bioreactor; extracellular enzyme; basidiomycete.

ÍNDICE

Resumo ..... **v**

Abstract ..... **vi**

Lista de Figuras ..... **viii**

Lista de Tabelas ..... **x**

Lista de Símbolos ..... **xi**

1.Introdução **1**

2. Objetivos **3**

2.1 - Objetivo geral 3

2.2 - Objetivos específicos 3

3. Revisão Bibliográfica **4**

3.1. Cogumelos comestíveis – espécies, produção e consumo 4

3.2. Hibridação de linhagens de Basidiomicetos 4

3.3. Instabilidade e manutenção de matrizes 8

3.4. Enzimas produzidas por fungos filamentosos Basidiomicetos 9

3.5. Biorreatores airlift operando com fungos filamentosos 10

3.6.Inóculo (Spawn) de fungos Basidiomicetos 11

3.7. *Lentinula edodes* (Berk.) Pegler 12

3.8. Métodos e parâmetros para o cultivo de *Lentinula edodes* 13

4.Resultados **17**

Artigo I - GROWTH KINETICS AND MODELING OF LENTINULA EDODES (BERK.) PEGLER CULTURES IN AN EXTERNAL LOOP AIRLIFT BIOREACTOR. **18**

Artigo II (short communication) - PHYSICO-CHEMICAL PROPERTIES OF THE SUBSTRATES AND MYCELIAL GROWTH RATE DETERMINATION IN LENTINULA EDODES (BERK.) PEGLER DIKARYOTIC STRAINS **.35**

Artigo III - The axenic cultivation of *lentinula edodes* (berk.) pegler: compost optimization and evaluation of dikaryotic strains. **43**

Apêndice - EVALUATION OF HYBRID LENTINULA EDODES (BERK.) PEGLER STRAINS BY INTRASPECIFIC MONOSPORIC CROSSES **63**

5. Considerações finais **70**

6. Perspectivas **73**

7. Referências bibliográficas **74**

8. Anexos **78**

LISTA DE FIGURAS

Figura 1 - Ciclo de vida de um fungo Basidiomiceto 5

Figura 2 - Sistema mating-type tetrapolar governado por dois locus gênicos na hibridização de micélios monocarióticos de fungos Basidiomicetos. 7

Figura 3 – Modelos esquemáticos de biorreatores airlift. (A) circulação interna; (B) circulação externa; (C) eixo profundo; (a) entrada de gás durante a operação; (b) entrada de gás para implante de stent. 10

Figura 4 - Sistemas de cultivo de Shiitake: (a) método em toras de madeira; (b) método axênico em serragem suplementada. 14

Figura 5 – Escurecimento do meio de cultura na cinética de crescimento de Lentinula edodes (Led-08) no cultivo em biorreator airlift de circulação externa. As condições foram: pH controlado a 5,0; temperatura de 25 ° C; e taxa de aeração de 0,20 vvm. 72

Figura 6 – Cromatograma do perfil do extrato bruto do cultivo no biorreator airlift de circulação externa com um detector de índice de refração e uma coluna Bio-Rad HPX-87H (300 mm x 7,8 milímetros) com 5 mM de ácido sulfúrico como eluente a 45 ° C, taxa de 0,6 mL.min-1 e volumes de amostra de 20 μ L. As condições foram: pH controlado a 5,0; temperatura de 25 ° C; e taxa de aeração de 0,20 vvm. 72

Figura 7 – Produção de *Lentinula edodes* em sistema axênico. Linhagens dicarióticas: (a) Led-08; (b) Led-T1; (c) Led-T2; (d) Led-BP; (e) Led-OTS. 78

Figura 8 – Hifas monocarióticas. Linhagens dicarióticas: (a) Led-08; (b) Led-T1; (c) Led-T2; (d) Led-BP 79

Figura 9 - Teor de proteínas solúveis do extrato bruto nos ensaios enzimáticos das linhagens dicarióticas e híbridas 79

Figure 10 - Growth kinetics of Lentinula edodes (Led-08) cultivation in the ELAB. Conditions were: temperature of 25°C, air flow of 0.16 vvm, and initial biomass of 1.0 $g.L^{-1}$. 80

Figure 11 - Growth kinetics of Lentinula edodes (Led-08) cultivation in the ELAB. Conditions were: temperature of 25°C, air flow of 0.16 vvm, and initial biomass of 2.0 $g.L^{-1}$. 80

Figure 12 - Growth kinetics of Lentinula edodes (Led-08) cultivation in the ELAB. Conditions were: temperature of 25°C, air flow of 0.20 vvm, and initial biomass of 1.0 $g.L^{-1}$. 81

Figure 13 - Growth kinetics of Lentinula edodes (Led-08) cultivation in the ELAB. Conditions were: temperature of 25°C, air flow of 0.20 vvm, and initial biomass of 2.0 $g.L^{-1}$. 81

Figure 14 - Growth kinetics of Lentinula edodes (Led-08) cultivation in the ELAB. Conditions were: temperature of 25°C, air flow of 0.24 vvm, and initial biomass of 1.0 $g.L^{-1}$. 82

Figure 15 - Growth kinetics of Lentinula edodes (Led-08) cultivation in the ELAB. Conditions were: temperature of 25°C, air flow of 0.24 vvm, and initial biomass of 2.0 $g.L^{-1}$. 82

GROWTH KINETICS AND MODELING OF LENTINULA EDODES (BERK.) PEGLER CULTURES IN AN EXTERNAL LOOP AIRLIFT BIOREACTOR

Figure 1. Diagram of external loop airlift bioreactor 29

Figure 2. Growth kinetics of Lentinus edodes Led-08 cultures in the ELAB. (a) Biomass, (b) CER, (c) pH, (d) dissolved oxygen saturation, and (e) glucose consumption during the cultivation. Culture conditions were: 25 °C, aeration rate 0.16, 0.20, or 0.24 vvm and inocula sizes (IS) of 1.0 $g.L^{-1}$ or 2.0 $g.L^{-1}$. 30

Figure 3. Growth kinetics of Lentinula edodes (Led-08) cultivation in the ELAB. Conditions were: pH controlled at 5.0; temperature of 25 ° C; and air flow of 0.20 vvm 31

Figure 4. Growth kinetics of Lentinula edodes (Led-08) cultivation in the ELAB and the modeling of experimental data. 32

THE AXENIC CULTIVATION of LENTINULA EDODES (BERK.) PEGLER: COMPOST OPTIMIZATION AND EVALUATION OF DIKARYOTIC STRAINS

Figure 1. a) Gel electrophoresis of the amplified rDNA internal transcribed sequence of ITS1-5.8S-ITS2 regions of the Lentinula edodes strains; (b) PCR product digested with enzymes HhaI, HinfI, and HaeIII at 37 ° C. 60

Figure 2. Surface response for biological efficiency (kg/kg of substate – a, b, c and d) and unit weight (grams – e, f, g and h), for the range of carbon/nitrogen 136.07/1 to 37.14/1 and culture time between 60 and 130 days. Strains: Led-08 (a and e); Led-T1 (b and f); Led-T2 (c and g) and Led-BP (d and h). 61

Figure 3. Enzyme assays of strains cultivated in test tubes containing 12 g of substrate and incubated at 25 ºC: (a) Total reducing sugar during; (b) Total amylolytic activity; (c) Total cellulolytic activity; (d) β-glucosidase activity; (e) Specific proteolytic activity; (f) pH. 62

EVALUATION OF HYBRID Lentinula edodes (BERK.) PEGLER STRAINS BY INTRASPECIFIC MONOSPORIC CROSSES

Figure 1- Enzyme assays of strains cultivated in test tubes containing 12 g of substrate at 25 ºC. Activities analyzed were total reducing sugar; total amylolytic activity; total cellulolytic activity; β-glucosidase activity; and specific proteolytic activity. 67

Figure 2- Yields of sporophores (kg.kg of substate^{-1}) during the first flush period (time in days after induction of the formation of pinning). Strains were grown in 57.62/1 carbon/nitrogen ratio for sporophores formation in 130 days. 69

LISTA DE TABELAS

GROWTH KINETICS AND MODELING OF LENTINULA EDODES (BERK.) PEGLER CULTURES IN AN EXTERNAL LOOP AIRLIFT BIOREACTOR

Table 1. Influence of aeration rate and initial biomass on the kinetic parameters of *L. edodes* Led-08 cultivation in the external loop airlift bioreactor. Biomass (X_f), Yield coefficients ($Y_{X/S}$, biomass/substrate; $Y_{P/X}$, CO_2/biomass; $Y_{P/S}$, CO_2/substrate), Biomass productivity (P_x), specific growth rate (μ_{max}), and oxygen mass transfer coefficient (k_la).....33

Table 2. Values of model parameters and the quadratic coefficient of determination (R^2) for *Lentinula edodes* Led-08 cultivation in the external loop airlift bioreactor at 25 °C, 0.16 vvm, and initial biomass of 1.0 $g.L^{-1}$.....34

PHYSICO-CHEMICAL PROPERTIES OF THE SUBSTRATES AND MYCELIAL GROWTH RATE DETERMINATION IN *Lentinula edodes* (BERK.) PEGLER DIKARYOTIC STRAINS

Table 1. Physico-chemical characteristics of substrates according to the proportion of sawdust and wheat bran 41

Table 2. Linear growth rate Kr ($mm.day^{-1}$) of dikaryotic parental strains according to the proportion of sawdust and wheat bran.....42

THE AXENIC CULTIVATION of LENTINULA EDODES (BERK.) PEGLER: COMPOST OPTIMIZATION AND EVALUATION OF DIKARYOTIC STRAINS

Table 1. Chemical composition of the substrates 56

Table 2. Actual and coded levels of the independent variables incubation time (X1) and percentage of wheat bran in relation to Eucalyptus saligna sawdust (X2) in the experimental design of the axenic cultivation of Lentinula edodes dikaryotic strains. The percentage of supplementation is represented as carbon/nitrogen ratio; the biological efficiency is given in kg of harvested mushroom (wet basis)/ kg of substrate (dry basis); the unit weight is given in grams by dividing the total mushroom mass divided by the total number of mushrooms formed in the first flush production.....57

Table 3. Regression coefficients of the variables and the regression for parameters biological efficiency (kg/Kg of substrate) and unit weight (g) of axenic cultivation of dikaryotic parental strains.....58

Table 4. Chemical composition of dikaryotic strains obtained when cultivated in wheat bran/ *Eucalyptus saligna* sawdust proportion to produce a carbon/nitrogen ratio of 57.26 for sporophores formation in 130 days. 59

EVALUATION OF HYBRID *Lentinula edodes* (BERK.) PEGLER STRAINS BY INTRASPECIFIC MONOSPORIC CROSSES

Table 1 - Yield of sporophores and unit mass of hybrid strains cultivated in carbon/nitrogen ratio 57.62/1 for sporophores formation in 130 days. 66

LISTA DE SÍMBOLOS

K_La – coeficiente volumétrico de transferência de oxigênio na presença de células [h^{-1}]

X – biomassa [$g.L^{-1}$]

X_f – biomassa final [$g.L^{-1}$]

Y – coeficientes de rendimento

$Y_{x/s}$ - biomassa/substrato [$g.g^{-1}$]

$Y_{P/X}$ – produção metabólica de CO_2/biomassa [$(g.L^{-1}).\ g^{-1}$]

$Y_{P/S}$ - produção metabólica de CO_2/substrato [$(g.L^{-1}).\ g^{-1}$]

P_X – produtividade de biomassa [$g.L^{-1}.h^{-1}$]

μ_{max} – taxa específica máxima de crescimento [day^{-1}]

Kss - constante de saturacao do modelo de Monod

αP – constante de formação de produto CO_2 metabólico

αH - constante de formação de íons H^+

KpO – constante de consumo de oxigênio dissolvido

P - produção metabólica de CO_2 [$g.L^{-1}$]

pO_2 – oxigênio dissolvido [%]

S – substrate [$g.L^{-1}$]

t – tempo de cultivo [h]

vvm - volume de ar por volume de meio

dx/dt – derivada dos valores de concentração de biomassa

dt – intervalo diferencial de tempo [h]

dx – biomassa formada no instante “dt”

dP – produto formado no intervalo “dt”

° C – graus Celsius

CO_2 – dióxido de carbono

HPLC – cromatografia líquida de alta eficiência

pH – potencial hidrogeniônico

nm – nanômetro

Kg – quilograma

g- grama

h – hora

L – litro

mL - mililitro

µL - microlitro

Kr - taxa linear de crescimento micelial [$mm.day^{-1}$]

$R_{C/N}$ – relação carnobo/nitrogênio

BE – eficiência biológica [kg of harvested mushroom (wet basis)/ kg of substrate (dry basis)]

$I_{(t)}$ – tempo de incubação [day]

Ψm – potencial matricial

S/WB – proporção de farelo de trigo (WB) em relação à serragem de *Eucalypitus saligna*

WD – densidade úmida [$g.L^{-1}$]

DD – densidade seca [$g.L^{-1}$]

DM – matéria seca [$g.100g^{-1}$]

TCSS - conteúdo total de sais solúveis [$g.L^{-1}$]

TP – porosidade total [$m^3.m^{-3}$]

AS – espaço de aeração [$m^3.m^{-3}$]

1. Introdução

O aproveitamento integral de subprodutos agroindustriais líquidos e sólidos como fonte de nutrientes para produção de micélio de fungos filamentosos viabiliza economicamente o uso destes microorganismos para diversas aplicações biotecnológicas. Por este motivo, o cultivo de cogumelos tem despertado interesse de produtores rurais, impulsionados pelo fato de que a cultura permite a diversificação dos negócios dentro da propriedade rural, detêm preços de venda atrativos, tem baixa sazonalidade de produção e estão acompanhados de um maior consumo destes tipos de alimentos por novos perfis de consumidores. Frente a estas demandas, muitos países, incluindo-se o Brasil, enfrentam um baixo nível de industrialização, desconhecem a biodiversidade comestível e carecem de conhecimento técnico, fazendo com que o rendimento dos cultivos destes países situe-se muito abaixo de outros que detém melhor hábito de consumo e de investimento em pesquisa e tecnologias.

Uma das justificativas para este fraco desempenho está relacionada à qualidade do inóculo e das linhagens propagadas. Embora a obtenção de material propagativo possa ser realizada pela clonagem de basidiomas, fatores de senescência celular poderão ser ligeiramente encontrados. O desconhecimento do estágio propagativo da cultura pode originar linhas celulares com idade avançada, proporcionando a obtenção de micélios degenerados, em virtude da alta taxa de divisões celulares mitóticas, levando a perda de vigor, baixo rendimento e mau-formação dos cogumelos. O isolamento do micélio nestas circunstâncias tem sido utilizado como referência na geração de inoculantes, sob propagação celular somática, e se baseia na seleção de características fenotípicas de interesse do cultivador. As características fenotípicas destas linhagens somente poderão ser mantidas em cultivo ao retornar-se à culturas-estoque e destas possam ser retirados materiais propagativos de linhas celulares mais jovens.

Os cogumelos são organismos que se reproduzem sexualmente estando submetidos a um número limitado de divisões celulares. Este ciclo inicia-se com a germinação de esporos seguido da união de hifas haplóides monocarióticas, formando uma hifa dicariótica. Estas células multiplicam-se até apresentarem condições para diferenciarem-se em basídios e basidiósporos, formados em basidiomas. A propragação é facilitada pela capacidade de regeneração do micélio após uma fragmentação mecânica, devido a algum agente abiótico ou biótico, ou mesmo sob manipulação artificial em laboratório. Cada fragmento formado pode potencialmente originar novos clones que serão geneticamente idênticos a matriz inicial e que preservarão características fenotípicas das linhagens. Entretanto, não ocorrendo a recombinação genética a expansão exponencial da massa micelial diminui seu vigor e eventualmente morre. Novas matrizes só apresentarão bons rendimentos ao serem geradas através da hibridização de micélios monocarióticos com consequente avaliação dos parâmetros de crescimento e formação de basidiomas. Esta prática pode contribuir para o aumento da produtividade dos cogumelos e é uma tecnologia promissora para fixar características de interesse na construção de novos genótipos.

O inóculo é tradicionalmente produzido através da propagação do micélio de forma vegetativa, originado da multiplicação em condições assépticas das células de uma cultura-mãe, pura, inoculada em grãos de cereais, método pelo qual é realizada sua distribuição comercial. Para chegar a este estágio, os isolados são utilizados na obtenção do inóculo desde que sejam realizados testes propagativos de viabilidade e pureza. Ao repicá-los

deverão apresentar viabilidade, estabilidade de crescimento, vigor e morfologia baseada nas características fenotípicas da linhagem em linhas celulares jovens. Posteriormente, são cultivados em grãos de cereais e comercializados imediatamente ou armazenados, após finalizada a colonização, de 1 a 3 meses sob refrigeração a 4ºC. Assim, a utlização de grãos como propágulos na produção do *spawn* requer um escalonamento contínuo a fim de garantir a repicagem do micélio no estágio de maior vigor propagativo. Em supensões micelianas, o *spawn* pode ser estocado sob refrigeração por um período ligeraimente maior, de até 4 meses.

O cultivo submerso de fungos filamentosos já foi abordado em diversos estudos e têm sido amplamente difundido para obtenção de produtos biológicos de interesse industrial. Do micélio podem ser extraídos polissacarídeos considerados importantes aliados no tratamento complementar de inúmeras doenças, agindo como imunomoduladores e incrementando a função e atividade do sistema imunológico natural. Do caldo fermentativo residual e das células podem ser extraídas enzimas intra e extracelulares. Além disto, a biomassa fúngica produzida em cultivo submerso pode servir como incremento de formulações alimentícias proteicas e como inóculo para o cultivo de cogumelos em meio sólido e este método possui vantagens aos métodos tradicionais, pois ocupa espaços reduzidos, diminui custos e riscos de contaminações, possibilita a mecanização da inoculação e, por esta razão, são consideradas fontes importantes na propagação de substratos axênicos.

Neste trabalho, utilizou-se a espécie *Lentinula edodes* (Berk.) Pegler como modelo para construção e avaliação de linhagens híbridas geradas a partir do cruzamento de hifas monocarióticas obtidas da proliferação de esporos de linhagens dicarióticas, correlacionando a sua eficiência biológica quando cultivada no sistema axênico com a atividade de enzimas extracelulares em ensaios de laboratório. O foco foram enzimas amiloliticas e totais do complexo celulolítico devido à natureza da biodegradação do substrato composto à base de serragem de *Eucalyptus saligna* e farelo de trigo. Além disto, em biorreatores airlift de circulação externa foram avaliadas o desenvolvimento dos processos de inoculantes líquidos para uso em sistema de cultivo axênico da referida espécie.

2. Objetivos

2.1 - Objetivo geral

Avaliar linhagens dicarióticas, desenvolver híbridos ativos de *Lentinula edodes* (Berk.) Pegler e obter inóculos líquidos através da propagação da biomassa em cultivo submerso descontínuo utilizando um biorreator airlift de circulação externa.

2.2 - Objetivos específicos

Resgate, preservação, avaliação e identificação molecular por meio da amplificação da região ITS1-5,8S–ITS2 do DNA ribossomal de linhagens comerciais de *L.edodes* recomendadas para o sistema axênico de produção.

Obtenção de isolados monocarióticos para a formação de matrizes aos cruzamentos monospóricos.

Padronizar a propagação do micélio em biorreator airlift de circulação externa para obtenção de inoculantes líquidos.

Realizar ensaios enzimáticos com as linhagens parentais e híbridas selecionadas.

Caracterizar o substrato serragem de eucalipto, variedade *Eucalyptus saligna* e a suplementação com farelo de trigo através da análise físico-química sob diferentes proporções de utilização.

3. Revisão Bibliográfica

3.1. Cogumelos comestíveis – espécies, produção e consumo

Cogumelo é o nome popular denominado aos corpos de frutificações de alguns fungos filamentosos, integrantes do subreino Dikarya, das divisões Basidiomycota e Ascomycota. (Hibbett D.S. et al., 2007). São alimentos protéicos e de baixo teor calórico reunindo aminoácidos essenciais à dieta humana, minerais, vitaminas, fibras e ácidos graxos insaturados em um único produto. Estima-se que possam existir 150.000 espécies diferentes de cogumelos no Reino Fungi, das quais foram identificadas aproximadamente 12.000, sendo pelo menos 2.000 destas reconhecidamente comestíveis. Um número muito menor é cultivado comercialmente, em torno de 35, das quais 20 já são produzidas em escala industrial. A espécie mais cultivada no mundo é o *Agaricus bisporus (champignon)*, seguido por *Lentinula edodes (shiitake)*, *Pleurotus spp (hiratake)*, *Auricularia aurícula*, *Flamulina velutipes* (enokitake) e *Volvariella volvacea* (Sanches C., 2004).

O histórico dos cultivos é bastante antigo. Datado em 500 anos AEA, os cogumelos foram cultivados na China e no Extremo Oriente sendo tradicões culinárias orientais até os dias de hoje. Na Europa, iniciaram-se no século XVII em Paris, quando foi implementado o primeiro cultivo de *Agaricus bisporus* (Stamets, 1983). Segundo dados da *Food and Agriculture Organization* (FAO) a produção mundial de cogumelos e trufas no ano de 2011 atingiu 11 milhões de toneladas em cogumelos frescos, produzidas em 67 países. A China é responsável pela maior parte, com cerca de 5 milhões de toneladas produzidas, 45 % do total.

Apesar da grande importância gastronômica, medicinal e nutricional, o cultivo de cogumelos ocorreu no Brasil inicialmente no ano de 1953 (Molena, 1986) e a sua utilização na dieta do brasileiro é relativamente recente. Não há registros oficiais da produção atual, entretanto, Furlan (2011) aponta que a estimativa esteja em torno de 1,5 mil toneladas anuais, das quais 62,5 % são representadas pelo cultivo de *A.bisoporus* e 15 % ao cultivo de A. *subrufescens*. O consumo ainda é muito pequeno se comparado a países europeus e asiáticos (Demiate e Shibata, 2003), estima-se sem dados oficiais que o consumo per-capita anual no Brasil seja de apenas 160 gramas por ano, comparado a 10 kg na China, 2,1 kg na França e 1,4 kg na Itália (Oliveira RS, 2010).

3.2. Hibridização de linhagens de Basidiomicetos

A hibridização em linhagens monocarióticas de fungos basidiomicetos visa a seleção de híbridos de crescimento estável e padronizado, de alta performance produtiva e resistentes às principais doenças de cultivo. Uma hibridização eficiente deve garantir que duas células monocarióticas de micélio que apresentem genes *mating-type* compatíveis se fundam através do processo de plasmogamia ou anastomose e seus dois núcleos passem a coexistir em um citoplasma comum, formando a fase dicariótica. O estágio dicariótico é um pré-requisito para que ocorra a reprodução sexual. O ciclo de vida se completa através da reprodução sexuada e consequente formação dos basídios que são estruturas reprodutivas microscópicas em forma de clava onde ocorre a cariogamia e a meiose, formando novamente células haploides denominadas basidiósporos. Os basídios podem se agrupar em um corpo de frutificação, constituindo o basidiocarpo, conhecido como cogumelo. (Moore-Landecker, 1996; Herrera Ulloa, 1998).

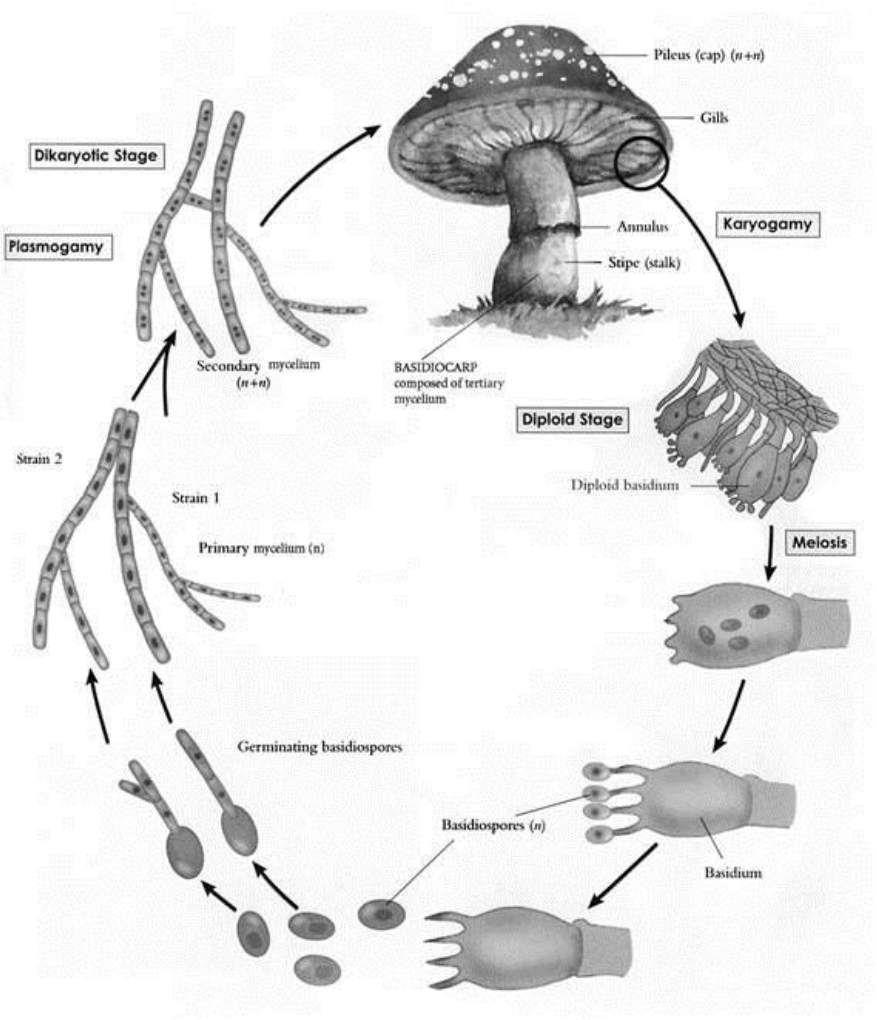

Figura 1: Ciclo de vida de um fungo Basidiomiceto (fonte: http://www.realmagick.com/ basidiomycota-life-cycle/)

Durante o ciclo o de vida dos basidiomicetos o micélio apresenta três fases de desenvolvimento denominadas tipos primários, secundários e terciários. O micélio primário formado a partir da germinação dos esporos consiste num estado de hifas cujo único núcleo da célula haplóide em cada compartimento é separado por uma estrutura chamada de septo ou dolipore. O micélio secundário, por sua vez, é composto de células binucleadas, dicarióticas ou deriva de micélio primário e é caracterizado pela presença de fíbulas ou grampos de conexão que são estruturas responsáveis pela migração nuclear entre hifas num processo contínuo de alongamento, dando origem finalmente ao micélio terciário que caracteriza-se por células organizadas em tecidos especializados que formam corpos de frutificação e é o tipo de micélio característico na reprodução sexual. (Koltin et al., 1972; Guzman et al., 1993; Herrera Ulloa, 1998).

Em basidiomicetos, existem dois tipos de sistemas de fertilidade denominadas sistema homotálico e heterotálico. A primeira é a forma mais comum de reprodução sexual entre os organismos do Reino dos Fungos, ainda que nos basidiomicetos o heterotalismo ocupe cerca de 90 %, dos quais um número estimado de 65 % do sistema utiliza acasalamento tetrapolar (Valencia-del Toro, 2002).

No sistema homotálico, o corpo de frutificação pode ser produzido por um micélio monocariótico e a transição da fase haplóide para dicariótica ocorre na ausência de uma interação compatível com outros micélios (Herrera Ulloa, 1998). Podendo haver dois tipos de sistemas. No homotalismo primário ou homocariótico o micélio é monocariótico e provém de um único núcleo meiótico, que pode progredir através de heterocariose até ao final do ciclo sexual, podendo ser citados *Coprinus sterquilinus, Volvariella Volvacea* e *Sistotrema brinkmanni* como exemplos de espécies capazes de completar a sua reprodução sexual neste sistema (Koltin et al., 1972; Guzman et al., 1993). No homotalismo secundário ou heterocariótico um micélio dicariótico fértil origina a partir de um esporo dois núcleos meióticos com dois tipos de acasalamento. Neste tipo de homotalismo, ocorre uma distribuição de combinações no basídio através da meiose de modo que ambos os núcleos tenham tipicamente genótipos complementares dentro de um único esporo e assim obtenham células heterocarióticas capazes de completar a reprodução sexual (Koltin et al, 1972; Valencia-del Toro, 2002).

Nas espécies de acasalamento heterotálico são necessários diferentes micélios homocarióticos para completar o ciclo sexual, sendo que nos basidiomicetos o heterotalismo é dividido em dois sistemas (Guzman et al, 1993; Herrera Ulloa., 1998), denominados de compatibilidade uni ou bipolar.

Compatibilidade por um fator (fator A) é controlada por um par de cromossomos homólogos em que o acasalamento sexual necessita dar origem a um par de genes alelos compatíveis (AxAy), enquanto que outras possíveis combinações (AxAx e AyAy) serão estéreis. *Agaricus bisporus* (Ramirez et al., 2000), *Auricularia aurícula* e *Pholiota nameko* (Guzman et al., 1993) são exemplos dessas espécies que apresentam este tipo de compatibilidade.

A compatibilidade de dois fatores (A e B é controlada) em cromossomos diferentes, devido à segregação meiótica. Cada fator tem um par de genes de alelos que controlam o mesmo tipo de caracteres em cada locus em posição idêntica no cromossomo e formam pares de genes homólogos (AxAy, BxBy). Estes *loci* possuem diferentes especificidades alélicas (x e y) formando através da recombinação quatro esporos geneticamentes distintos (AxBx, AxBy, AyBx, AyBy). A interação de micélios monocarióticos AxBx e AyBy formarão um micélio dicariótico capaz de formar corpos frutíferos. As demais combinações são incompatíveis e, portanto, inférteis conforme representado na Figura 2. Deste modo serão alcançadas 25 % de chances de ocorrerem interações bem sucedidas em cruzamentos aleatórios de um mesmo basidioma (Kothe, 2001). Estes genes de acasalamento (*mating-type*) são atualmente a indicação mais importante em programas de melhoramento sendo utilizados como marcadores moleculares para identificar rapidamente linhagens compatíveis em um determinado conjunto de progênies monocarióticas. (Kothe, 2001). Em adição ao gênero *Lentinula edodes*, Guzman et al. (1993) cita como exemplos de cogumelos comestíveis com este tipo de compatibilidade *Auricularia polytricha, Coprinus fimetarius, Flammulina velutipes, Lentinus boryanus, L. lepideus* e *Pleurotus ostreatus*.

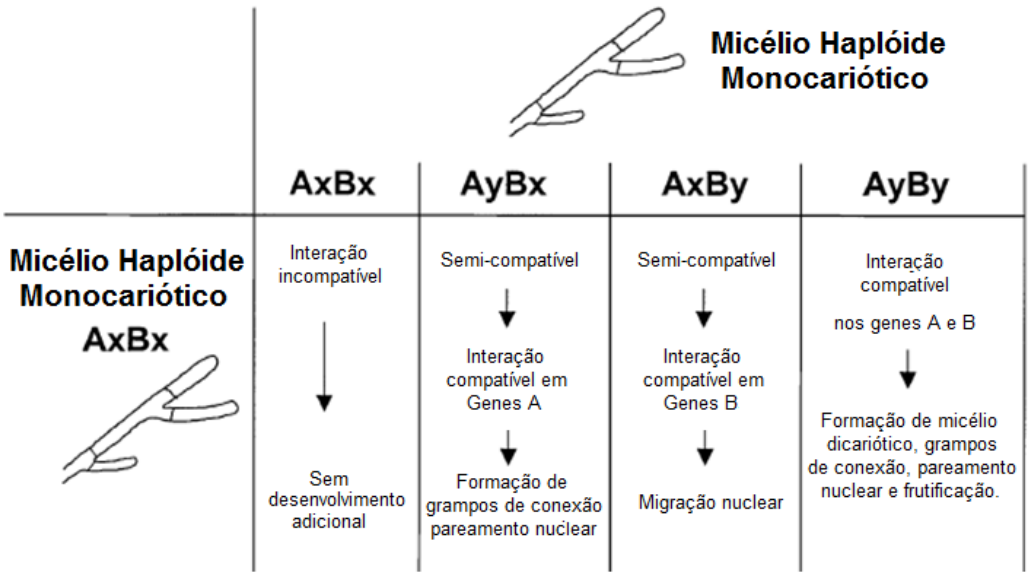

Figura 2: Sistema *mating-type* tetrapolar governado por dois *locus* gênicos na hibridização de micélios monocarióticos de fungos Basidiomicetos.

Adaptado de Kothe, 2001

As hibridizações de linhas monocarióticas de micélio apresentam boas perspectivas para o melhoramento genético de linhagens. Cogumelos híbridos possuem características mais estáveis e apresentam melhor resultado em rendimento. Estudos recentes evidenciam sucesso na obtenção de cruzamentos dicarióticos induzidos através da clonagem de genes *mating-type* introduzidos em micélios monocarióticos de linhagens de interesse (O'Shea et al, 1998). Entretanto, no modelo clássico estas linhagens são geradas através da fusão de hifas oriundas da germinação de basidiósporos puros e individuais através do isolamento em placa de Petri com ágar e antibióticos. Esta técnica é muito simples e permite a obtenção de esporos isolados em 5 minutos, sendo facilmente coletados com agulha fina. Após 36-48 h de incubação a 27 ± 1° C é possível verificar a proliferação do micélio monocariótico para aplicação em diversos cruzamentos ao acaso (Gupta et al., 2011).

Os marcadores moleculares e técnicas de biologia molecular são metodologias rápidas e confiáveis para determinar-se as identidades das coleções de micélio monocarióticas e dicarióticas e são ferramentas úteis para estudos taxonomicos (Singh et al. 2006, Gupta et al., 2011), evolutivos, ecológicos e filogenéticos em espécies de cogumelo mantidas em Banco Ativo de Germoplasma (Lynch & milligan, 1994). A amplificação da região do Espaço Interno Transcrito (ITS), seguido da digestão por enzimas de restrição é um método apropriado para discriminar espécies relacionadas ou até mesmo variedades de uma mesma espécie. Por serem sequências relativamente curtas e estarem em grande quantidade de cópias no genoma, as regiões ITS são amplificadas com facilidade via reação em cadeia da polimerase (PCR) utilizando primers universais baseados em sequências conservadas do DNA ribossômico (rDNA).

O levantamento de informações genéticas e morfológicas associadas à eficiência biológica em sistemas de cultivo permite ampliar-se o conhecimento das espécies existentes podendo serem garantidas a conservação de características de interesse para estudos futuros de melhoramento com estes microorganismos.

3.3. Instabilidade e manutenção de matrizes

O fenômeno de instabilidade das matrizes é frequentemente encontrado no cultivo de cogumelos. Ele está relacionado com o envelhecimento e mutação genética das linhagens proporcionado pela divisão celular mitótica excessiva. Isto ocorre quando o micélio é propagado continuadamente de forma vegetativa (Li.A. et al.,1994) e quando os isolados clonados são selecionados de cogumelos mantidos constatemente sob cultivo. Setorização, instabilidade de crescimento, malformação do micélio e do corpo de frutificação, incapacidade para colonizar um substrato e de produzir primórdios, atraso no período de frutificação e uma crescente susceptibilidade a doenças são sintomas clássicos desta degeneração (Stamets, 2000).

Os cogumelos são organismos que se reproduzem sexualmente estando submetidos a um número limitado de divisões celulares no estágio dicariótico. O ciclo vital inicia-se com a germinação de esporos seguido da união de hifas haplóides monocarióticas formando uma hifa dicariótica. Estas células multiplicam-se até apresentarem condições para diferenciarem-se em basídios e basidiósporos formados em corpos frutíferos (Kothe, 2001). A propragação vegetativa é facilitada pela capacidade de regeneração do micélio após uma fragmentação mecânica, devido a algum agente abiótico ou biótico, ou mesmo sob manipulação artificial em laboratório. Cada fragmento formado pode potencialmente originar novos clones que serão geneticamente idênticos a matriz inicial que preservarão características fenotípicas das linhagens (Rinker, 1993). Entretanto, não ocorrendo a recombinação genética a expansão exponencial da massa micelial diminui seu vigor e eventualmente morre. A propagação limita-se diferentemente para cada espécie, sendo para fins comerciais a recomendação de até 10 tranferências sucessivas de uma cultura inicial (isolada de um cogumelo silvestre ou obtida da hibridização de esporos), considerando uma repicagem de 1 cm^2 de meio de cultura para uma placa de Petri de 100 x 15 mm. Muitas linhagens de *Morchella esculenta*, *Lentinula edodes* e *Stropharia rugosoannulata*, por exemplo, expressam mutações acima destes níveis sugeridos. Ao não ser mais possível retornar às culturas de reserva, para linhas de células jovens, novas matrizes deverão ser geradas através da germinação de esporos para se obterem bons resultados de produtividade e sucesso na frutificação (Stamets, 2000).

Uma cultura padrão mantida em placa de Petri 100 x 15 mm pode ser suficiente para inocular 50 a 100 tubos de ensaio de 100 x 20 mm. Após a completa colonização do meio de cultura estes podem ser mantidos a 4 ° C por um período de até 5 anos ou mais. Subculturas são utilizadas para gerenciar a idade das culturas e devem ser realizadas periodicamente para para serem avaliadas em função de sua taxa de crescimento, morfologia e fisiologia (Stamets, 1983). Pequenas variações podem não afetar o rendimento e a qualidade dos cogumelos. Entretanto, fornecedores de *spawn* selecionam características constantes de colonização, descartando culturas que demonstrem sinais de setorização grave ou áreas excessivas com hifas aéreas, sintomas que são geralmente seguidos por formação de um estroma em alguma região do micélio (Sonnenberg, 2000).

Uma coleção de culturas pode ser mantida sob criopreservação em nitrogênio líquido a 196°C. Sob este método o metabolismo celular é reduzido até o congelamento de toda água intracelular evitando reações bioquímicas do micélio. Sobre as células são adicionadas soluções estéreis crioprotectoras, como o glicerol a 10 % (fração volumétrica). Quando forem propagadas as culturas são lentamente descongeladas e inoculadas em placas de Petri com meio de cultura para serem avaliadas (Chang, 2008). Não há nenhum teste *in vitro* para determinar-se o estágio propagativo da cultura. Uma série de ensaios de incubação, de frutificação e de análises dos corpos frutíferos deverão ser realizados a fim de estipular a viabilidade de propagação de uma linha celular.

Também não há nenhuma indicação de que o armazenamento sobre estes métodos levem a qualquer alteração na taxa de crescimento do micélio ou no rendimento e qualidade dos cogumelos (Sonnenberg, 2000).

3.4. Enzimas produzidas por fungos filamentosos Basidiomicetos

A atividade enzimática dos Basidiomicetos está relacionada principalmente ao tipo de resíduo agroindustrial utilizado no cultivo. A concentração enzimática secretada pelo fungo é alterada pelo uso substratos variados, podendo serem produzidas celulases (endoglicanase, exoglicanase, β-glicosidase), pectinases, hemicelulases e lignases (lacase, lignina peroxidase, manganês peroxidase). O uso de substratos lignocelulósicos por estes fungos é dependente de sua capacidade de sintetizar enzimas hidrolíticas e oxidativas que convertam polímeros de celulose, hemicelulose e lignina em compostos de baixo peso molecular para serem assimilados nutricionalmente (Buswell et al. 1993). Estas enzimas são secretadas no meio extracelular e estão, portanto, envolvidas com o processo da degradabilidade do substrato. Na conversão da celulose a açúcares fermentescíveis é empregado um complexo enzimático celulolítico. Este metabolismo nutricional primário do fungo atua a partir da hidrólise destes compostos através da ação sinérgica de endoglicanases, exoglicanases e β-glicosidases (Sun and Cheng, 2002).

As endoglicanases hidrolisam as ligações β-1,4-glicosídicas das cadeias de celulose de forma aleatória, produzindo quebras na fibra. Nas extremidades tanto redutora como não redutoras da cadeia, as exoglicanases podem se ancorar e em seqüência liberar celobioses ou glicoses solúveis. As β-glicosidases podem hidrolisar as celobioses, formando glicoses e assim diminuir a produção destas enzimas em inibição por substrato (Zhang and Lynd, 2004).

A degradação da lignina ocorre no final deste crescimento primário, através de um metabolismo secundário quando há deficiência de nutrientes (Pointing, 2001). Um processo oxidativo aeróbio de compostos fenólicos, metóxidos e alifáticos da lignina são oxidados ocasionado a ruptura de anéis aromáticos com consequente formação de novos grupos carbônicos. Lignina peroxidase, manganês peroxidase, lacase e H_2O_2 peroxidase são enzimas extracelulares produzidas por fungos que estão envolvidas neste processo. Estas mudanças na estrutura da lignina resultam na sua despolimerização e na produção de dióxido de carbono (Kirk & Farrell 1987).

As amilases são responsáveis pela degradação da molécula de amido e estão amplamente distribuídas na natureza. O amido é encontrado principalmente em sementes de cereais como milho, cevada, trigo e arroz, e em tubérculos ou raízes como batata e mandioca. Possui em sua constituição média 25 % de amilose e 75 % amilopectina (Moraes, 2004). A amilose é um polímero linear constituído de cerca de 6000 resíduos de glicose unidos por ligações glicosídicas do tipo α-1,4. A amilopectina consiste de pequenas cadeias laterais de 15 a 45 resíduos unidos por ligações do tipo α-1,6 (Buléon, et al., 1998). Segundo Gupta (2003), as endoamilases hidrolizam de forma aleatória o interior da molécula do amido. Essa ação causa a formação de ramos lineares de oligossacarídeos de cadeias de amilose ou amilopectina de vários comprimentos.

A produção destas enzimas pelo micélio do fungo é uma etapa crucial do processo de colonização do substrato e apresenta-se com um fator determinante para o rendimento produtivo dos cogumelos. Ferramentas bioquímicas e moleculares podem ser utilizadas para mensurar o perfil de síntese destes complexos enzimáticos

produzidos por fungos filamentosos no decorrer da fermentação e podem indicar características importantes das linhagens a serem propagadas.

3.5. Biorreatores airlift operando com fungos filamentosos

Airlift é um tipo de biorreator empregado em bioprocessos aeróbios (Figura 3). São preferencialmente utilizados para formação de biomassa de fungos filamentosos por apresentarem menores tensões de cisalhamento celular em comparação a biorreatores de tanque agitado (*stirred tank reactor* - STR). A fluidização do meio de cultura é promovida unicamente pela injeção pontual de oxigênio, aliada às geometrias próprias desses equipamentos (Chisti, 1989), resultando na circulação do fluido com escoamentos em duas regiões distinas: ascendente (*riser*) e descendente (*downcomer*) (Chisti & Moo-oung, 1987). Esta configuração apresenta vantagens para aumento da escala de operação em relação a outros biorreatores, pois os modelos não possuem partes móveis (Onken e Weiland, 1983) e são projetos de construção simples e de barata manutenção (Moresi, 1981).

Modelos de predição para velocidade e tempo de circulação, tempo de mistura, retenção gasosa, coeficiente volumétrico de transferência de oxigênio (k_La) e velocidade de cisalhamento devem ser analisados como critérios de desempenho destes modelos de biorreator (Chisti, 1989), principalmente na fase exponecial da fermentação. Nesta etapa, o crescimento celular eleva a demanda de oxigênio do processo em função de mudanças na viscosidade aparente do caldo acabando por dificultar a transferência de oxigênio da fase gasosa para a líquida, reduzindo o k_La (Badino et al., 2001).

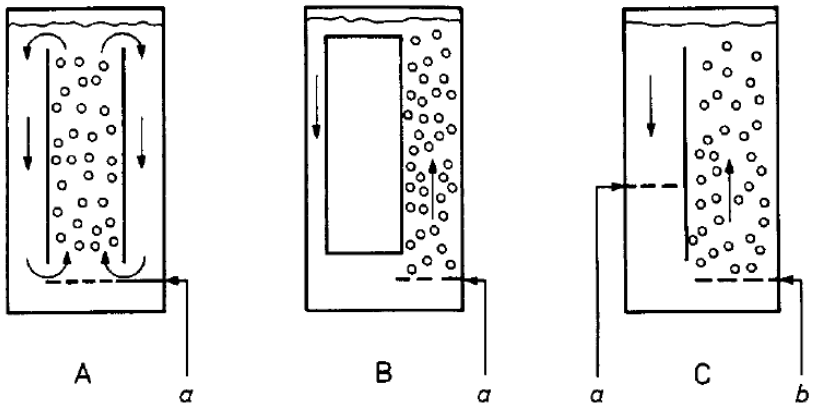

Figura 3 – Modelos esquemáticos de biorreatores airlift. (A) circulação interna; (B) circulação externa; (C) eixo profundo; (a) entrada de gás durante a operação; (b) entrada de gás para implante de stent.

Adaptado de Onken and Träger, 1990

A taxa de crescimento (μ) e a morfologia dos fungos filamentosos em fermentação submersa operados em biorreatores airlift variam de acordo com as condições de cultivo podendo apresentar-se sob formas filamentosas ou agregadas em peletes. Esta complexidade de crescimento torna a medida de formação biomassa ao longo do cultivo um problema crítico nestas fermentações, principalmente quando são utilizados meios de

cultura de natureza heterogênea (Koutinas et. al, 2003). Métodos indiretos de mensuração têm sido empregados para contornar esta situação e baseam-se na medida de algum componente da biomassa ou medidas de alguma atividade metabólica. Nestes sistemas a medida indireta de produção metabólica de CO_2 demonstrou-se ser uma alternativa à medida de peso seco por ser uma técnica online e não-destrutiva (Raimbault, 1998) podendo ser parametrizada a um modelo matemático relacionando-a com o perfil de biomassa fúngica formada (Mitchell, 1992).

3.6. Inóculo (Spawn) de fungos Basidiomicetos

Spawn pode ser definido como uma matriz de propagação vegetativa do micélio (Stamets, 2005) envolvendo essecialmente a obtenção de culturas puras clonadas de células somáticas de um cogumelo ou através da hibridização de esporos.

Em 1894, foram obtidas na França as primeiras culturas puras de *spawn* por dois pesquisadores (Costanin e Matruchot - Pasteur Institute) através de esporos germinados em composto a base de esterco de cavalo. Mais tarde, em 1905, um americano (Duggar) realizou a primeira cultura de micélio através da clonagem de células somáticas de um cogumelo, sendo propagados até a década de 30 sob a forma comercial de compostos esterilizados a base de esterco de cavalo (Rinker, 1993).

O processo de produção envolvendo grãos esterilizados foi iniciado na Pennsylvania State University pelo pesquisador Prof. J.W. Sinden, em 1932. A utilização de grãos para veiculação do micélio no composto de produção obteve vantagens operacionais sobre o uso do esterco, pois são facilmentes distribuídos, apresentam maiores quantidades de pontos de crescimento e fornecerem melhor aporte nutricional para o desenvolvimento fúngico (Beyer, 2003). Para serem produzidos, fragmentos de meio de cultivo em ágar contendo culturas puras são inoculados em frascos contendo grãos de cereais como o trigo, o milheto e o sorgo para futura expansão em embalagens plásticas contendo mais grãos esterilizados. A manutenção do crescimento sob condições adequadas é de fundamental importância para que sejam reduzidos os períodos de incubação e a energia utilizada para sua manutenção, portanto, o inóculo é mantido durante seu crescimento em salas com controle de temperatura e de disponibilidade de oxigênio com base no desenvolvimento ótimo do micélio (Rinker, 1993). Após serem totalmente colonizados pelo fungo recomenda-se realizar a propagação imediata do inóculo a fim de evitar o envelhecimento celular e propagá-lo em seu estágio de maior vigor de recuperação. Este crescimento pode ser interrompido pela manutenção do *spawn* sob refrigeração a 4°C. O período de armazenagem em estado sólido é variado para cada espécie e está relacionado principalmente com o vigor de degradabilidade do substrato, podendo ser mantido de 1 (*Pleurotus spp.*) a 3 (*Agaricus bisporus, Lentinula edodes, por exemplo*) meses sem perder viabilidade (Chang, 2008).

Quando ocorre o processo de envelhecimento o micélio torna-se mais compacto, forma peles, cascas e caroços, produz exudados coloridos com um odor desagradável caracterizado pela autólise das células. Nesta fase o micélio ainda poderá ser propagado, entretanto, ocorre perda de vigor e aumentam as chances de contaminações, principalmente as de origem bacteriana (Chang & Miles, 1989).

Micélio cultivado em fermentação submersa também pode ser utilizado para produção de *spawn*. A utilização deste procedimento é recomendada para mecanizar o processo de inoculação de grãos e substratos. Neste sistema foram relatadas vantagens por diversos autores (Kawai, 1996; Silveira, 2006; Yang, 2007) em

relação ao estado sólido. O período de incubação em substratos axênicos pôde ser reduzido devido ao fato de ocorrer a melhor distribuição das células inoculadas, aumentanto a proporção de pontos de crescimento fúngico. A produção pode ser realizada em menor espaço e em período mais curto de tempo. Também permite controlar melhor as condições de cultivo como pH, oxigênio dissolvido, temperatura e concentração de nutrientes e, além disto, o período de armazenagem é ligeraimente maior, em torno de 4 meses. Entretanto, vantagens como diminuição dos riscos de contaminação bacteriana em função da ausência de água livre, o baixo custo das instalações, a amplitude de fontes nutricionais disponíveis (grãos e resíduos lignocelulósicos) e o fluxograma simplificado são apresentadas pelo estado sólido quando comparado ao cultivo submerso (Rossi, 2006).

A imobilização de fungos filamentosos em polímeros sólidos demonstrou ser uma alternativa importante para produção de inoculantes de fungos de controle biológico e ectomicorrízicos. As principais vantagens são o aumento da vida útil do inóculo mesmo sob longos períodos de armazenamento, em torno de 18 meses (Oliveira et al., 2004), a proteção física do micélio, facilidade de distribuição, armazenamento e transporte. A técnica consiste na mistura de uma suspensão miceliana com alginato de sódio, usualmente de concentração 2 a 4 % (fração volumétrica). Esta mistura é gotejada em uma solução de cloreto de cálcio. O íon Ca^{2+} promove a formação de ligações iônicas, que resultam na formação de um gel consistente e insolúvel, o qual imobiliza o microrganismo (Wang et al., 2005). A natureza química porosa do gel permite o crescimento dos microrganismos tanto dentro das esferas quanto em direção ao exterior destas (Kuek et al., 1992).

Outra tecnologia promissora envolve a produção de peletes através da mistura de alginato de cálcio, vermiculita, *hygramer* e diferentes concentrações de proteína de soja (de 0,5 a 8 %). Nesta metodologia, a imobilização do micélio em polímeros pode ser ampliada em volume através da utilização de suportes inorgânicos. Apresenta-se como uma grande alternativa para redução de custo da imobilização celular, aumento do número de pontos de crescimento fúngico inoculados ao composto de produção. Quando foram utilizados como inóculo no cultivo de *Agaricus bisporus*, o desempenho foi comparável aos propágulos em grãos (Romaine & Schlagnhaufer, 1992).

3.7. *Lentinula edodes* (Berk.) Pegler

Cogumelos Shiitake foram encontrados inicialmente na China, Japão e Coréia (Chang & Miles, 2004). Descobertas recentes demonstraram a ocorrência de espécies do gênero *Lentinula* em habitats naturais nos Estados Unidos, sendo conhecido como “Shiitake das Américas” podendo ser encontrado em madeira morta de muitas espécies arbóreas florestais nas regiões subtropicais e tropicais do continente americano, do sudoeste dos Estados Unidos até a América do Sul (Pegler, 1983; Guzmán et al., 1993). Crescem principalmente em climas temperados e são colonizadores de madeiras densas, em particular, Shii (*Pasania spp.*), carvalho (*Quercus spp.*), e outros carvalhos asiáticos (Stamets, 2000).

O nome do cogumelo shiitake é derivado de palavras japonesas: "Shii", que significa a madeira de *Pasania spp.* e "take" que significa cogumelo. Shiitake é o nome popular mais difundido no mundo para este cogumelo, mas também tem sido reconhecido por “*black forest mushroom*”, nos EUA. Na China, Shiitake é conhecido como Xiang-gu (cogumelo perfumado), Dong-gu, (cogumelo do inverno), e hua-gu, (cogumelo flor) (Chen, 2001).

O nome científico *Lentinula edodes* (Berkeley) Pegler é classificado no gênero *Lentinula*, pertencente a família de Tricholomataceae, da ordem Agaricales e subfilo Basidiomiceto. Até 1975 era conhecido como *Lentinus edodes* (Berk.) Singer, a partir do qual Pegler propôs a transferência para o gênero *Lentinula*. A justificativa desta transferência foi baseada em observações microscópicas do micélio que apresenta hifas monomíticas no gênero *Lentinula*, diferentemente de hifas dimiticas encontradas no gênero *Lentinus edodes* (Berk.) Singer, acompanhadas de estudos moleculares destes gêneros (Pegler, 1983).

O ciclo de vida Shiitake é do tipo heterotálico tetrapolar. Em um ambiente favorável, basidiósporos produzidos pela reprodução sexual através da meiose, germinam em hifas monocarióticas, mononucleadas. Havendo compactibilidade entre *mating-types* as hifas se fundem através plasmogamia para produzir hifas dicarióticas as quais são capazes de dar origem à frutificação dos cogumelos (Chen, 2004).

3.8. Métodos e parâmetros para o cultivo de *Lentinula edodes*

Shiitake é tradicionalmente cultivado em madeira de várias espécies de árvores. A espécie de cultivo primário utilizada no Japão foi a árvore Shii (*Castanopsis cuspidata*). No entanto, o maior volume produtivo mundial de shiitake no sistema de "toras de madeira" é encontrado nos Estados Unidos, onde são utilizadas espécies de carvalho *Quercus, Castanopsis, Lithocarpus*, ou *Carpinus* (Oei, 2003). No Brasil, o cultivo de shiitake em toras é efetuado principalmente em troncos de espécies de Eucalipto, as quais são perfuradas, com auxílio de uma furadeira elétrica e inoculadas com o micélio do fungo e vedadas com parafina fundida. Posteriormente, as toras são submetidas à incubação durante um período de 6-8 meses, sob condições controladas de temperatura e umidade até que o micélio as colonize totalmente, quando então estas são induzidas a produzir basidiomas (Minhoni et al., 2005).

Existem inúmeras vantagens de se utilizar o sistema de produção axênico de shiitake em "blocos de serragem" quando comparado ao método em toras (Silva, 2005). O ciclo se completa cerca de 3 meses desde a inoculação até a colheita e foram registradas eficiências biológicas superiores em 75 % a 125 % em relação ao cultivo nas toras dependendo unicamente do substrato e da linhagem cultivada (Chen, 2005). Permite o fornecimento constante do mercado através da produção durante todo o ano. Além disso, o cultivo em substratos é um meio de utilizar subprodutos agrícolas (Chang and Miles, 2004) para gerar alimentos de alto valor agregado (Babcock, 2004).

Serragem é o ingrediente basal mais popular para cultivo de shiitake em sacos suplementados à base de materiais nitrogenados. A introdução da suplementação pode representar de 10 a 60 % do peso seco da formulação do composto e podem ser utilizados diversos subprodutos agroindustriais, tais como farelo de trigo, farelo de arroz, milho, centeio. Estes suplementos servem como nutrientes para fornecer uma faixa ótima de relação C/N para cada linhagem produzida. Outros suplementos inorgânicos, adicionados em menores quantidades incluem $CaCO_3$ e gesso, com a finalidade de corrigir propriedades químicas e físicas do substrato (Chen, 2005). Os ingredientes são combinados num misturador com adição de água para atingir-se o teor de umidade ideal. Os sacos são feitos de polipropileno resistente ao calor e contêm um *patch filter* microporoso que permite o metabolismo aeróbio do fungo. Após preenchidos com substrato os sacos são esterilizados durante 2 h a 121°C e após resfriados e inoculados com *spawn* (2-3 % do peso do substrato úmido) em sala asséptica para posteriormente serem transferidos para a sala de colonização (Chen, 2004).

(a)

(b)

Figura 4 - Sistemas de cultivo de Shiitake: (a) método em toras de madeira (fonte: http://www.technologychaoban.com/index.php; (b) método axênico em serragem suplementada (cortesia: Martin Van de Vorle, 2014).

A nutrição do Shiitake são fontes de carbono e de nitrogênio, das quais são estabelecidas em uma relação C/N, minerais e vitaminas. Durante a fase de propagação o Shiitake produz micélio através de um crescimento saprofítico. O micélio absorve diretamente pequenas moléculas de nutrientes, como sais minerais e outras substâncias solúveis a partir do xilema e do floema da madeira, ou cliva moléculas alimentares complexas através da secreção de enzimas lignocelulolíticas, amilolíticas e proteolíticas, dependendo da natureza do substrato (Stamets, 2000).

Materiais com uma relação Carbono/Nitrogênio de 25/1 são recomendados para o melhor crescimento micelial vegetativo, enquanto que materiais com uma C/N de 40/1 são as faixas mais indicadas para a fase de produção de cogumelos (Chen 2005). Nitrogênio em excesso pode permitir que o micélio cresça vigorosamente, mas não virá a produzir cogumelos de boa qualidade. A concentração de nitrogênio mais adequada durante a fase de propagação do micélio é de 016-0,064 %, enquanto que 0,02 % é a mais indicada para a fase reprodutiva (Wu, 2000).

Os principais elementos minerais, tais como fósforo, enxofre, cálcio, magnésio e potássio são utilizados nas atividades fisiológicas da célula para produção de energia assim como para manter equilibrada a pressão osmótica celular. Fósforo e potássio, em particular, não só são benéficos para o crescimento do micélio, mas também para a formação de basidiomas. Os microelementos (Os Fe, Cu, Zn, Mn, B, Mo) são indispensáveis, pois atuam como catalisadores da ação de enzimas específicas (Bisko, 2004). Compostos inorgânicos como KH_2PO_4, K_2HPO_4, gesso, fosfato de magnésio, em concentrações de 100-500 mg/L, são geralmente utilizados na formulação de substratos, visando incrementar estes nutrientes (Oei, 2003). A vitamina B1 (tiamina) é necessária para o crescimento micelial e frutificação, estando presente em farelos como os de arroz ou de trigo, entretanto,

esta vitamina é sensível ao calor. Decompõe-se acima de 120°C e, por este motivo o sobreaquecimento durante a esterilização do substrato deve ser evitado (Chen, 2005).

Shiitake são fungos de climas temperados (Ting, 1994). Quando as temperaturas são muito elevadas, as enzimas podem perder a sua viabilidade. Quando a temperatura é demasiadamente baixa torna-se reduzida a assimilação de nutrientes, visto que a atividade enzimática torna-se menor e a taxa de respiração diminui. Isto resulta em uma diminuição do crescimento micelial (Stamets, 2000). A faixa de temperatura ideal para o crescimento micelial do shiitake é de 24-27°C. Entretanto, a espécie pode ser adaptada para crescer numa grande gama de temperaturas de 5 a 32°C. Entretanto, são vulneráveis às altas temperaturas e a morte celular ocorre acima de 40°C. A faixa ideal de germinação dos esporos fica entre 22 a 26°C, mas este processo pode ocorrer em uma amplitude de 15 a 28°C. A frutificação pode ocorrer em faixas de temperatura de 5 a 25°C, entretanto a faixa ótima é de 15 ± 1-2°C e é dependente da cepa utilizada (Chen, 2005).

Durante a colonização do micélio no substrato a faixa ótima de umidade relativa do ar (UR) fica entre 50 e 55 % e durante a frutificação entre 85 e 95 % de umidade. A manutenção da UR na etapa da frutificação abaixo de 30 % dificulta a formação dos cogumelos, enquanto que UR acima de 95 % são produzidos cogumelos de baixa qualidade e suscetíveis ao apodrecimento (Chang and Miles, 1989).

A umidade do substrato promove a dissolução dos nutrientes, a fim de serem absorvidos pelo micélio. Do mesmo modo, os resíduos metabólicos precisam ser dissolvidos em água, a fim de ser eliminados. O crescimento micelial pode ocorrer com umidade do substrato variando entre 55 % e 75 %. Abaixo de 50 % e acima de 65 % a velocidade de extensão do micélio diminui.

Shiitake são fungos aeróbios (Urben et al., 2001). Durante seu metabolismo, a disponibilidade de oxigênio é importante para que compostos orgânicos sejam oxidados através da respiração. A energia é liberada e armazenada na forma ATP para ser utilizada no crescimento micelial e frutificação. (Stamets, 1983). De maneira geral, é necessário mais oxigênio durante a fase da reprodução do que durante a fase de crescimento do micélio vegetativo. Em altas concentrações de CO_2 ocorre malformação dos cogumelos. Uma concentração de CO_2 maior do que 10.000 ppm inibe o desenvolvimento de corpos de frutificação e induz de forma precoce a abertura do píleo dos cogumelos (Chen, 2005). Quando os níveis de CO_2 chegarem a 50.000 ppm não é observada a formação de corpos de frutificação (Stamets, 2000).

A luz é necessária para o shiitake na formação de corpos de frutificação e na formação dos esporos e dispersão, entretanto, na fase vegetativa, sob luz fraca, difusa ou na ausência desta o micélio cresce de forma mais vigorosa do que sob a luz forte e direta. Na escuridão, o micélio cresce 3-4 vezes mais rápido do que em 500 lux. O nível de iluminação ideal é 50-100 lux de luz difundida durante a frutificação (Chen, 2001).

Enzimas extracelulares atuam em um intervalo de pH específico quando hidrolisam o substrato. O Shiitake pode crescer em uma ampla faixa de pH (3 a 7) com a ótima faixa de pH situando-se em um ambiente ácido de 4.5- 5.5. A melhor faixa para a formação do primórdio é num intervalo de pH 3,5 - 4,5 (Silva, 2005). O pH inicial do substrato geralmente situa-se entre 5 - 6. Com o crescimento do micélio, ácidos orgânicos são produzidos, diminuindo o pH do substrato. K_2HPO_4 e KH_2PO_4 são adicionados ao substrato para tamponar e estabilizar o pH. As serragens de madeira utilizadas para o cultivo de shiitake têm um valor de pH adequado e

não necessitam ser ajustadas, entretanto, deve-se ser dada atenção quando a água disponível é de natureza alcalina (Tarui, 1997).

O período de colonização do substrato depende da densidade de semeadura (Minhoni, 2005). A média é de 20 a 25 dias, executado em 21 h no escuro e 4 h de luz por dia. Os sacos são espaçados aproximadamente 1 cm de distância para facilitar a circulação e resfriamento do ar. Com este método, os sacos são retirados do substrato após a completa colonização e são expostos a um ambiente propício para escurecimento do substrato. A oxidação da superfície do micélio é caracterizada pela formação de um "acastanhamento" caracterizado pela formação de uma camada de hifas que é resistente à dessecação (Chen, 2005). Durante o período de escurecimento que dura em média 30 dias os blocos, são mantidos a uma temperatura de 19°C, enquanto que os níveis de CO_2 são mantidos entre 2.200 e 3.000 ppm (Stamets, 1983). A manutenção de CO_2 a estes níveis exige menor uso de energia e promove o escurecimento mais rápido das superfícies do bloco (Chen, 2005).

Com o escurecimento o processo de incubação do substrato está praticamente concluído, iniciando-se a formação de primórdios, visíveis como rachaduras formadas na camada de hifas de proteção de cerca de 1-2 mm abaixo da superfície (Urben et al., 2001). Ao escurecer o bloco fora do saco são produzidas hifas mais firmes e blocos de serragem mais resistentes à quebra durante o manuseio e, além disto, a qualidade dos cogumelos tende a ser melhor quando os blocos de serragem são escurecidos fora do saco. A desvantagem é a gestão operacional que exige regas diárias e manutenção de umidade relativa do ar superior, de modo que as superfícies do bloco não ressequem, aumentando assim a incidência de parasitas como *Trichoderma spp*. (Stamets, 2000).

Para estimular a maturação de primórdios quando o escurecimento é concluído, os blocos podem ser imersos em água durante 3-4 horas. Para os blocos que são escurecidos dentro do saco a imersão no primeiro fluxo produtivo não se faz necessária porque a água disponível é suficiente para sustentar os primeiros cogumelos formados. No entanto, a imersão dos blocos nos fluxos subsequentes é necessária (Oei, 2003).

Após a imersão, os blocos são colocados em prateleiras e os cogumelos começam a surgir e, aproximadamente 7-11 dias após a imersão, estão prontos para a colheita. Os cogumelos são torcidos a partir da superfície e o substrato residual é removido (Minhoni, 2005).

As chaves para o cultivo bem-sucedido de shiitake incluem um substrato de boa qualidade com uma suplementação equilibrada. Em cada cultivo deve-se atentar para os estágios diferentes da incubação do substrato e da frutificação. Mantendo as condições ideais e linhagens recomendadas permite ao produtor produzir altos rendimentos e cogumelos de melhor qualidade (Chen, 2005).

4. Resultados

Os resultados deste trabalho estão apresentados no formato de artigos científicos, diagramados conforme respectivo periódico científico e intitulados como:

Artigo I - GROWTH KINETICS AND MODELING OF LENTINULA EDODES (BERK.) PEGLER CULTURES IN AN EXTERNAL LOOP AIRLIFT BIOREACTOR. Submetido para o periódico: *Process biochemistry.*

Artigo II (short communication) - PHYSICO-CHEMICAL PROPERTIES OF THE SUBSTRATES AND MYCELIAL GROWTH RATE DETERMINATION IN LENTINULA EDODES (BERK.) PEGLER DIKARYOTIC STRAINS. A ser submetido.

Artigo III - The axenic cultivation of *lentinula edodes* (berk.) pegler: compost optimization and evaluation of dikaryotic strains. A ser submetido para o periódico: *Food and bioprocess technology*.

Os resultados adicionais estão apresentados no formato Apêndice, intitulado:

EVALUATION OF HYBRID LENTINULA EDODES (BERK.) PEGLER STRAINS BY INTRASPECIFIC MONOSPORIC CROSSES.

GROWTH KINETICS AND MODELING OF LENTINULA EDODES (BERK.) PEGLER CULTURES IN AN EXTERNAL LOOP AIRLIFT BIOREACTOR

Diego Melo Pereira[a], Barbara Alves Zolet[a], Débora Jung Luvizetto Faccin[b], Marco Antônio Záchia Ayub[a,*]

[a]Biotechnology & Biochemical Engineering Laboratory (Bioteclab), & [b]Chemical Engineering Department, Federal University of Rio Grande do Sul, Av. Bento Gonçalves, 9500, P.O. Box 15090, ZC 91501-970, Porto Alegre, RS, Brazil

* Corresponding author:
Tel.: +55 51 3308 6685; Fax + 55 51 3308 7048
E-mail address: mazayub@ufrgs.br (M.A.Z. Ayub)

Abstract

In this work, we developed a submerged culture system for mycelium production of Shiitake (*Lentinula edodes* (Berk.) Pegler) using an external loop airlift bioreactor designed by us. Aeration conditions (0.16, 0.20, and 0.24 vvm) and inocula sizes (1.0 $g.L^{-1}$ and 2.0 $g.L^{-1}$) were tested. The maximum biomass (dry weight) achieved in the airlift bioreactor was approximately 15.5 $g.L^{-1}$, reached after 10 days of cultivation under the operating conditions of 1 $g.L^{-1}$ of inoculum and 16 vvm of aeration. The specific growth rate μ_{max} was 0.516 d^{-1}, and yield factors for biomass ($Y_{x/s}$), and productivity (P_X) were 1.06 $g.g^{-1}$, and 0.061 $g.L^{-1}.h^{-1}$, respectively. A mathematical model approach was employed in order to describe the culture kinetics based on the mass balances for biomass, CO_2 metabolic production, glucose consumption, and acidification of the culture medium by the production of H^+ of the *L. edodes*, results producing congruent fitting between model and experimental data.

Keywords: *Lentinula edodes*; shiitake; liquid spawn; airlift bioreactor; growth kinetics modeling; basidiomycete.

1. Introduction

Lentinula edodes (Berk.) Pegler, commonly known as Shiitake, is the second most cultivated edible mushroom in the world (Czarnecka et al. 2014), having a great economic importance for the food industry. Saprophyte basidiomycetes filamentous fungi are able to degrade lignocellulosic components of wood, fact that is explored for their commercial production, in which logs and sawdust are used as source of nutrients and support for growing this species (Rossi et al. 2003; Royse and Sanchez 2007; Philippoussis et al. 2007). However, the axenic cultivation method, when the microorganism is inoculated in a previously sterilized substrate, has advantages over cultivation in logs and has been widely adopted by some producers, allowing optimized production, reducing the incubation time, and providing better production performances of the mushroom (Chen 2005; Philippoussis et al. 2011). This system of cultivation involves four specific steps that are, (a) the manufacture of sawdust substrate, (b) the development of inoculation of spawn, (c) the spawn run, and (d) the full fruiting (Kawai 1996; Chang and Miles 1989). The traditional technology has been to inoculate the mycelia mass to colonize cereal grains when using previously sterilized substrate for inoculation. More recently, however, the inoculation of a liquid culture of mycelia has been developed allowing the use of submerged cultures of this fungus, which has been demonstrated to produce better results than the solid inoculation process for the production of spawn in the axenic cultivation of shiitake (Kirchhoff and Lelley 1991; Kawai 1996; Chen 2005). Submerged inocula preparation for mushroom cultivation on solid media (Silveira et al. 2006) has several advantages over the traditional methods based on grains inoculation because it needs simpler and smaller equipment (vessel or bioreactors), reduced costs of substrate manipulation, and reduced risks of contamination, thus being considered an important development in the spread of axenic substrates (Chang 1993; Wuest 1989; Kawai 1996).

The successful development of submerged cultures of filamentous fungi depends on the correct choice of bioreactor type, including its geometry, as well as on the parameters of operating conditions. The airlift bioreactor, commonly used in aerobic bioprocesses of yeasts and bacteria (Chisti 1989), appears to be an ideal configuration to be used for filamentous fungi biomass formation because of the lower shear stress imposed on cells, when compared with stirred tank bioreactors (STR). Fluidization of the culture medium in airlifts is promoted only by the occasional injection of air and as a consequence of its own geometry (Chisti and Moo-Young 1987; Chisti 1989). Moreover, airlifts have an unique advantage when increasing the operational scale because they have no turbines or other moving parts (Onken and Weiland 1983), which require energy, thus being of simpler construction and of cheap operation and maintenance (Moresi 1981).

On the other hand, mathematical kinetic models have been used as tools in bioprocess, applied for the prediction of microbial growth, thus allowing to analyze, to design, to improve, and to facilitate the control of cultures. Although several literature reports have been published to describe non-structured models of bacterial cultures, very few were specifically developed for filamentous fungi (Papagianni 2004). These mathematical models can be used to describe the environmental conditions within the bioreactor as a function of time (Viniegra et al. 1994; Mitchell at al. 2004). For the spawn development of *L. edodes* in airlift submerged cultures in particular, there are no proposed models that could be used to predict growth or any other kinetic parameter.

In this context, the aims of this study were to develop a consistent submerged culture of *Lentinula edodes* in an in-house projected airlift bioreactor and to study the effects of the size of the inocula and the influence of the aeration rate on cell growth. Mathematical modeling of this system was carried out using the EMSO software (Soares and Secchi 2003). Parameters of the model were estimated according experimental data at the best culture conditions.

2. Materials and methods

2.1 Strain and materials

The basidiomycete fungus *Lentinula edodes* (Berk.) Pegler, strain Led-08, was used in this study. This microorganism was isolated from tissue culture of sporophore and identified by amplification of the ITS1-5.8S-ITS2 regions of ribosomal DNA, presenting a DNA fragment of 734 base pairs (bp) and cleavage sites matching restriction enzymes *HhaI* (two overlapping bands of 367 bp), *HaeIII* (two bands of 702 and 32 bp), and *HinfI* (three bands of 399, 327, and 8 bp), being considered *L. edodes* when compared to the access GQ866860.1 (735 bp) of the National Center for Biotechnology Information (NCBI) and similar cleavage using NebCutter V2.0 tool (New England Biolabs). The Led-08 strain was selected because this strain is recommended for axenic culture systems. Working stocks were kept in test tubes at 4 ^{o}C (Microbial collection of the BiotecLab (Food Science and Technology Institute, UFRGS, Porto Alegre, Brazil). Unless otherwise indicated, all reagents and media components were bought from either Sigma-Aldrich (St. Louis, USA), or Vetec (Rio de Janeiro, Brazil).

2.2 Media and culture conditions

Cultures pre-inocula were obtained as static mycelium cultures by inoculating 8 mm diameter agar discs in 20 mL of MCM culture medium (Mushroom Complete Medium) in 250 mL Erlenmeyer flasks, and grew at 25° C. After 12 and 21 days of incubation, 1 $g.L^{-1}$ and 2 $g.L^{-1}$ of mycelia biomass were usually obtained. The Mushroom Complete Medium was composed of (in $g.L^{-1}$): meat peptone, 2; yeast extract, 2; glucose, 20; $MgSO_4.7H_2O$, 0.5; KH_2PO_4, 0.46; K_2HPO_4, 1; supplemented with trace minerals solution containing (in $g.L^{-1}$): $MnSO_4.5H_2O$, 0.5; NaCl, 1; $FeSO_4.7\ H_2O$, 0.1; $CoCl_2.6\ H_2O$, 0.1; $ZnSO_4.7H_2O$, 0.1; $CuSO_4.5H_2O$, 0.01; $AlK(SO_4)_2$, 0.01; H_3BO_3, 0.01; $NaMoO_4.2H_2O$, 0.01; the pH was adjusted to 5.8. The biomass obtained was filtered through a sieve (0.4 mm mesh), washed with sterile distilled water and subsequently fragmented for 20 s on a sterilized blender, added of 200 mL of MCM medium and immediately inoculated in to the airlift bioreactor containing 1.3 L of MCM medium. Culture temperature was always maintained at 25 ◦C and the initial culture pH was adjusted to 5.6 using 1 M H_3PO_3 1 M or 2 M NaOH.

2.3 External loop airlift bioreactor (ELAB)

All cultures were performed in an in-house projected and constructed 1.8 L airlift bioreactor (ELAB), with 1.5 L working volume (Figure 1). Bioreactor operation was controlled using a BIOSTAT B computer unit (Braun Biotech International, Germany), connected to a remote PC-computer for data acquisition, monitoring,

and control. The addition of the inoculum, reagents for pH control (1 M H_3PO_3 or 2 M NaOH), and antifoam were carried out through ports on the top cover. A condenser mounted on the cover provided the exhaustion of gas and minimizing water loss by evaporation. The operating temperature was kept at 25 ºC by a cooling water jacket. The bioreactor was sterilized at 121 ºC, 20 min, and let cool prior to inoculations.

2.4 Analytical methods

On-line measurements of CO_2 evolution rate (CER) during cultivations were monitored using a Bluesens Gas Sensor (Herten, Germany). Aeration varied from 0.16 vvm to 0.24 vvm, providing 100 % oxygen saturation of the liquid culture at the beginning of cultivations. CER was calculated using Eq. (1), described by Koutinas (2003):

$$\mathrm{CER} = MW.[(F.(A - A_0).0.01)/BV] \quad (1)$$

Where CER is the CO_2 evolution rate (g CO_2.(L.h)$^{-1}$), F is the aeration rate (L.h^{-1}), A is the percentage of CO_2 in the exit gas (on a volume basis), A_0 is the percentage of CO_2 in the inlet gas (on a volume basis), MW is the molecular weight of CO_2 (44), V is the working volume of the bioreactor (1.5 L), and B is the molecular volume of CO_2 (22.4 L).

Biomass dry weight was determined by centrifuging 4.0 mL of samples (4 500 *g*, 15 min), drying at 75 ºC until reaching constant weight. The supernatant was used to analyze glucose concentration by HPLC (Shimadzu, Japan), equipped with a refractive index detector and a Bio-Rad HPX-87H column (300 mm (Shimadzu, Japan) using 5 mM sulfuric acid as eluent at 45 ºC, flow rate of 0.6 mL min^{-1} and sample volumes of 20 **µ**L.

All assays were performed in duplicate. Results were evaluated by analysis of variance (ANOVA) and Tukey test ($p \leq 0.05$).

2.5 Mathematical model for the determination of fungal growth

The mathematical models representing *L. edodes* cultures proposed in this research were basead on the mass balances for biomass (X), CO_2 metabolic production (P), glucose consumption (S), and acidification of the culture medium by the production of H^+, as demonstrated in Equations 2 to 6. The expression for specific growth rate (µ) was the Monod Model applied to the growth-limiting substrate.

Total Biomass: $\frac{dX}{dt} = \mu.X$ (2)

Substrate: $\frac{dS}{dt} = -\left(\frac{1}{Yx/s}\right)\mu.X$ (3)

Specific growth rate: $\mu = \mu max\left(\frac{S}{S+Kss}\right)$ (4)

CO_2 Production: $\frac{dP}{dt} = \alpha P.\mu.X$ (5)

(H^+): $\frac{dH}{dt} = \alpha H.\mu.X$ (6)

Where X is biomass dry weight, S is the glucose concentration, μ is the specific growth rate, μmax is the maximum specific growth rate, Kss represents the saturation constant of Monod model, αP is the slope of the CO_2 metabolic production, and αH is the slope of the formation of H^+.

It was also applied the mass balance to oxygen equilibrium in the gas and liquid phases (eq. 7 and 8) in order to describe oxygen consumption.

Oxygen balance in the gas phase: $\frac{dCg}{dt} = \frac{Far}{Vg}(Cgo - Cg) - k_l a(Cle - Cl).\frac{Vl}{Vg}$ (7)

Oxygen balance in the liquid phase: $\frac{dCx}{dt} = k_l a(Cle - Cl) - kpO.\mu.X$ (8)

Where Cl is the concentration of dissolved oxygen in the liquid phase ($g.L^{-1}$); Cle is the liquid-phase oxygen concentration in equilibrium with the gas ($g.L^{-1}$); Cg is the concentration of oxygen in the gas phase ($g.L^{-1}$); k_la is the gas-liquid mass transfer coefficient of the oxygen (h^{-1}); Far is the air flow rate injected in the bioreactor ($L.h^{-1}$); Vg is the internal volume of gas in the bioreactor (L); Vl is the volume of liquid of the bioreactor (L); Cgo is the oxygen concentration in the air feeding flow ($g.L^{-1}$), and kpO is the constant of oxygen consumption by microorganisms.

Parameters estimation were carried out using the software EMSO (Soares, 2003), using the Flexible Polyhedron method (Nelder, 1965), based on data of the best bioreactor operation results. The coefficient of determination (R^2), was used to compare the goodness of fit of the tested models.

3. Results and discussion

3.1 Performance of L. edodes Led-08 *cultivation in the airlift bioreactor*

Microbial biomass formation is essentially influenced by the oxygen supply of the system. Therefore, definition of variables in this set of experiments was carried out to understand the effect of aeration rate and inoculum size on the growth of *L. edodes* Led-08 in submerged batch cultivation in the external loop airlift bioreactor designed by our research group. In Figure 2 are presented the kinetic profiles of cultivations of *L. edodes* Led-08 as functions of aeration rate and inoculum size, whereas in Table 1 are presented the calculated parameters of growth, yields, and productivities. Best results in terms of biomass formation and yields were obtained when the bioreactor was operated at 0.16 vvm aeration rate and 1 $g.L^{-1}$ of inoculum. Increasing aeration rates to 0.20 or higher negatively affected these values, probably because the strong aeration allowed for a much faster growth rate (Table 1), which is known to cause metabolic fluxes impairments, consequently affecting biomass formation. This result is confirmed by the increased CO_2 productions (Table 1 and Figure 2 B) under the highest aeration conditions and the marked decrease in the values of pH (Figure 2 C).

Acidification of the culture medium for the growth of fungi is related to the production of organic acids (Yang and Liau 1998; Makela et al. 2002; Shu and Lung 2004), with several authors reporting changes in pH during culture growth, for several species of Basidiomycetes (Chao et al. 2011; Enman et al. 2008; Go et al. 1984; Lelik et al. 1997; Furlan et al. 1997). This pH reductions acts on metabolism of membrane proteins and is

a critical factor hampering mold growth because it will affect the function of the cell membrane, cell morphology, the solubility of intra and intermembrane salts, and the ionic state of substrates, compromising the absorption of various nutrients by the cell, the cell enzyme activity, and the biosynthesis of products **(**Elisashvili 2012; Kim et al. 2002**).**

The best pH value reported in the literature for the growth of mycelium of *L. edodes* is in the range of 4.8 to 5.0 (Chang and Miles 1989). However, in our work, the pH control at 5.0 produced detrimental effect on cell growth and metabolism when the dissolved oxygen fell to critical concentrations, in the case of *L. edodes*, when the dissolved oxygen in the culture medium fell below 40 % (Figure 3), when we observed darkening of the culture medium and cell death. The biomass obtained under these conditions was 7.4 g. L^{-1} in 144 h. The negative effect of the combination of pH control and decrease in the concentration of oxygen in the culture was not observed when the pH was not controlled (Table 1, aeration of 0.16 vvm and inoculum size of 1 g. L^{-1}), where the formation of biomass was kept up to 24 % of dissolved oxygen (Figure 2 a and d). Below this value of oxygen saturation growth was interrupted because of mass transfer problems due to the high cellular density (Elisashvili, 2012).

At high aeration rates, and in especial when the inoculum size was 2.0 g.L^{1}, we could notice a high turbulence in the bioreactor, resulting in the formation of large pellets of cell, containing regions of inactive cells in this mass, similar to what has been demonstrated by Rossi (2002) during cultivation of *Pisolithus microcarpus* in airlift bioreactors. The size and type of inoculum, agitation, composition of the medium, temperature, cellular viability, dissolved oxygen concentration, mechanical stress, and pH are the main factors influencing the formation of pellets. (Enman et al. 2008; Go et al. 1984; Lelik et al. 1997; Furlan et al. 1997). During the submerged cultivation of *Ganoderma lucidum* in airlift bioreactor without pH control, Fang and Zhong (2002) observed a change in mycelia morphology following acidification of the culture medium, from initial pH 6.0 to 2.6. The authors suggested that the pH reduction by cell metabolism decreased the viscosity of the liquid.

Elisashvili et al. (2004) demonstrated that exopolysaccharides formation in submerged cultivation of *L. edodes* is strongly affected by the carbon source used, especially glucose. The stress caused by the initial acidification of the culture medium for this fungus significantly inhibited mycelia growth, whereas for higher initial pH better cell growth was observed. This behavior was also observed in several studies for other Basidiomycetes (Elisashvili 2012; Stanbury et al. 1995; Kim et al. 2002). A possible explanation for this metabolic behavior could be that fungi have the ability to change environmental pH during growth, which is related to the maintenance of a suitable internal ionic equilibrium (Cooke 1993). In General, fungi growth is best under slightly acid conditions, with pH values between 4 and 6, values that may inhibit bacteria growth (Yang and Liau 1998).

Enman et al. (2008) studied the formation of *L. edodes* biomass in submerged cultures in flasks under agitation and without pH control and compared results with cultures in STR bioreactors, with or without pH control, operated the 25° C, 1.0 vvm and agitation of 50, 150, and 250 rpm. The results showed a pronounced

decrease in pH of cultures in flasks and bioreactors without pH control. The speed of agitation in bioreactors presented a positive effect on biomass production, regardless of the pH control.

Surprisingly, in the experiments where an inoculum of 2 $g.L^{-1}$ was employed there was a significant reduction of the **μ**m (Table 1), as well as biomass formation, consequently affecting all yields. In order to obtain the necessary amount of mycelium for a 2 $g.L^{-1}$ inoculum, a 21 days cultivation in static cultures were required, and we noticed the formation of small stromal cells in the pre-inoculum, containing inactive cell fractions, and the drop in pH (Figure 2 C) was accentuated in these cultures, possibly affecting the respiratory condition of the fungus.

Based on the results obtained in this set of experiments, the conditions of 0.16 vvm of aeration and 1.0 $g.L^{-1}$ of inoculum were chosen to carry out the experiments for the modeling of the cultivation of *L. edodes* Led-08.

3.2 Model development using the EMSO software

Proposing a mathematical model for the growth of *L. edodes* in submerged bioreactor cultures could be an important tool in scaling-up the process for the production of industrial inocula of this fungus. Therefore, the parameters of the model were estimated using the experimental data based on a fermentation under the best conditions previously described here. For the construction of the model, the parameters μ_{max}, k_la, and $Y_{x/s}$ were determined by calculations based on the experimental data (Table 1), in order to adjust them to the model prediction. In Figure 4 are presented the kinetics of experimental data and the predicted models. In Table 2 are listed the estimated parameters Kss, αP, αH, and kpO. The model showed a good fit for biomass (X), dissolved oxygen (pO_2), and pH, represented by the quadratic regression. For the substrate (S) and the metabolic production of CO_2 (P), R^2 values were somewhat less predictive, but still satisfactory according to the model was not fit to represent the product and biomass increment in the final stages of cultivation (Figure 4, from 192 to 240 h). This behavior was associated with the negative impact on mass transfer rates resulting from the high cell density, which is also confirmed by the decrease in metabolic CO_2 evolution rate (Figure 2 c), reduction of dissolved oxygen (Figure 2 d), and the consumption of glucose.

Kinetic models of growth of filamentous fungi presented in the literature suggest that the growth curve should be separated into several stages, with a different equation for each phase of cultivation (lag, exponential growth, linear growth, slowdown, stationary and decline) (Mitchell et al. 2004; Sanchez 2004). The batch process represented by the mathematical model described in this work does not provide the deceleration of cell growth because we did not observe a specific phase of decline or cell death. This is confirmed by the metabolic production of CO_2, the consumption of oxygen and glucose, and the formation of H^+ ions by decreasing the pH, parameters considered in the derived equations as growth associated, thus accompanied by the increase of biomass until the closing stages of cultivation (Mitchell et al., 2004; Mazaheri et al. 2013). Therefore, it is considered that the kinetics of *L. edodes* described by the model represents only the estimation of the exponential growth and stationary phases, confirming the prediction of the model with respect to empirical equations and reducing the negative effect of the experimental data affected by mass transfer problems. The Monod model

proposed in this work for *L. edodes*, and the mass balance between gas and liquid phases for oxygen (equations 7 and 8), have proven reliable parameters for scaling up studies because the predictive results for X, pO2, and pH were statistically significant.

The model for *L. edodes under* the operating conditions used in this work proposes a kinetic based on the metabolic pathways of carbon balance, using glucose for the formation of CO_2 as a metabolic product, and expressed in terms of mass of cell, and following the oxygen consumption rate and the acidification of the culture medium. These data are essential for the optimization and scale-up of this bioprocess, because there is no published data in the literature for the cultivation of this fungus in airlift bioreactors of external circulation. The model might also be used as precursor to models in which the products of metabolism are not growth associated or to the models predicting cell growth impairment by accumulation of biomass.

Conclusion

The development of submerged cultivation of *Lentinula edodes* in airlift bioreactors as the one designed in this research may help in the scaling-up production of industrial inocula for the production of mushroom. Optimal operating conditions were achieved using a moderate aeration rate and small inoculum size. Strong aeration conditions showed a negative influenced for the culture because it stimulates fast growth rates, impairing metabolic fluxes and reduction of biomass production. The mathematical model developed using the EMSO software allowed to reproduce the parameters of biomass, respiration rates, and pH variation of cultures at different stages of growth of this microorganism. These results might be applicable to bioreactor engineering aimed at the production of liquid inoculants for mushroom cultivation, in especial to *Lentinula edodes* under the axenic method.

Acknowledgements

The authors wish to thank Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Fundação de Amparo a Pesquisa do Rio Grande do Sul (FAPERGS) for the funding of the research and the scholarships of the first two authors.

References

Chang ST, Miles PG (1989). Edible mushrooms and their cultivation. CRC Press, Boca Raton, 345 p

Chang, ST (1993) Mushroom biology: The impact on mushroom production and mushroom products, In: Mush biol and mush prod, (ed. by Chang, S., Buswell, J.A. and Chiu, S.), pp. 3-20

Chao CH, Wu HJ, Lu MK (2011). Promotion of fungal growth and underlying physiochemical changes of polysaccharides in *Rigidoporus ulmarius*, an edible basidiomycete mushroom. Carbohydr Polymers (85): 609–614

Chen, AW (2005) What is shiitake. In: Gush R (ed) Mushroom growers' handbook 2. Mush World, Seoul, pp 1–11

Chisti MY (1989). Air-Lift Bioreactors, Elsevier, New York, 345 p

Chisti MY, Moo-Young M (1987) Airlift reactors: characteristics, applications and design considerations. Chem Eng Commun (60): 195-242

Cooke RC, Whipps JM (1993) Ecophysiology of fungi. Blackwell Scientific, Oxford, UK, 348 p

Czarnecka AB, Kreft B, Marsch WC (2014). Flagellate dermatitis after consumption of Shiitake mushrooms. Adv in Dermat and Allerg (3): 187–190. http://doi.org/10.5114/pdia.2014.40929

Elisashvili V (2012). Submerged cultivation of medicinal mushrooms: bioprocesses and products (review). Int J Med Mushrooms (3): 211-239

Elisashvili V, Wasser S, Tan KK, Chichua D, Kachlishvili E (2004). Extracellular polysaccharide production by culinary medicinal shiitake mushroom *Lentinus edodes* (Berk.) Singer and *Pleurotus* (Fr.) P. Karst. species depending on carbon and nitrogen source. Int J Med Mushr. (6): 165–727

Enman J, Hodge D, Berglund KA, Rova U (2008). Production of the Bioactive Compound Eritadenine by Submerged Cultivation of Shiitake (*Lentinus edodes*) Mycelia. J Agric Food Chem (56): 2609–2612

Fang FQH, Zhong JJ (2002) Effect of initial pH on production of ganoderic acid and polysaccahride by submerged fermantation of *Ganoderma lucidum*. Process Bioch, (37): 769- 774

Furlan SA, Virmond LJ, Miers, DA, Bonatti M, Gern RMM, Jonas R (1997). Mushroom strains able to grow at high temperatures and low pH values. World Jour of Microb & Biotech, (13): 689-692

Go SJ, You CH, Park YH (1984). Effect of temperature, pH, carbon and nitrogen nutrition on mycelial growth of *Pleurotus sajor-caju* (Fr.) Sing. and *Pleurotus ostreatus* (Fr). quél. Korean Jour of Myc, (12): 15-20

Kawai G, Kobayashi H, Fukushima Y, Ohsaki K (1996). Effect of liquid mycelial culture used as a spawn on sawdust cultivation of shiitake (*Lentinula edodes*). Mycoscience 37: 201-207

Kim SW, Hwang HJ, Park JP, Cho YJ, Song CH, Yun JW (2002). Mycelial growth and exo-biopolymer production by submerged culture of various edible mushrooms under different media. Lett Appl Microbiol. 34(1): 56-61

Kirchhoff B, Lelley J (1991). Investigations of shiitake (*Lentinus edodes* (Berk.) Sing.) bag-log cultivation to increase the yield in Germany. In: Science and cultivation of edible fungi, (ed. by Maher, M. J.), pp. 509-516. Balkema, Rotterdam

Koutinas AA, Wang R, Webb C (2003). Estimation of fungal growth in complex, heterogeneous culture. Bioch Eng Jour (14): 93–100

Lelik, L, Vitanyi G, Lefler J, Hegoczky, J, Nagy-Gasztonyi M, Vereczkey G (1997). Production of the mycelium of Shiitake (*Lentinus edodes*) mushroom and investigation of its bioactive compounds. Acta Aliment (26): 271–277

Makela M, Galkin S, Hatakka A, Lundell T (2002), Production of organic acids and oxalate decarboxylase in lignin-degrading white rot fungi. Enz and Microb Techn, (30): 542-549

Mazaheri D, Shojaosadati SA (2013). Mathematical Models for Microbial Kinetics in Solid-State Fermentation: A Review. Iran. J. Biotech (3) 156 - 167

Mitchell DA, Meien O, Krieger N, Diba F, Dalsenter H (2004). A review of recent developments in modeling of microbial growth kinetics and intraparticle phenomena in solid-state fermentation. Bioch Eng Jour (17): 15–26

Mitchell DA, Meien OFV, Krieger N, Dalsenter FDH (2004) A review of recent developments in modeling of microbial growth kinetics and intraparticle phenomena in solid-state fermentation. Bioch Eng Jour (17): 15–26

Monod J (1942) Recherches sur la croissance des cultures bacteriennes. Hermann et Cie, Paris

Moresi M (1981). Optimal design of airlift fementers. Biotechnol Bioeng (23): 2537-2560

Nelder JA, Mead R (1965). A simplex method for function minimization. Comput Jour (7): 308-313

Onken U, Weiland P (1983) Airlift Fermenters: Construction, Behavior, and Uses, Adv. Biotechnol Processes pp. 67-95

Papagianni M (2004). Fungal morphology and metabolite production in submerged mycelial processes. Biotech Adv (22): 189-259

Philippoussis A, Diamantopoulou P, Israilides C (2007) Productivity of agricultural residues used for the cultivation of the medicinal fungus Lentinula edodes. Int Biodeterior Biodegrad 59(3):216–219

Philippoussis A, Diamantopoulou P, Papadopoulou K, Lakhtar H, Roussos S, Parissopoulos G, Papanikolaou S (2011). Biomass, laccase and endoglucanase production by Lentinula edodes during solid state fermentation of reed grass, bean stalks and wheat straw residues. World Jour of Microb and Biotech, v. 27, (2): 285-297. http://dx.doi.org/10.1007/s11274-010-0458-8

Rossi IH, Monteiro AC, Machado JO, Andrioli JL, Barbosa JC (2003). Shiiatke *Lentinula edodes* production on a sterilized bagasse substrate enriched with rice bran and sugarcane molasses. Braz J Microbiol 34: 66–71

Rossi MJ, Souza JA, Oliveira VL (2002) Inoculum production of the ectomycorrhizal fungus *Pisolithus microcarpus* in an airlift bioreactor. Appl Microbiol Biotechnol. (59): 175-181

Royse DJ, Sanchez JE (2007) Ground wheat straw as a substitute for portions of oak wood chips used in Shiitake (*Lentinula edodes*) substrate formulae. Biors Technol 98: 2137–2141

Sanchez C (2004). Mini review: modern aspects of mushroom culture technology. Appl Microbiol Biot 64(1):756-762

Shu CH, Lung MY (2004). Effect of pH on the production and molecular weight distribution of exopolysaccharide by *Antrodia camphorata* in batch cultures. Process Biochem 2004 39:931–37

Silveira M, Wisbeck E, Ninow J, Gern RMM, Furlan SA (2006). *Pleurotus* cultivation in submerged culture and nutritional value of fruit bodies cultivated in solid culture. In: Larroche C, Pandey A, Dussap CG (Orgs.). Current topics on bioprocesses in food industry (1): 197-203

Soares RDP, Secchi AR (2003) EMSO: A new environment for modelling, simulation and optimisation. Comput Aided Chem Eng (14): 947–952

Stanbury PF, Whitaker A, Hall SJ (1995). Principles of Fermentation Technology, Pergamon Press, New York, USA 357 p

Viniegra GG, Larralde CCP, Lopez FI (1994) A new approach for modelling the kinetics of mycelial cultures, in: E.Galindo, O.T. Ramirez (Eds.), Adv in Biopro Eng, Dordrecht, pp. 183–189

Wuest PJ (1989). Shiitake growing in sawdust. In: Shiitake mushrooms, pp. 47-52. University of Minnesota, Minnesota

Yang FC, Liau CB (1998) Effects of cultivating conditions on the mycelial growth of *Ganoderma lucidum* in submerged flask cultures. Biopro Eng (19): 233-236

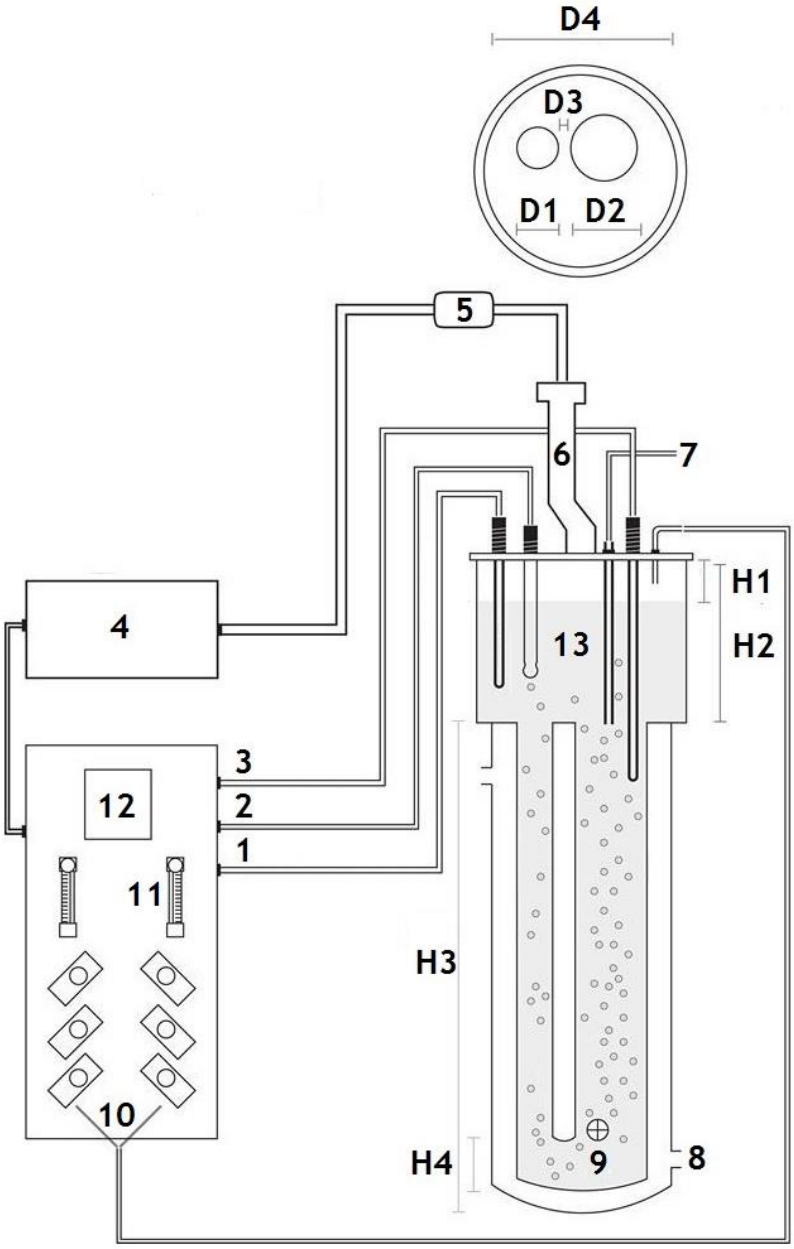

Figure 1 – Diagram of external loop airlift bioreactor: (1) temperature probe; (2) pH sensor; (3) dissolved oxygen probe; (4) gas analyzer; (5) filtered outlet gases; (6) condenser; (7) port for harvesting cells; (8) cooling jacket; (9) sparger; (10) pumping of acid and base; (11) rotameter; (12) monitoring unit; (13) sterile nutrient medium; (H1) gas head space (30 mm); (H2) disengagement zone height (115 mm); (H3) down comer and riser height (355 mm); (H4) communicating vessels height (30 mm); (D1) down comer diameter (30 mm); (D2) riser diameter (48 mm); (D3) distance from the riser and down comer (18 mm); (D4) disengagement zone diameter (130 mm).

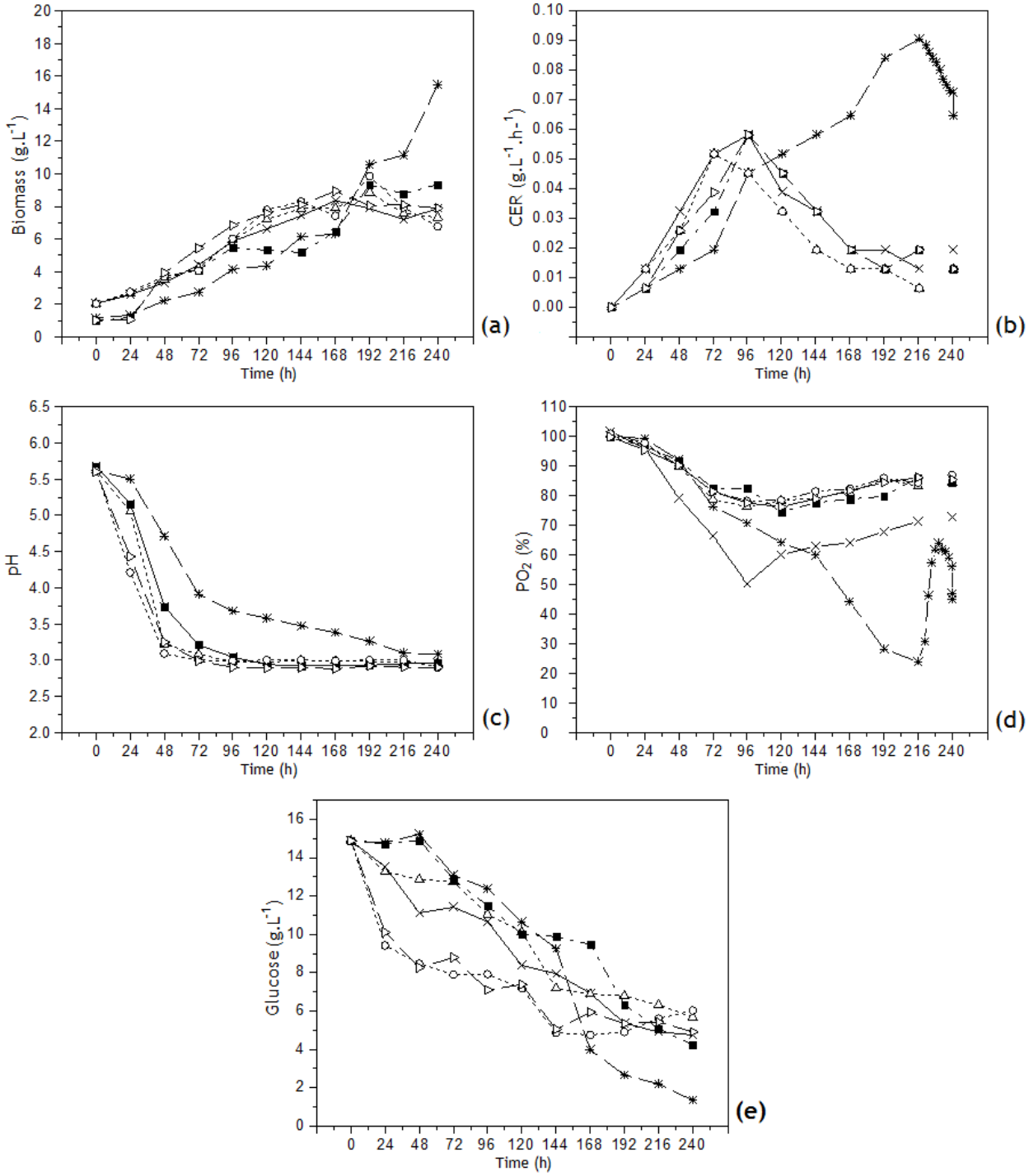

Figure 2. Growth kinetics of *Lentinus edodes* Led-08 cultures in the ELAB. (a) Biomass, (b) CER - CO_2 evolution rate, (c) pH, (d) PO_2 - dissolved oxygen saturation, and (e) glucose consumption during the cultivation. Culture conditions were: 25 °C, aeration rate 0.16, 0.20, or 0.24 vvm and inocula sizes (IS) of 1.0 $g.L^{-1}$ or 2.0 $g.L^{-1}$. (—×— 0.16 vvm, 2.0 $g.L^{-1}$ IS); (···✳··· 0.16 vvm,1.0 $g.L^{-1}$ IS); (··△·· 0.20 vvm, 2.0 $g.L^{-1}$ IS); (···■··· 0.20 vvm, 1.0 $g.L^{-1}$ IS); (···○··· 0.24 vvm, 2.0 $g.L^{-1}$ IS); (—▷— 0.24 vvm, 1.0 $g.L^{-1}$ IS).

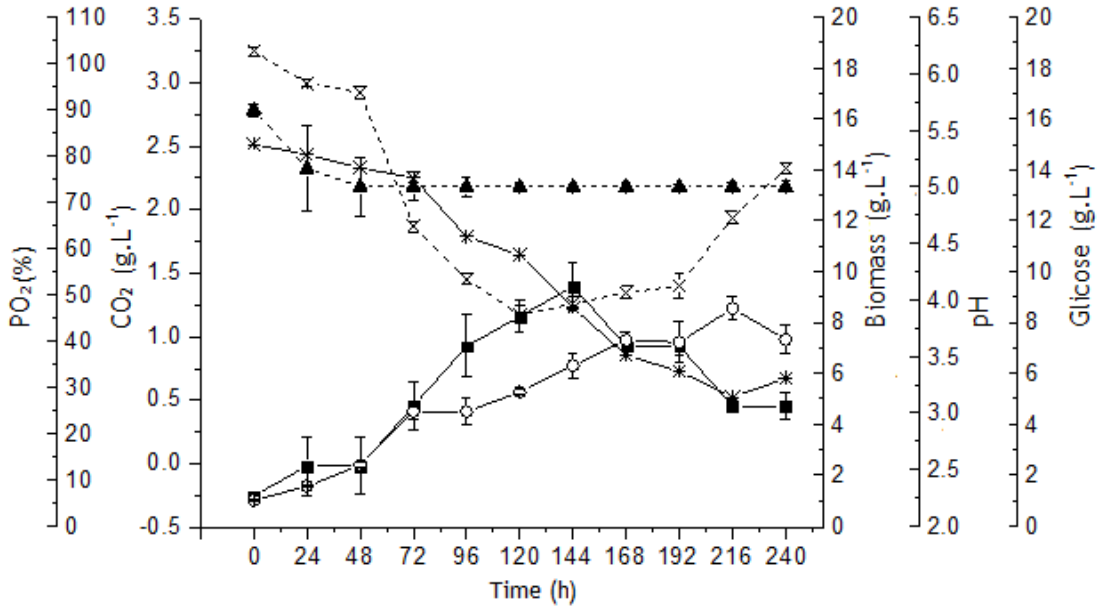

Figure 3. Growth kinetics of *Lentinula edodes* (Led-08) cultivation in the ELAB. Conditions were: pH controlled at 5.0; temperature of 25 ° C; and air flow of 0.20 vvm. (—○—) biomass; (—■—) metabolic production of CO_2; (···▲···) pH ; (···✕···) dissolved oxygen saturation; and (—✳—) glucose.

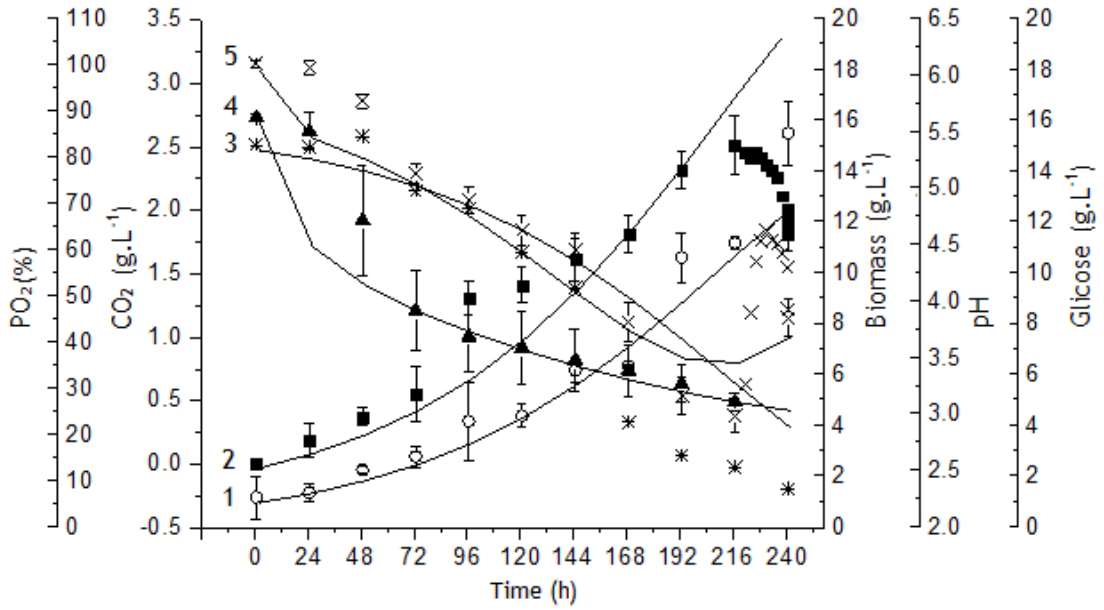

Figure 4. Growth kinetics of *Lentinula edodes* (Led-08) cultivation in the ELAB and the modeling of experimental data. Conditions were: temperature of 25°C, air flow of 0.16 vvm, and initial biomass of 1.0 $g.L^{-1}$. Experimental data: (○) biomass; (■) metabolic production of CO_2; (▲) pH; (✕) dissolved oxygen; and (✳) glucose consumption. Modeled parameters (lines): (1) biomass; (2) metabolic production of CO_2; (3) glucose; (4) pH; (5) dissolved oxygen.

Table 1. Influence of aeration rate and initial biomass on the kinetic parameters of *L. edodes* Led-08 cultivation in the external loop airlift bioreactor. Biomass (X_f), Yield coefficients ($Y_{X/S}$, biomass/substrate; $Y_{P/X}$, CO_2/biomass; $Y_{P/S}$, CO_2/substrate), Biomass productivity (P_x), specific growth rate (μ_{max}), and oxygen mass transfer coefficient (k_la).

Aeration rate	X_f [$g.L^{-1}$]	$Y_{X/S}$ [$g.g^{-1}$]	$Y_{P/X}$ [$(g.L^{-1}).\ g^{-1}$]	$Y_{P/S}$ [$(g.L^{-1}).\ g^{-1}$]	P_X ($g.L^{-1}.h^{-1}$)	μ_{max} [day^{-1}]	k_la [h^{-1}]
Initial Biomass 1.0 $g.L^{-1}$							
0.16 vvm	15.47 ± 1.27^a	1.06^a	0.485^d	0.140^c	0.061^a	0.516^c	5.04
0.20 vvm	$9.35 \pm 0{,}45^b$	0.78^b	0.601^c	0.199^c	0.049^c	1.138^b	10.08
0.24 vvm	$8.94 \pm 0{,}31^c$	0.62^c	0.516^d	0.154^c	0.053^b	1.315^a	45.63
Initial Biomass 2.0 $g.L^{-1}$							
0.16 vvm	8.36 ± 0.24^d	0.56^d	0.505^d	0.235^b	0.050^c	0.287^e	4.86
0.20 vvm	8.86 ± 0.81^c	0.67^c	0.666^b	0.364^a	0.046^c	0.340^e	9.88
0.24 vvm	9.85 ± 0.67^b	0.70^b	0.710^a	0.394^a	0.051^c	0.394^d	39.93

Data are the mean duplicates. Means with different superscript letters (a–e) at same column are significantly different at the $p \leq 0.05$ level (Tukey test).

$Y_{P/X}$ and $Y_{P/S}$ were calculated at time when the fungus was at maximun CO_2 production (CER - CO_2 evolution rate);
$Y_{X/S}$ was calculated at maximum biomass formed;
P_X was calculated for the complete kinetics;
μ_{max} was calculated during the exponential growth phase.

Table 2. Values of model parameters and the quadratic coefficient of determination (R^2) for *Lentinula edodes* Led-08 cultivation in the external loop airlift bioreactor at 25 °C, 0.16 vvm, and initial biomass of 1.0 $g.L^{-1}$

Kss	11.857 ± 1.70
αP	0.3 ± 0.09
αH	$7.321 \times 10^{-5} \pm 5.07 \times 10^{-5}$
KpO	0.353 ± 0.06

	X	P	pO_2	pH	S
R^2	0.958	0.686	0.937	0.931	0.848

PHYSICO-CHEMICAL PROPERTIES OF THE SUBSTRATES AND MYCELIAL GROWTH RATE DETERMINATION IN LENTINULA EDODES (BERK.) PEGLER DIKARYOTIC STRAINS

Diego Melo Pereira & Marco Antônio Záchia Ayub*

Biotechnology & Biochemical Engineering Laboratory (Bioteclab), Federal University of Rio Grande do Sul, Av. Bento Gonçalves, 9500, P.O. Box 15090, ZC 91501-970, Porto Alegre, RS, Brazil.

*Corresponding author:
Tel.: +55 51 3308 6685; Fax + 55 51 3308 7048
E-mail address: mazayub@ufrgs.br (M.A.Z. Ayub)

Abstract

The mycelium growth rate is a determinant propriety to select mushroom strains. In this work, we determined the mycelial growth rate of five dikaryotic strains of *Lentinula edodes* under five levels of carbon/nitrogen ratio ($R_{C/N}$), ranging from 124.89/1 to 27.82/1. We used *Eucalyptus saligna* sawdust supplemented with wheat bran as the basis for the growth of *Lentinula edodes*. The measurements of mycelial growth, associated with determination of the physical chemical properties of the substrate, revealed the best levels of $R_{C/N}$ (65.90/1 and 45.01/1), as well as the best densities of substrate (wet density ranging from 308 to 318 $g.L^{-1}$ and the dry density from 166 to 178 $g.L^{-1}$). Our results indicate that extension of mycelia is related to the bioavailability of nitrogen and the density obtained for each formulation.

Keywords: *Lentinula edodes*; shiitake; mycelium growth rate; basidiomycete

Introduction

Scientific data suggest that the determination of the mycelium extension rates and the biomass production are important factors in order to achieve successful cultivations of *L. edodes* (Leatham 1985; Silva et al. 2005; Elisashvili et al. 2008; Philippoussis et al. 2011). On the other hand, the physical properties of the substrate are also considered of great importance, in special the substrate density, its porosity, the interspace aeration, and the water retention capacity. An ideal substrate for filamentous fungal cultivation must simultaneously have the characteristics of allowing good aeration (oxygen diffusion), good water storage capacity, and low penetration resistance of the fungal hyphae (Royse and Sanchez 2001; Abad et al. 2004).

Based on these considerations, the mycelial growth rates of five dikaryotic parental strains of *L. edodes* commercially available for the production of mushroom were determined for five levels of carbon/nitrogen ratios in the substrate composition, based on the combination of wheat bran and *Eucalyptus saligna* sawdust. We also evaluated the physical properties of the substrates when different proportions of supplements were added.

2. Materials and methods

2.1. Fungal strains

Lentinula edodes (Berk.) Pegler, strains Led-08 (Fungibras spawn company, Botucattu, São Paulo, Brazil), Led-T1, Led-T2, and Led-BP (Funghi & Flora spawn company, Valinhos, São Paulo, Brazil), and Led-OTS (Commercial producer of mushrooms, Porto Alegre, Rio Grande do Sul, Brazil) were used in this work. Stock cultures were kept in test tubes at 4°C in Mushroom Complete Medium at the microbial collection of the BiotecLab (Food Science and Technology Institute, UFRGS, Porto Alegre, Brazil).

2.2. Formulation of substrates

Substrates were formulated by mixing different proportions of wheat bran (WB) and eucalyptus sawdust (S): (S/WB) 90/10; 80/20; 70/30; 60/40; and 50/50, combinations that resulted in the following carbon/nitrogen ratios: 124.89/1, 65.90/1, 45.01/1, 34.36/1, 27.82/1.

2.3. Physico-chemical characterization of the substrate

The water content of the substrate was determined by dry weight at 65 °C, until constant weight. The wet and dry densities of the substrate were determined according to the method of the

Union of German Agricultural Research Institutions (VDLUFA, Hoffmann, 1970) based on the self-compression of the material. Briefly, the method consists in measuring the volume of compaction of samples placed in a transparent plastic beaker after letting it fall from a height of 10 cm, for 10 consecutive times.

The total porosity (TP) and the aeration space (AS) of the substrate were determined using the voltage table method (Kiehl, 1979). Aluminum rings of 57 mm of diameter were filled with 118.75 cm^3 of substrate and let to drain after complete water saturation, using a glass square tension table (0.16 m^2) with a 0.5 cm center hole attached to the hose connected to a system of communicating vessels equivalent to a water column of 10 hPa, enough to remove the free water present in the solid material. This measurement is defined as the matric potential (Ψm) and it was measured for each formulation. Total porosity (TP) was defined as the water content present in saturated samples (0 hPa), defined according to equation 1. The aeration space (AS) was defined as the volume of air present in the substrate drained under conditions equivalent to 10 hPa and the difference between the total porosity and water content released, according to equation 2.

$$TP = 100 \cdot \frac{(mass\ of\ saturated\ sample - mass\ of\ the\ dry\ sample\ (65°\ C))}{ring\ volume\ (mL)} \quad (1)$$

$$AS = 100 \cdot \frac{(mass\ of\ saturated\ sample - mass\ of\ the\ sample\ under\ 10hPa)}{ring\ volume\ (mL)} \quad (2)$$

Conductivity and pH of substrates were measured using a potentiometer (Quimis, Q-400A, São Paulo, Brazil), using 1:5 (volume fraction) substrate suspension in deionized water.

2.4. Mycelial growth rate determination

Linear growth rate tests of mycelia were performed using test tubes (180 mm x 20 mm) filled with 12 g of substrate and inoculated with agar discs of 8 mm diameter, as described elsewhere (Philippoussis et al. 2001). The fungal growth was recorded in a daily basis for the measurement of penetration in the substrate by visible mycelium colonization in two perpendicular directions. The linear extension rate, Kr, given in ($mm.day^{-1}$) was calculated after the upper mycelium reached 60 mm of extension.

2.5. Statistical analysis

Results obtained in this work were evaluated by analysis of variance (ANOVA) and the Tukey test ($p \leq 0.05$).

3. Results and discussion

The physico-chemical characteristics of the substrates used in our experiments are presented in Table 1. The increase in the wheat bran content in the formulation ($R_{C/N}$ reduces from 124.89/1 to 34.36/1), negatively correlates with Ψm, thus decreasing AS and PT.

It has been reported that substrates with carbon/nitrogen ratios smaller than 40/1 to be used in the cultivation of *L. edodes* are unfavorable for formation of primordia (Chen, 2005). In this work, it was observed that below this level occurs a significant decrease ($p \leq 0.05$) of the growth of hyphae for all strains tested, results represented by the Kr (Table 2). Surprisingly, it was observed an expected variation for Ψm values in the treatment with smaller carbon/nitrogen ratio (50:50, $R_{C/N}$ 27.82/1). The differences observed for TP and AS for this condition may have been caused by the particle size distribution of substrates, which, in turn, affects the pore size distribution of the material, therefore affecting mass transfer rates. (Abad et al. 2004).

When $R_{C/N}$ decreased from 45.01/1 to 34.36/1, it was observed a decrease in the extent of hyphae growth, Kr, for all strains. This result is in agreement with the literature and can be explained by the increased density of the substrate (WD and DD), which affects the formation of macropores (Abad et al. 2004). Hyphae growth was best for carbon/nitrogen ratio $R_{C/N}$ 45.01/1 (sawdust/wheat bran, 70:30), DD of 178 $g.L^{-1}$ and WD of 318 $g.L^{-1}$.

The increase in pH and the decrease in the content of soluble salts, were observed when higher levels of wheat bran were incorporated to the substrate. According to Chang and Miles (1989), the ideal pH range for *L. edodes* cultivations is in between 4.8 and 5.0, condition that was observed at low levels of incorporation of wheat bran. Therefore, as the formulations presented different proportions of minerals and initial pH values, the activity of extracellular enzymes produced by the fungi may have varied, decreasing the initial degradability of the substrate and affecting the value of Kr according to the treatment (Silva et al. 2005; Elisashvili et al. 2008; Philippoussis et al. 2011).

Our results suggest that the substrate can be supplemented up to a carbon/nitrogen ratio of 45.01/1, within the ranges studied, without compromising the development of fungal biomass, reaching conditions statistically identical ($p \leq 0.05$), with the exception of strain Led-OTS, which showed statistaical difference from the others and best growth conditions being defined as carbon/nitrogen ratio between 65.90/1 to 45.01/1.

Based on these results, the extension of mycelium of *L. edodes* showed better growth profiles within the interval of $R_{C/N}$ of 65.90/1 and 45.01/1, wet density ranging from 308 to 318 $g.L^{-1}$, and the dry density varying from 166 to 178 $g.L^{-1}$, when using the combination of eucalyptus sawdust and wheat bran.

4. Conclusion

The measurements of mycelial growth, associated with the physical-chemical properties of the substrates, revealed the best levels of $R_{C/N}$ for *L.edodes* growth when using *Eucalyptus saligna* sawdust supplemented with wheat bran. The intrinsic characteristics of the *L. edodes* strains used in this work

did not produce a considerable effect on the colonization of the substrate, indicating that the mycelium growth was related to the bioavailability of nitrogen and the density of the substrate obtained for each formulation. These results can help in the design of substrate formulation for the cultivation of *L. edodes.*

Acknowledgements

The authors wish to thank Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Fundação de Amparo a Pesquisa do Rio Grande do Sul (FAPERGS) for the funding of the research and the scholarships of the first author.

5. References

Abad MB, Nogueira PM, Carrión CB (2004) Los sustratos en los cultivos sin suelo. Tratado de cultivo sin suelo, Mundi-Prensa, Madrid, pp 113-158

Chang ST, Miles PG (1989). Edible mushrooms and their cultivation. CRC Press, Boca Raton, 345 p

Chen, AW (2005) What is shiitake. In: Gush R (ed) Mushroom growers' handbook 2. Mush World, Seoul, pp 1–11

Elisashvili V et al. (2008) Lentinus edodes and Pleurotus species lignocellulolytic enzymes activity in submerged and solid-state fermentation of lignocellulosic wastes of different composition. Bioresour. Technol. 99: 457-462

Hoffmann G (1970). Verbindliche Methoden zur Untersuchung von TKS und Gärtnerischen Erden. Mitteilungen der VDLUFA 6: 129-153

Kiehl, EJ. Manual de Edafologia: Relações Solo-Planta (1979). São Paulo: Ed. Agronômica Ceres Ltda, 262p

Leatham GF (1985) Extracellular enzymes produced by the cultivated mushroom Lentinus edodes during degradation of a lignocellulosic medium. Appl Environ Microbiol 50:859–867

Philippoussis A, Diamantopoulou P, Papadopoulou K, Lakhtar H, Roussos S, Parissopoulos G, Papanikolaou S (2011). Biomass, laccase and endoglucanase production by Lentinula edodes during solid state fermentation of reed grass, bean stalks and wheat straw residues. World Jour of Microb and Biotech, v. 27, (2): 285-297. http://dx.doi.org/10.1007/s11274-010-0458-8

Philippoussis A, Zervakis G, Diamantopoulou P (2001) Bioconversion of lignocellulosic wastes through the cultivation of the edible mushrooms Agrocybe aegerita, Volvariella volvacea and Pleurotus spp. World J Microbiol Biotechnol 17(2):191–200

Royse DJ, Sanchez-Vazquez JE (2001) Influence of substrate wood-chip particle size on shiitake (Lentinula edodes) yield. Bior Tech, v. 76(3): 229-233

Silva EM, Machuca A, Milagres AMF (2005) Effect of cereal brans on Lentinula edodes growth and enzyme activities during cultivation on forestry waste. Lett Appl Microbiol 40: 283–288

Table 1. Physico-chemical characteristics of substrates according to the proportion of sawdust and wheat bran

Carbon/Nitrogen ratio	124.89/1	65.90/1	45.01/1	34.36/1	27.82/1
Proportion sawdust / wheat bran	(90/10)	(80/20)	(70/30)	(60/40)	(50/50)
Matric potential (Ψm) water retention 10 cm	0.5 ± 0.005	0.45 ± 0.006	0.41 ± 0.013	0.39 ± 0.010	0.44 ± 0.005
WD ($g.L^{-1}$)	312 ± 4.047	308 ± 6.288	318 ± 5.184	335 ± 2.011	346 ± 3.387
DD ($g.L^{-1}$)	170 ± 4.610	166 ± 1.881	178 ± 0.281	186 ± 0.305	193 ± 2.514
DM ($g.100\ g^{-1}$)	54	55	55	56	56
pH	4.99	5.45	5.54	6.25	6.42
TCSS ($g.L^{-1}$)	1.897	1.440	1.415	0.792	0.662
TP ($m^3.m^{-3}$)	0.76 ± 0.017	0.76 ± 0.020	0.72 ± 0.008	0.71 ± 0.008	0.71 ± 0.012
AS ($m^3.m^{-3}$)	0.25 ± 0.023	0.31 ± 0.026	0.31 ± 0.004	0.33 ± 0.001	0.27 ± 0.018

WD = wet density; DD = dry density; DM = dry matter; pH determined in water dilutions of 1: 5; TCSS = total content of soluble salts, measured by conductivity in water dilutions of 1: 5; TP = total porosity; AS = aeration space.

Standard deviation of the analyses in triplicates.

Table 2. Linear growth rate Kr (mm.day^{-1}) of dikaryotic parental strains according to the proportion of sawdust and wheat bran

Carbon/Nitrogen ratio		124.89/1	65.90/1	45.01/1	34.36/1	27.82/1
Proportion sawdust / wheat bran		(90/10)	(80/20)	(70/30)	(60/40)	(50/50)
Kr (mm.day^{-1})	Led-08	0.404 ± 0.055^{b}	0.433 ± 0.137a	0.440 ± 0.113^{a}	0.348 ± 0.152^{c}	0.342 ± 0.082^{c}
	Led-T1	0.410 ± 0.136^{b}	0.435 ± 0.028a	0.446 ± 0.217^{a}	0.396 ± 0.046^{c}	0.402 ± 0.051^{c}
	Led-T2	0.392 ± 0.075^{b}	0.417 ± 0.036a	0.415 ± 0.041^{a}	0.373 ± 0.021^{c}	0.373 ± 0.137^{c}
	Led-BP	0.458 ± 0.102^{b}	0.481 ± 0.077a	0.471 ± 0.173^{a}	0.394 ± 0.130^{c}	0.402 ± 0.022^{c}
	Led-OTS	0.358 ± 0.065^{c}	0.400 ± 0.062^{c}	0.421 ± 0.043^{a}	0.350 ± 0.126^{c}	0.344 ± 0.072^{c}

Standard deviation of the analyses in triplicates.

Data are the mean from independent repeats. Means with different superscript letters (a–c) at same line for Kr (mm.day^{-1}) are significantly different at the $p \leq 0.05$ level (Tukey test).

The axenic cultivation of *lentinula edodes* (berk.) pegler: compost optimization and evaluation of dikaryotic strains

Diego Melo Pereira, Giovanni Neves Mendes da Silva, Barbara Alves Zolet, Daiane Dias Lopes, Marco Antônio Záchia Ayub*

Biotechnology & Biochemical Engineering Laboratory (Bioteclab), Federal University of Rio Grande do Sul, Av. Bento Gonçalves, 9500, P.O. Box 15090, ZC 91501-970, Porto Alegre, RS, Brazil

*Corresponding author:
Tel.: +55 51 3308 6685; Fax + 55 51 3308 7048
E-mail address: mazayub@ufrgs.br (M.A.Z. Ayub)

Abstract

We determined the yields performance of axenic cultivation of sporophores of five strains of *Lentinula edodes* (Berk.) Pegler, using sawdust of *Eucalyptus saligna* and wheat bran as substrates. Central composite design (CCD) was used to evaluate the independent variables incubation time $I_{(t)}$, and percentage of wheat bran in relation to Eucalyptus sawdust (representing the carbon/nitrogen ratio ($R_{C/N}$)), having the biological efficiency (BE) and the unit weight of strains as target results. The best values of BE for all strains were obtained in cultivations with $R_{C/N}$ 57.62/1, with formation of primordia after $I_{(t)}$ 130 days of induction. Fungi obtained under these operating conditions had their centesimal composition determined. Total amylolytic and cellulolytic, β-glucosidase, and specific protease enzymatic activities were performed in order to correlate the synthesis of these enzymes with yields of sporophores. The CCD confirmed that BE and unit weight were influenced by the $R_{C/N}$ and the $I_{(t)}$, being strain dependent, whereas productivity was related to the best synthesis of extracellular enzymes. The technique of amplification of the Internal Transcribed Spacer (ITS1 and ITS2 rDNA) regions, followed by digestion with restriction enzymes (*HhaI, HinfI,* and *HaeIII)* was used to confirm the genetic profile of the five strains used in this work as *Lentinula edodes*.

Keywords: *Lentinula edodes*; shiitake; substrate formulation; ITS region analysis; extracellular enzyme; basidiomycete.

Introduction

Lentinula edodes, an important commercial fungus, shows a two-phases life cycle, characterized by the mycelium growth and the production of sporophores, both affected by the proportions of cellulose, hemicellulose, and lignin in relation to the nitrogen content of substrates used for growth (Philippoussis et al. 2003; Mata and Savoie 2005). Supplements containing sources of carbohydrate and nitrogen readily available are usually added to substrates to accelerate growth and to increase the production of mushrooms (Ohga and Royse 2001; Silva et al. 2007). Hardwood (preferably oak-wood) sawdust, supplemented of 20-30 % (dry mass fraction) of wheat or rice bran is the most frequently used synthetic substrate formulation for the commercial production of *L. edodes* (Miller and Jong 1986; Royse and Bahler 1986; Pire et al. 2001).

In the axenic cultivation for Shiitake production, the strains used, and the incubation time, were identified as important variables for an efficient production (Royse 1985; Kalberer 1995; Philippoussis et al. 2001), and the cultivation method using agricultural by-products or residues as substrates has been developed and improved in recent decades (Royse 2004). Short culture times and greater biological efficiency are the main advantages offered over the traditional cultivation in logs. (Royse 2004; Philippoussis et al. 2007). The efficacy of the bioconversion of residues on the mushroom productivity is assessed using the biological efficiency (BE), considering the characterization of type of substrate and strain used, while the yield response is determined by the duration of the period of incubation.

Scientific data suggest that the determination the biomass production and the activity of extracellular enzymes are important factors in order to achieve successful cultivation of *L. edodes* (Leatham 1985; Philippoussis et al. 2003; Silva et al. 2005; Elisashvili et al. 2008). Several studies demonstrate that the type and composition of lignocellulosic substrate determines the type and amount of enzymes produced by basidiomycetes during vegetative growth (Baldrian and Valaskova 2008; Gaitán-Hernández et al. 2011; Kwon et al. 2008; Kwon et al. 2015) and their high yields may also be attributed to their high water-soluble sugars contents.

The species *L. edodes* is an efficient wood decomposer and can be grown on several agro-industrial lignocellulosic materials, such as sawdust and cereal straws (Mishra and Leatham 1990; Mata and Savoie 1998; Philippoussis et al. 2003; Silva et al. 2005; Silva et al. 2007). The amount of nitrogen present in the formulations of the substrates is an important factor associated with the number, morphological structure and nutritional composition of mushrooms (Royse 1996; Silva et al. 2005; Philippoussis et al. 2007).

Taking into account these considerations, the aims of this research were to use five strains of *Lentinula edodes* (Berk.) Pegler to establish a correlation between the biological efficiency of strains growing under the axenic system and the extracellular activities of amylolytic and total cellulolytic enzyme complex because of the nature of the required biodegradation of the substrate, which was composed of sawdust of *Eucalyptus saligna* and wheat bran. These substrates were characterized in their chemical composition and we proposed best formulations for each of the tested strains based on a Central Composite Design (CCD) used to evaluate the independent variables incubation time ($I_{(t)}$), and percentage of wheat bran in relation to Eucalyptus sawdust on the biological efficiency and unit weight

of mushrooms. Enzymatic assays and centesimal composition was performed to compare the five strains. The genus and species of strains of *Lentinula edodes* (Berk.) Pegler were confirmed by amplification of ITS1 and ITS2 rDNA regions, followed by digestion with restriction enzymes and compared to NCBI accessions.

Materials and methods

Dikaryotic parental strains

The basidiomycete fungus *Lentinula edodes* (Berk.) Pegler, strains Led-08 (Fungibras spawn company, Botucattu, São Paulo, Brazil), Led-T1, Led-T2, and Led-BP (Funghi & Flora spawn company, Valinhos, São Paulo, Brazil), and Led-OTS (Commercial producer of mushrooms, Porto Alegre, Rio Grande do Sul, Brazil) were used in this work. Stock cultures were kept in test tubes at 4 °C in Mushroom Complete Medium at the microbial collection of the BiotecLab (Food Science and Technology Institute, UFRGS, Porto Alegre, Brazil).

ITS sequence of rDNA

L. edodes strains were cultured in 20 mL of Mushroom Complete Medium, pH 5.8, until reaching approximately 0.5 mg/mL of biomass (dry weight). Cells were recovered by centrifugation (3 000 *g*, 15 min) and pellets were used to extract the total DNA using the Wizard purification kit Genomic DNA (Promega, Brazil). PCR amplifications of ITS1-5.8S-ITS4 regions of rDNA (ITS1 primer 5'-TCC GTA GGT GAA CCT GCG G - 3' and ITS4 primer 5' - TCC TCC GCT TAT TGA TAT GC - 3') were performed in a thermo cycler (BioRad T100, Brazil) under the following conditions: denaturation temperature of 95 °C for 5 min, followed by 30 cycles of denaturation 95 °C for 1 min, annealing at 55 °C for 45 sec, 72 °C extension for 1 min, and final extension at 72 °C for 5 min. PCR products were digested using *HhaI*, *HinfI*, and *HaeIII*, resolved by electrophoresis (3 V/cm, 1.3 % agarose gel stained with Safe DNA Dye, Kasvi), and photographed under ultraviolet light for analysis and comparison with existing database at the National Center for Biotechnology Information (NCBI), and NebCutter V2.0 tool (New England Biolabs). The data generated were analyzed using the Gel-Pro Analyzer Version 3.1.

Chemical composition of the substrates

The chemical compositions of substrates *Eucalyptus saligna* and wheat bran were determined and the results of macro and micronutrients are presented in Table 1. Gravimetric moisture was determined by sample drying in an oven at 105 °C. The total amount of nitrogen was determined using the semi-micro Kjeldahl method (Tedesco et al. 1995). The total organic carbon was determined by the dichromate oxidation method, proposed by Walkley & Black (1934). The elements P, K, Ca, Mg, S, Cu, Zn, Fe, Mn, and Na were determined in the extract of the nitrogene digestion, by induced plasma spectrometry (ICP-OES), whereas the determination of B was carried out by dry digestion, as proposed

in the methodology by Jackson (1965). The pH of substrates was measured using a potentiometer (Quimis, Q-400A, São Paulo, Brazil), using 1:5 (volume fraction) substrate suspension in deionized water.

Experimental designs for sporophores production

Central composite design was used to evaluate the independent variables incubation time $I_{(t)}$, and percentage of wheat bran in relation to Eucalyptus sawdust (representing the carbon/nitrogen ratio ($R_{C/N}$)), having the biological efficiency (BE) and the unit weight of mushrooms as the target results. Eleven experiments were carried out, according to a 2^2 full-factorial central composite design with three central and four axial points (Table 1). The behavior of the system was fitted to a second order equation (3):

$$Y_i = b_0 + b_1X_1 + b_2X_2 + b_{12}X_1X_2 + b_{11}X_1^2 + b_{22}X_2^2 \quad (3)$$

Where Y_i represents the dependable variables; b_n are constant regression coefficients; X_1 and X_2 are the coded levels of independent variables (X_1, incubation time, and X_2, percentage of wheat bran in relation to Eucalyptus sawdust, respectively). Surface-response methodology was employed to evaluate the effects of the independent variables on the biological efficiency (BE, expressed as kg of harvested mushrooms wet basis/kg of substate dried basis), and the unit weight of strains (expressed as the total mass/number of mushrooms formed on the first flush of production). Based on the results of organic carbon, nitrogen, and moisture content in substrates (Table 1), we defined the $R_{C/N}$ used in the experiments presented in Table 2.

The experimental procedure was as follows: Five dikaryotic strains were inoculated (3.4 % mass fraction of inoculum) in the different media compositions according to the CCD matrix. After inoculation, substrate block cultures were kept static at 24 °C and 1 500 ppm CO_2 atmosphere. Formation of primordia was induced at 16 °C and, after 10 days of incubation, whole mushrooms were collected for the analyses.

Centesimal analysis of the fungi

The water content of the fungi was determined as dry weight as explained above. Ashes were determined by incineration in a muffle furnace at 550 ± 5°C, for 12 h. Protein was quantified by the Kjeldahl method, considering the factor 4.38 to convert nitrogen to protein. Lipids were determined by extraction in a Soxhlet, following the Soxhlet methodology. Total fiber content was determined by the Weende method, and carbohydrates were determined by subtraction of all other components from the initial biomass. All analyzes for chemical composition were determined according to the AOAC, 1997.

Enzyme extraction

Twelve g of solid substrate with 55 % (mass fraction) of moisture, pH 5.5, were added to test tubes and autoclaved at 121 ^{o}C for 30 min. The substrate was then inoculated with agar discs of 8 mm diameter and incubated in a BOD incubator at 25 °C for 150 days. Samples were taken every two days for assessment of the enzymatic activity. The contents of the test tubes were placed into an Erlenmeyer flask and extracted using 100 mL of deionized water by incubation in a shaker at 180 rpm, 30 °C for 1 h. The crude enzymatic extract was then twice centrifuged at 4 500 *g* for 15 min. The supernatant collected was analyzed for enzyme activities described below.

Enzyme assays

The DNS method (3,5-dinitrosalicylic acid, Miller 1959), measuring the total reducing sugars, was used to determine both the cellulolytic and amylolytic activities of the crude enzymatic extracts. Total cellulolytic activity of the crude extracts was determined by incubating 1 mL of the extract, 1 mL of 50 mM sodium citrate buffer (pH 4.8), and 50 mg of Whatman filter paper n° 1. The enzymatic reaction was conducted at 50 °C in a water bath for 60 min. Similarly, the total amylolytic activity was determined by incubating 1 mL of the crude enzyme extract with 1 mL of 0.5 % starch solution in acetate buffer (pH 4.8) at 50 ºC for 60 min. The reaction was subsequently terminated by the addition of 1 mL of DNS, heated at 100 °C for 5 min and the absorbance read at 540 nm. One unit of enzyme activity was defined as the amount of enzyme necessary to produce 1 μmol of reducing sugars per min. All measurements were performed in triplicate and the standard errors were calculated.

Extracellular β-glucosidase was determined by measuring the amount of chromogenic substrate p-nitrophenol released in the reaction of mixtures containing 90 μL of sodium citrate buffer 100 mM, pH 4.8, 10 μL p-nitrophenyl-glucopyranoside, and 100 μL of crude extract. Mixtures were kept at 37 °C for 30 min and, after addition of 1 mL 1 M sodium carbonate, the p-nitrophenol released was measured by spectrophotometry at 405 nm. One unit of enzyme activity was expressed as the amount of enzyme required to form 1 *p*mol of product (p-nitrophenol)/min under the assay conditions.

Specific proteolytic activity was measured using the azocasein method (Sarath et al. 1989). One enzymatic activity unit (U) was defined as the amount of enzyme needed to produce one unit of absorbance under the conditions of the method. Specific activity was expressed as U/mg protein. Protein in crude extracts was determined using the Lowry method and bovine serum albumin as standard (Lowry et al. 1951).

Statistical analysis

Results obtained in this work, when required, were evaluated by analysis of variance (ANOVA) and the Tukey test ($p < 0.05$). Statistica 7.0 software (Statsoft, USA) was used for regression analysis of the data and CCD. The significance of the regression coefficients was determined by Student's t-test and the second order model equation by Fisher's F-test. The variance explained by the model is given by the multiple coefficient of determination, R^2.

Results and discussion

Genetic characterization of the dikaryotic strains

In this study, amplification of the rDNA ITS region were used to characterize strains that could be useful to be crossed in order to create new strains and also to confirm that the strains we were using were, effectively, *Lentinula edodes*. Therefore, the profile cleavage of the amplified rDNA ITS regions of each strain by restriction enzymes (*HhaI, HinfI,* and *HaeIII)* were compared with the cleavage sequences of NCBI accessions using the software NebCutter (Figures 1a and 1b). The pair of strains Led-T1 and Led-T2 and the pair Led-08 and Led-BP did not show polymorphism from each other. The results show that DNA digestion of restriction enzymes produced the same banding profile for all strains compared to NCBI AY683196.1 access (banding on 724 bp) and GQ866860.1 access (banding on 735 bp), confirming that the strains are *L. edodes*.

Experimental designs for sporophores production

The CCD was used to obtain the best conditions for BE and unit weight of *L. edodes* strains, and these conditions were not previously described in the literature. Analysis of variance (ANOVA) was employed for the determination of significant parameters and to estimate BE and unit weight of *L. edodes* strains as function $R_{C/N}$ and $I_{(t)}$. Coefficients estimates by the regression model are shown in Table 3. The response surface (Figure 2) presents the data of percentage of wheat bran in relation to *Eucalyptus saligna* sawdust plotted against the respective fraction of carbon/nitrogen ratio ($R_{C/N}$). The experimental design of different percentage of wheat bran in relation to Eucalyptus sawdust and $I_{(t)}$ during the axenic cultivation of dikaryotic strains are shown in Table 2.

The best results in terms of BE for all strains were obtained for a $R_{C/N}$ of 57.62/1 and 130 days of $I_{(t)}$ (assay 6). The predicted quadratic regression by the model (0.767 to 0.977, depending on the strain), in relation to BE, presented a good fit in the 95 % confidence interval, showing that most of the variability in the response for BE can be assigned to the independent variables. These results confirm that the values of $R_{C/N}$ and $I_{(t)}$ have a strong impact on the performance of mushroom cultivations for all strains. The significance of second-order model was also explained by Fisher's test that showed the computed F-values (F_{calc}) higher than the F-value in statistic tables (F_{tab} 4.53) for all strains, demonstrating the significance of $R_{C/N}$ and $I_{(t)}$ on BE response (Box and Witson 1951; Box 1953).

The response surface generated based on the proposed model (Figure 2) demonstrates that smaller incubation times than 130 days $I_{(t)}$ may be adopted using higher $R_{C/N}$, however, long incubation periods significantly affect the unit weight of all tested strains (Figure 2 e, f, g, and h). It has also been observed in this work that incubation times longer than 120 days $I_{(t)}$ promoted excessive cellular fluids exudates causing the presence of free water in the substrate (Rossi et al. 2003). The excess of water reduces empty spaces in the substrate, negatively impacting the mass transfer of oxygen and carbon

dioxide (Zadrazil 1993; Royse and Sanchez 2007) and contributes to bacterial contaminations (Moyson and Verachtert 1991).

Concerning the result as unit weights, the model showed good fit for strains Led-T1 and Led-T2, with $F_{calc} > F_{tab}$ (p-value <0.0009 and p<0.0295 respectively), strains showing higher unit weights, obtained for the incubation time for induction of primordia in between 80 and 90 days (Figure 3 b). However, this period of incubation contradicts the best predictions in the literature in terms of productivity (Ando 1974; Tan and Moore 1992; Pacumbaba and Pacumbaba, 1999). Moreover, it is not the best results concerning those observed for BE obtained in this work. The interaction of independent variables had a negligible effect concerning unit weight for strains Led-08 and Led-BP ($p \leq 0.05$). However, all interactions were kept in the equation because of the representativeness of the response for these strains, with R^2 of 0.802 and 0.760, respectively.

For all five strains, the best yields were obtained using the substrate in the proportion 30 % of wheat bran to Eucalyptus sawdust ($R_{C/N}$ 57.62/1), for induced fructification in 130 days of $I_{(t)}$. Several works reported successful production of *L. edodes* on hardwood sawdust after approximately 3 to 4 months of $I_{(t)}$ after inoculation of the substrate (Ando 1974; Tan and Moore 1992; Pacumbaba and Pacumbaba, 1999). Chen (2005) reported the optimal $R_{C/N}$ for production sporophores to be around 40/1, whereas Kües and Liu (2000), stressing the extreme importance of keeping the balance between C and N sources for fructification of sporophore, have shown thatthe substrate supplementation with protein-rich materials improved the yields of *L. edodes* strains. Rossi et al. (2003) produced *L. edodes* mushrooms on sugarcane bagasse and reported increased production and higher productivity when the medium was supplemented with 25 to 30 % (mass fraction) of rice bran. Finally, this relationship was also confirmed by cultivating *L. edodes* in high nitrogen-content medium using corn-cobs and wheat straw to supplement oak-wood sawdust (Philippoussis et al. 2007).

The higher biological efficiency (BE) was obtained for the cultivation of LED-T2 strain, showing conversions of 0.495 kg/kg of substate of fresh sporophores in the first flush production (Figure 2 a). Under the same conditions, the other strains presented biological efficiencies of 0.438 (Led-T1); 0.359 (Led-08), and 0.236 (Led-BP) kg/kg of substate of fresh sporophores.

We observed that higher $I_{(t)}$ and lower $R_{C/N}$ combinations produced high numbers of sprouting mushrooms. In this case, the sporophores were competing for nutrients and space and, possibly, there was a detrimental condition for the mass transfer of oxygen and aeration, as it has been suggested by Chen (2005), interfering with the formation of basidioms, which open the pileus for smaller sizes of sporophores (Chang and Miles, 1989). This result was confirmed for Led-BP strain cultivation, in which high $I_{(t)}$ associated with low $R_{C/N}$ produced an excessive number of sporophores per block, showing 69 mushrooms in the first flush (mushroom unit weight of 5.15 g on average). This large amount of sporophores into a single flush affects the morphological standard for this mushroom (Royse and Sanchez 2001).

Regarding unit weight, the best results were dependent on the strain cultivated (Figure 2 b). The strains Led-T1 and Led-BP presented small standard unit weights, reaching 21.44 g and 14.00 g

respectively. Strains Led-T2 formed mushrooms of 28.95 g, whereas strain Led-08 formed mushrooms up to 40 g. In general, we observed that the unit weight was inversely proportional to the number of mushrooms formed and by the long incubation periods. However, enhanced BE was observed under this condition for all strains. This relationship was also demonstrated to other strains of *L. edodes* (Philippoussis et al. 2011; Rossi et al. 2003).

The Led-OTS strain did not produce sporophores under any of the tested conditions. The physiological results of enzymatic degradation of the substrate by this strain demonstrated a low extracellular enzymatic activity, characterized by low production of reducing sugars in the crude extract. Despite this, the Led-OTS strain showed characteristics similar to the other strains regarding the speed of colonization, represented by the variation of pH (Figure 3 f), and the browning of the cultivation block, characteristic for the species.

Centesimal composition of dikaryotic strains

The chemical (centesimal) composition of whole mushrooms of dikaryotic strains, obtained in the axenic cultivation using $R_{C/N}$ 57.62/1 and induced primordia at 130 days of $I_{(t)}$ (the best results of BE), is presented in Table 4. The Led-08 mushrooms showed significant lower protein content than all other strains. The high protein content of Led-BP strain was correlated with lower synthesis of fibers and carbohydrates and higher content of ashes. The ratio of protein synthesis was inversely proportional to lipid synthesis in sporophores for all strains, with Led-08 strain showing highest lipid content compared to all strains. The water content of all strains was above 90 %, compatible with literature data.

In studies conducted for *L. edodes* grown on *Eucalyptus grandis* logs (Andrade (2008), Cheung (1996) and Yang, (2001)), whole mushroom samples ranged on averages from 18.67 to 22.67 % of protein, 2.01 % to 3.46 % for lipids, 3 to 3.88 % for ashes and 8.04 to 11.44 % for fiber, with such variations been dependent on the cultivated strain. These values of protein are lower than the results obtained in our study (Table 4). These comparisons show the importance of the experimental design in assessing the best conditions of substrate formulation and induction time on the nutritional quality of the product obtained.

Enzymatic activities of dikaryotic strains

We also compared the activity of some enzymes that are important in the hydrolysis of lignocellulosic materials present in the substrates. Therefore, total activities of amylases, cellulases, β-glucosidases, and the specific proteolytic activities of strains cultivations were followed and correlated with variations in the liberation of total reducing sugars and pH in the medium (Figure 3). Led-T2 strain produced the highest activities of cellulases (FPA 5.73 U/kg of substate in 13 days of incubation, Figure 3 c), and amylases (8.27 U/kg of substate in 31 days of incubation, Figure 3 b). These results correlate well with the high amount of reducing sugars liberated in the crude extract of Led-T2 cultivations. Low molecular weight sugars are easily assimilated by the mycelium, favoring the mushroom growth (Royse 1990; Philippoussis et al. 2002; Pereira et al. 2003). This profile of reducing sugars remained high during the first 30 days of cultivation. Several authors reported similar behavior concerning liberation of

reducing sugars in the crude extract during the early stages of cultivation, remaining constant along short incubation periods (Tokimoto 1987; Ohga and Royse 2001; Kwon et al. 2008; Philippoussis et al. 2011). This strain also produced one of the highest specific proteolytic activities (Figure 3 e) in comparison to other strains. Our results suggest that it would be useful to run these enzymatic tests as a quick way to evaluate *L. edodes* strains concerning their potential in the axenic cultivations.

All strains showed similar enzymatic profiles concerning *β*-glucosidase activities (Figure 3 c), peaking at 13th day of cultivation (Led-BP, 2.54 U/kg of substate) and 19th day of cultivation (Led-T2, 2.61 U/kg of substate), and again at the end of cultivations (Led-BP, 3.43 U/kg of substrate). Led-BP strain, the one with highest protein content of sporophores, and lower carbohydrate, showed the highest β-glucosidase activity. This enzyme plays a fundamental biochemical process in the carbon metabolism (Lynd et al. 2002) and studies have shown that *β*-glucosidase activities have negative correlation with the concentration of low-molecular weight carbohydrates (Morais et al. 2001; Mfombep et al. 2013). This fact confirms the results of this work, as the Led-BP strain had the lowest levels of reducing sugars in the crude extract (Figure 3 a). The formation of biomass of Led-BP strain was characterized by a mycelia mass thicker than the other strains and presenting excessive free water removal from the substrate during incubation. In addition, this strain presented the slowest blackening of the substrate.

Conclusion

Lentinula edodes (Berk.) Pegler is a commercially important mushroom and the development of axenic cultivations for this species would help its production. Testing strains is also important, but they must be tested to their stability and productivity along time. We tested four dikaryotic strains for their potential as mushroom production. All strains belong to the species *Lentinula edodes* (Berk.) Pegler based on the amplification of ITS1 and ITS2 rDNA regions. The CCD revealed the best conditions of $R_{C/N}$ and $I_{(t)}$ when using *Eucalyptus saligna* sawdust supplemented with wheat bran as the basis for the cultivation of *L. edodes*. The comparisons of centesimal compositions of strains, cultivated under the best conditions predicted by the experimental design, showed the interrelation between protein content, biological efficiency, and enzymatic activities of hydrolytic enzymes for all strains. Our results suggest useful tools for selecting strains of *L. edodes* of high standard productivity to be used in the axenic cultivation of this species of mushroom.

Acknowledgements

The authors whish to thank Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Fundação de Amparo a Pesquisa do Rio Grande do Sul (FAPERGS) for the research funding and the scholarships of the first two authors.

References

Ando, M (1974) Fruit-body formation of *Lentinula edodes* (Berk.) Sing. on the artificial media. Mush Sci, 9(1): 415-422

Andrade MCN, Minhoni MTA, Zied DC (2008). Caracterização bromatológica de oito linhagens de *Lentinula edodes* (Shiitake) cultivadas em toras de *Eucalyptus grandis*. Ciênc Tecnol Aliment, v.28, n.4

Association of Official Analytical Chemists – AOAC (1997). Official Methods of Analysis of A.O.A.C., 16th ed, ver. 3, Gaithersburg: AOAC International

Baldrian P, Valaskova V (2008). Degradation of cellulose by basidiomycetous fungi. FEMS Microbiol Rev. (32): 501-521

BOX GEP (1953) Non-normality and tests on variances. Biometrika, (40): 318-335

Box GEP, Witson KB (1951). On the experimental attainment of optimum conditions. JRoy Stat Soc (13): 1-45

Chang ST, Miles PG (1989). Edible mushrooms and their cultivation. CRC Press, Boca Raton, 345 p

Chen, AW (2005) What is shiitake. In: Gush R (ed) Mushroom growers' handbook 2. Mush World, Seoul, pp 1–11

Cheung PCK (1996) Dietary fiber content and composition of some edible mushroom fruiting bodies and mycelia. Jour of agric food chem, v. 44, (2): 468-471

Elisashvili V, Penninckx M, Kachlishvili E, Tsiklauri N, Metreveli E, Kharziani T, Kvesitadze G (2008) *Lentinus edodes* and *Pleurotus* species lignocellulolytic enzymes activity in submerged and solid-state fermentation of lignocellulosic wastes of different composition. Bioresour. Technol. (99): 457-462

Gaitán-Hernández R, Cortés N, Mata G (2011). Quantitative changes in the biochemical composition of lignocellulosic residues during the vegetative growth of *Lentinula edodes*. Braz. J. Microb (42): 30-40

Jackson ML (1965). Soil chemicals analysis. Englewood, Prentice-Hall, 370p

Kalberer PP (1995). An investigation of the incubation phase of a shiitake (*Lentinus edodes*) culture. Mush Sci, (14): 375-383

Kües U, Liu Y (2000). Fruiting body production in basidiomycetes. App Microb and Biotech, 54(2): 141-152

Kwon HW, Back IJ, Ko HG, You CH, Kim SH (2008). Extracellular enzyme activities of the monokaryotic strains generated from basidiospores of shiitake mushroom. Mycobiology, 36(1):74-76

Kwon HW, Choi MA, Yun YH, Oh YL, Kong WS, Kim SH (2015). Genetic and biochemical characterization of monokaryotic progeny strains of button mushroom (*Agaricus bisporus*). Mycobiology, 43(1): 81–86

Leatham GF (1985) Extracellular enzymes produced by the cultivated mushroom *Lentinus edodes* during degradation of a lignocellulosic medium. Appl Environ Microbiol (50): 859–867

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. Jour of Biol Chem, (19): 265-275

Lynd, LR, Weimer PJ, Van Zyl WH, Pretorius IS (2002). Microbial Cellulose Utilization: Fundamentals and Biotechnology. Microbiology and Molecular Biology Reviews, 66(3): 506–577

Mata G, Savoie JM (1998). Extracellular enzyme activities in six *Lentinula edodes* strains during cultivation in wheat straw. World. Microbiol. Biotechnol. (14): 513-519

Mata G, Savoie JM (2005) Whet straw. In: Gush R (ed) Mushroom growers' handbook 2. Mush World, Seoul, pp 105–109

Mfombep PM, Senwo1 ZN, Isikhuemhen OS (2013). Enzymatic activities and kinetic properties of *β*-glucosidase from selected white rot fungi. Adv in Biol Chem (3): 198-207

Miller GL (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 31(3): 426–428

Miller MW, Jong SC (1986). Commercial cultivation of shiitake in sawdust filled plastic bags. In: Proceed. Int. Sym. Scientific and Technical Aspects of Cultivating Edible Fungi. Penn State Univ., PA, USA

Mishra C, Leatham, GF (1990). Recovery and fractionation of the extracellular degradative enzymes from *Lentinula edodes* cultures cultivated on a solid lignocellulosic medium. Jour of Ferm Eng (69): 8-15

Morais H, Ramos C, Forgacs E, Cserhati T, Oliviera J, Illes T (2001). Three-dimensional principal component analysis employed for the study of the beta-glu- cosidase production of *Lentinus edodes* strains. Chem and Intel Lab Syst, (57): 57-64

Moyson E, Verachtert H (1991). Growth of higher fungi on wheat straw and their impact on the digestibility of the substrate. Appl Microbiol Biotechnol (36): 421-424

Ohga S, Royse DJ (2001). Transcriptional regulation of laccase and cellulose genes during growth and fruiting of *Lentinula edodes* on supplemented sawdust. FEMS Microbiol Lett (201):111–115

Pacumbaba RP, Pacumbaba RO Jr (1999). Shiitake mushroom growth on the formulated culture media, production of spawn and basidiocarps in the laboratory. HortTech 9(1): 85-90

Pereira Júnior JA, Correia MJ, Oliveira NT (2003) Cellulase activity of a *Lentinula edodes* (Berk.) Pegl. strain grown in media containing carboximetilcellulose or microcrystalline cellulose. Braz Arch Biol Technol 46(3): 333–337

Philippoussis A, Diamantopoulou P, Israilides C (2007) Productivity of agricultural residues used for the cultivation of the medicinal fungus *Lentinula edodes*. Int Biodeterior Biodegrad 59(3):216–219

Philippoussis A, Diamantopoulou P, Papadopoulou K, Lakhtar H, Roussos S, Parissopoulos G, Papanikolaou S (2011). Biomass, laccase and endoglucanase production by *Lentinula edodes* during solid state fermentation of reed grass, bean stalks and wheat straw residues. World Jour of Microb and Biotech, v. 27, (2): 285-297

Philippoussis A, Diamantopoulou P, Zervakis G (2002) Monitoring of mycelium growth and fructification of *Lentinula edodes* on several agricultural residues. In: Sanchez JE, Huerta G, Montiel E (eds) Mushroom biology and mushroom products. UAEM, Cuernavaca, pp 279–287

Philippoussis A, Diamantopoulou P, Zervakis G (2003) Correlation of the properties of several lignocellulosic substrates to the crop performance of the shiitake mushroom *Lentinula edodes*. World J Microbiol Biotechnol 19(6): 551–557

Philippoussis A, Zervakis G, Diamantopoulou P (2001) Bioconversion of lignocellulosic wastes through the cultivation of the edible mushrooms *Agrocybe aegerita*, *Volvariella volvacea* and *Pleurotus spp*. World J Microbiol Biotechnol 17(2): 191–200

Pire DG, Wright JE, Alberto E (2001). Cultivation of shiitake using sawdust from widely available local woods in Argentina. Micol. Apl. Int. 13(2): 87-91

Rossi IH, Monteiro AC, Machado JO, Andrioli JL, Barbosa JC (2003). Shiiatke *Lentinula edodes* production on a sterilized bagasse substrate enriched with rice bran and sugarcane molasses. Braz J Microbiol (34): 66–71

Royse DJ (1985). Effect of spawn run time and substrate nutrition on yield and size of the shiitake mushroom. Mycologia. (77): 756–762

Royse DJ (1996) Yield stimulation of shiitake by millet supplementation of wood chip substrate. Mush Biol and Mush Prod. Penn State Univ Press; Pennsylvania: 277–283

Royse DJ (2004) Specialty mushrooms, mushroom fact sheet. Mushroom Spawn Laboratory, Penn State University, Pennsylvania

Royse DJ, Bahler BD, Bahler CC (1990). Enhanced yield of shiitake by saccharide amendment of the synthetic substrate. Appl. Environ. Microbiol. 56(2): 479-482

Royse DJ, Bahler CC (1986). Effects of genotype, spawn run time and substrate formulation on biological efficiency of shiitake. Appl. Environ. Microbiol. 52(6): 1425-1427

Royse DJ, Sanchez JE (2007) Ground wheat straw as a substitute for portions of oak wood chips used in Shiitake (*Lentinula edodes*) substrate formulae. Biors Technol (98): 2137–2141

Royse DJ, Sanchez-Vazquez JE (2001) Influence of substrate wood-chip particle size on shiitake (*Lentinula edodes*) yield. Bior Tech, v. 76(3): 229-233

Sarath G, de la Motte RS, Wagner FW (1989) Proteases assay methods. In: Beyon, R.J. Bond, J.S. (eds). Proteolytc enzymes: a practical approach, IRL, Oxford, pp 259

Silva EM, Machuca A, Milagres AMF (2005) Effect of cereal brans on *Lentinula edodes* growth and enzyme activities during cultivation on forestry waste. Lett Appl Microbiol (40): 283–288

Silva ES, Cavallazzi JRP, Muller G (2007) Biotechnological applications of *Lentinus edodes*. J Food Agric Environ 5(3): 403– 407

Tan YH, Moore D (1992). Convenient and effective methods for in vitro cultivation of mycelium and fruiting bodies of Lentinus edodes. Myc. Res. 96(12): 1077-1084

Tedesco MJ, Gianello C, Bissani CA, Bohnen H, Volkweiss SJ (1995). Análise de solos, plantas e outros materiais. Porto Alegre, Universidade Federal do Rio Grande do Sul, Boletim Técnico, 5: 174p

Tokimoto K, Fukuda M, Kishimoto H, Koshitani H (1987) Activities of enzymes in bed logs of *Lentinus edodes* during fruit body development. Reports of the Tottori Mycological Institute (25): 24-56

Walkley A & Black, IA (1934). An examination of the Degtjareff method for determining soil organic matter and a proposed modification of the chromic acid titration method. Soil Sci, (37): 29-38

Yang JH, Lin HC, Mau JL (2001) Non-volatile taste components of several commercial mushrooms. Food Chemistry, v. 72, (4): 465-471

Zadrazil, F (1993). Lentinula (=Lentinus) edodes: physiology and conditions of industrial production. Mush. Inf. 6: 5-27

Table 1. Chemical composition of the substrates

Determinations**	*Eucalyptus saligna* Sawdust	Wheat bran	Applied methodology	Detection limit
Humidity - (kg/m^3)	22	13	Gravimetric	-
pH	4.7	6.4	sample:water ratio 1:5	Potentiometric
Wet density	252	388	-	-
Organic carbon - % (mass fraction)	42	41	Wet combustion/Walkey Black	0.01 %
Nitrogen (TKN) - % (mass fraction)	0.03	2.8	Kjeldahl	0.01 %
Phosphorus* - % (mass fraction)	0.02	1.3	Nitric-perchloric wet digestion ICP-OES	0.01 %
Potassium* - % (mass fraction)	0.07	1.2	Nitric-perchloric wet digestion ICP-OES	0.01 %
Calcium* - % (mass fraction)	0.53	0.15	Nitric-perchloric wet digestion ICP-OES	0.01 %
Magnesium* - % (mass fraction)	0.04	0.51	Nitric-perchloric wet digestion ICP-OES	0.01 %
Sulfur* - % (m/m)	0.01	0.19	Nitric-perchloric wet digestion ICP-OES	0.01 %
Copper* - (mg/kg)	2	14	Nitric-perchloric wet digestion ICP-OES	0.6 mg/kg
Zinc* - (mg/kg)	4	123	Nitric-perchloric wet digestion ICP-OES	2 mg/kg
Iron* - (mg/kg)	142	338	Nitric-perchloric wet digestion ICP-OES	4 mg/kg
Manganese* - (mg/kg)	362	142	Nitric-perchloric wet digestion ICP-OES	4 mg/kg
Sodium* - (mg/kg)	164	178	Nitric-perchloric wet digestion ICP-OES	10 mg/kg
Boron* - (mg/kg)	4	3	Dry digestion ICP-OES	1 mg/kg

**Results expressed in dried basis, except for pH and density. All results are mean of duplicates.

Table 2. **A**ctual and coded levels of the independent variables incubation time (X_1) and percentage of wheat bran in relation to *Eucalyptus saligna* sawdust (X_2) in the experimental design of the axenic cultivation of *Lentinula edodes* dikaryotic strains. The percentage of supplementation is represented as carbon/nitrogen ratio; the biological efficiency is given in kg of harvested mushroom (wet basis)/ kg of substrate (dry basis); the unit weight is given in grams by dividing the total mushroom mass divided by the total number of mushrooms formed in the first flush production.

Assay	Coded variables		Uncoded Variables		Biological efficiency (kg/kg of substrate)				Unit weight (g)			
	X1	X2	Incubation time (days)	% of wheat bran in relation to Eucalyptus sawdust (Carbon/nitrogen ratio)	LED-08	LED-T1	LED-T2	LED-BP	LED-08	LED-T1	LED-T2	LED-BP
1	-1	-1	70	10 (136.07/1)	0.085	0.272	0.258	0.052	35.00	21.44	28.95	10.44
2	1	-1	120	10 (136.07/1)	0.091	0.303	0.313	0.071	40.00	17.04	12.09	8.00
3	-1	1	70	50 (40.76/1)	0.178	0.324	0.341	0.063	28.00	11.00	16.22	8.44
4	1	1	120	50 (40.76/1)	0.212	0.364	0.408	0.122	29.48	11.99	9.02	5.15
5	-1.41	0	60	30 (57.62/1)	0.116	0.129	0.245	0.043	30.00	16.00	14.20	10.01
6	1.41	0	130	30 (57.62/1)	0.359	0.438	0.495	0.236	37.39	11.80	10.50	14.00
7	0	-1.41	95	1.8 (496.69/1)	0.083	0.147	0.216	0.000	21.01	19.77	23.70	0.00
8	0	1.41	95	58.2 (37.14/1)	0.281	0.391	0.452	0.052	41.94	12.17	8.44	12.27
9	0	0	95	30 (57.62/1)	0.189	0.404	0.462	0.113	22.00	16.15	17.44	12.10
10	0	0	95	30 (57.62/1)	0.192	0.409	0.475	0.106	25.00	16.97	16.46	12.80
11	0	0	95	30 (57.62/1)	0.219	0.406	0.464	0.102	24.00	16.43	16.99	12.80

Table 3. Regression coefficients of the variables and the regression for parameters biological efficiency (kg/kg of substrate) and unit weight (g) of axenic cultivation of dikaryotic parental strains.

Coefficient	Led-08				Led-T1				Led-T2				Led-BP			
	Yield of sporophores		Unit weight		Yield of sporophores		Unit weight		Yield of sporophores		Unit weight		Yield of sporophores		Unit weight	
	Coefficient	p	Coefficient	p	Coefficient	P	Coefficient	P	Coefficient	p	Coefficient	p	Coefficient	p	Coefficient	p
β_0	-1.2660	0.041	43.759	0.015	-1.0762	0.017	26.742	0.001	-1.212	0.00015	124.450	0.002	-0.7368	<0.0001	-33.791	0.272
β_1	0.0215	0.006	0.341	0.485	0.0177	0.002	-0.542	0.092	0.022	<0.0001	-1.195	0.018	0.0068	0.00014	-1.107	0.039
β_{11}	<0.0001	0.008	-0.004	0.397	-0.0003	0.004	0.001	0.669	<0.0001	<0.0001	0.008	0.056	-0.0001	<0.0001	-	-
β_2	0.0260	0.043	-0.053	0.703	0.0231	0.015	-	-	0.027	0.00011	-1.919	0.005	0.0150	<0.0001	1.371	0.049
β_{22}	-0.0001	0.047	-	-	-0.0001	0.021	-0.001	0.276	<0.0001	0.00013	0.009	0.008	-0.0001	0.00011	-0.009	0.024
β_{12}	-	-	-0.004	0.386	-	-	0.003	0.293	-	-	0.005	0.126	-	-	0.010	0.060
Regression																
p-Value	0.042		0.075		0.009		0.0009		<0.0001		0.030		0.0001		0.052	
F	4.943		4.062		9.64		22.077		65.119		33.292		46.736		5.299	
R^2	0.767		0.802		0.865		0.936		0.977		0.903		0.969		0.760	
p- Value LOF*	0.003		0.094		0.046		0.351		0.139		0.113		0.854		0.040	

*Lack of fit

Table 4. Chemical composition of dikaryotic strains obtained when cultivated in wheat bran/*Eucalyptus saligna* sawdust proportion to produce a carbon/nitrogen ratio of 57.26 for sporophores formation in 130 days.

Strains	Humidity	Lipids*	Proteins*	Ash*	Carbohydrates*	Fibers*
Led-08	93.26 ± 0.44	3.46 ± 0.00^{a}	26.49 ± 0.07^{c}	7.63 ± 0.04^{b}	51.46 ± 0.01^{a}	10.95 ± 0.04^{b}
Led-T1	93.58 ± 0.29	3.01 ± 0.06^{b}	28.18 ± 0.15^{b}	6.72 ± 0.02^{c}	50.16 ± 0.19^{a}	11.93 ± 0.04^{a}
Led-T2	92.90 ± 0.10	2.66 ± 0.01^{b}	27.35 ± 0.01^{b}	7.62 ± 0.22^{b}	51.92 ± 0.51^{a}	10.44 ± 0.27^{b}
Led-BP	93.48 ± 0.11	2.01 ± 0.00^{c}	32.02 ± 0.26^{a}	8.87 ± 0.02^{a}	48.11 ± 0.25^{b}	8.98 ± 0.02^{c}

(*) Results expressed on dry basis (mg/100 mg)

Standard deviation of the analyses in duplicates.

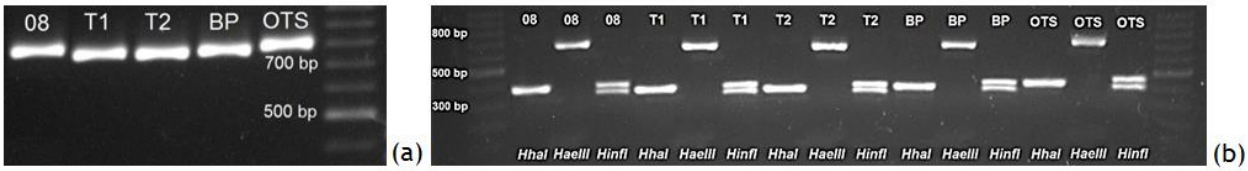

Figure 1. a) Gel electrophoresis of the amplified rDNA internal transcribed sequence of ITS1-5.8S-ITS2 regions of the *Lentinula edodes* strains; (b) PCR product digested with enzymes *HhaI*, *HinfI*, and *HaeIII* at 37 ° C.

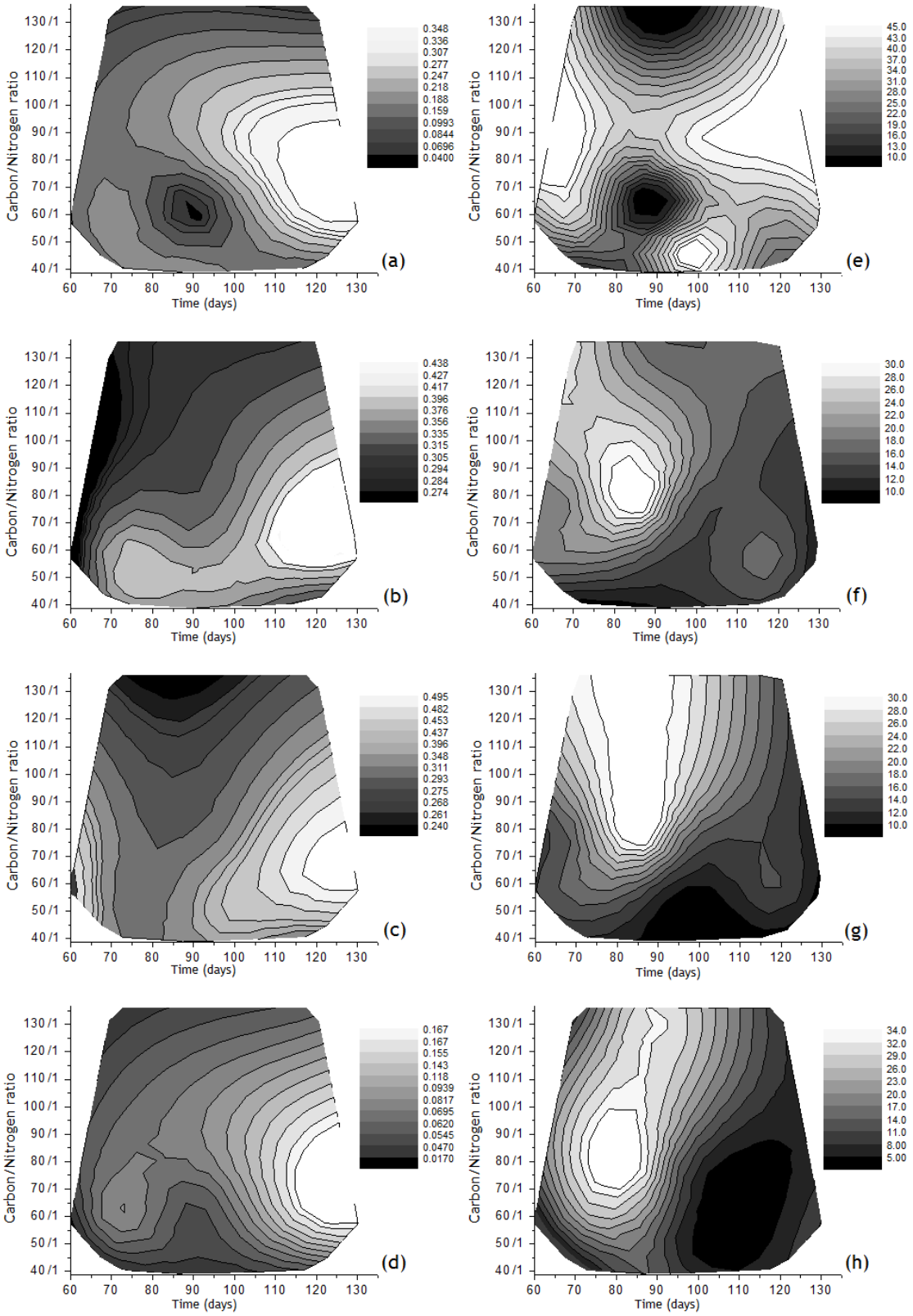

Figure 2. Surface response for biological efficiency (kg/kg of substate – a, b, c, and d) and unit weight (grams – e, f, g and h), for the range of carbon/nitrogen 136.07/1 to 37.14/1 and culture time between 60 and 130 days. Strains: Led-08 (a and e); Led-T1 (b and f); Led-T2 (c and g) and Led-BP (d and h).

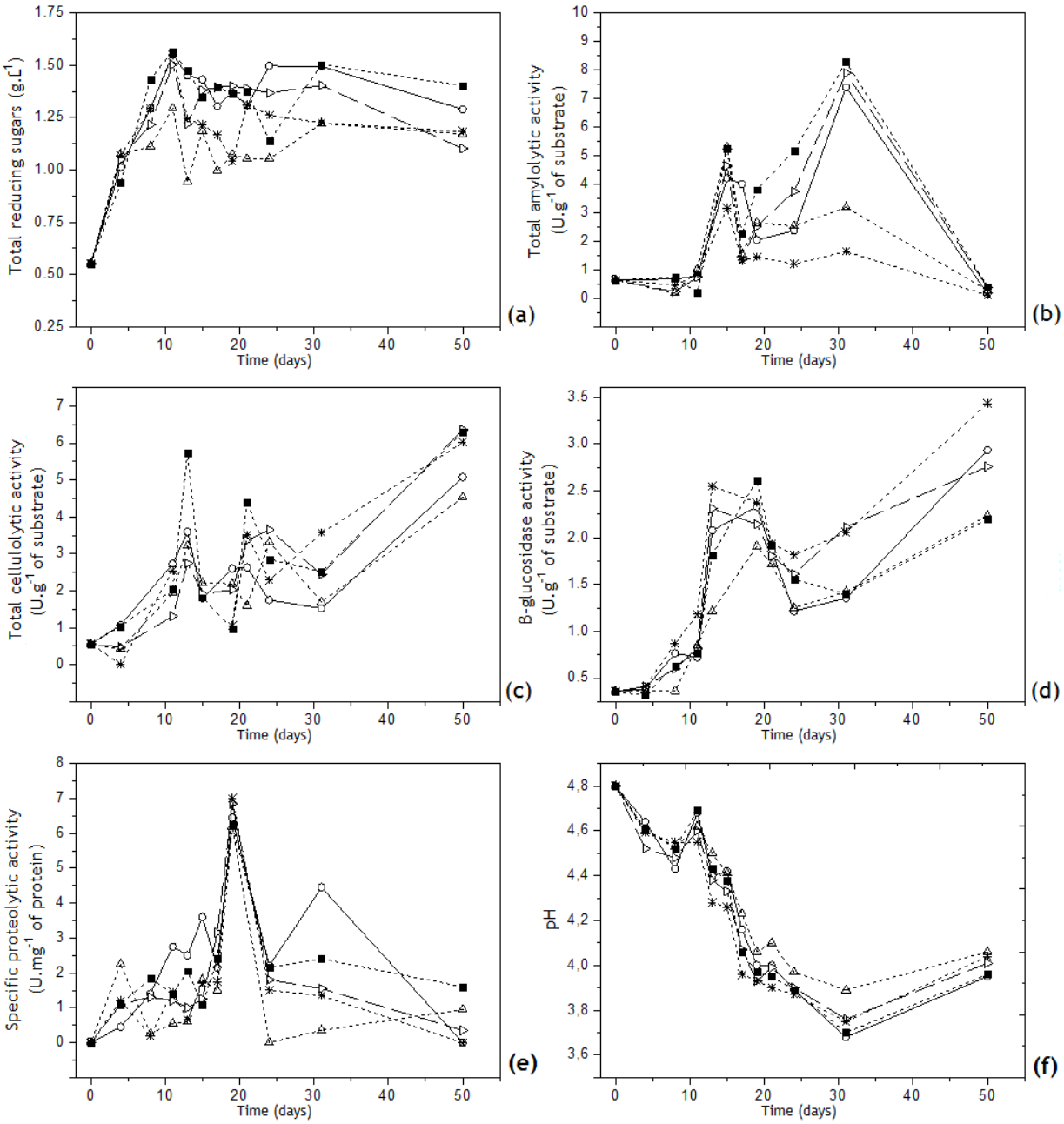

Figure 3. Enzyme assays of strains cultivated in test tubes containing 12 g of substrate and incubated at 25 ºC: (a) Total reducing sugar during; (b) Total amylolytic activity; (c) Total cellulolytic activity; (d) *β*-glucosidase activity; (e) Specific proteolytic activity; (f) pH. Dikaryotic strains: (—○— - Led-08); (···▷··· - Led-T1); (··■·· - Led-T2) (··✳·· - Led-BP) (··△·· - Led-OTS)

EVALUATION OF HYBRID LENTINULA EDODES (BERK.) PEGLER STRAINS BY INTRASPECIFIC MONOSPORIC CROSSES

1. Introduction

Hybridization of monokaryotic mycelium has good prospects for genetic improvement of commercial strains, discriminating working cultures that have stable production characteristics, firm texture and higher productivity (Kwon et al, 2015). Mushroom hybrid strains are generated by crossing monokaryotic hyphae, which are derived from germination of individual basidiospores (Gupta, 2011). In this work, we used three strains of *Lentinula edodes* (Berk.) Pegler to create hybrid strains generated by intraspecific crosses of monokaryotic hyphae obtained from the spread of spores of dikaryotic strains and evaluating the new hybrid strains concerning their yields of sporophores and enzymatic activities for total amylolytic and cellulolytic, β-glucosidase, and specific protease.

2. Materials and methods

2.1 Dikaryotic parental strains

Lentinula edodes (Berk.) Pegler, dikaryotic strains Led-08 (Fungibras spawn company, Botucatu, São Paulo, Brazil), Led-T2, and Led-BP (Funghi & Flora spawn company, Valinhos, São Paulo, Brazil), were used in this work. Stock cultures were kept in test tubes at 4 °C in Mushroom Complete Medium (MCM) at the microbial collection of the BiotecLab (Food Science and Technology Institute, UFRGS, Porto Alegre, Brazil).

2.2. Mating test

The isolation of basidiospores for intraspecific crossing between strains was based on the method described by Gupta, 2011. Mature sporophores were used for the isolation of individual basidiospores by printing in 2 % agar medium in Petri dishes containing ampicillin (100 $mg.mL^{-1}$). After 5 min of impression of basidiospores, sporophores were removed and the plates were incubated at 25 °C for 48 h allowing germination and hyphal proliferation. Vegetative colonies of monokaryotic mycelium were isolated in Mushroom Complete Medium (MCM) and arranged at both ends of the Petri dish in order to allow the fusion of the mycelium in the center. Compatible monokaryons were identified by the production of clamp connections.

2.3 Yield parameters and enzymatic assays

In order to evaluate the hybrid strains, axenic cultivations were carried out under conditions defined as the best, obtained in the CCD experimental design (Chapter 4, article III), using a carbon/nitrogen ratio ($R_{C/N}$) of 57.62/1, with a period of 130 days of incubation. Whole mushrooms were

collected for the analyses of yields and enzymatic assays of total amylolytic and cellulolytic activities, *β*-glucosidase, and specific protease activity, which were used as the parameters of comparison against the dikaryotic strains.

3. Results and discussion

Intraspecific monokaryotic crossing was carried out assuring that the hybridizations were between different strains. The Led-BP strain was selected in two different monokaryons, labeled BP-1 and BP-2, and the others received the identification of their parental strains and the identification "1" (Led-08-1 and Led T2 -1).

3.1 Enzyme activities and yields of hybrid strains

In Figure 1 are presented the results obtained for the enzymatic activities of the hybrid strains. Table 1 shows the yields of hybrid and parental strains and in Figure 2 are depicted the yields of sporophores during the first flush period.

The enzymatic activities of hybrid strains were higher than those obtained by the parental strains, except for the cellulolytic and total amylolytic activities of cross Led-BP-1 x Led-T2-1. The proteolytic activities of all hybrid strains showed a unique profile and were higher during the first 25 days of cultivation. These high proteolytic activities suggest that other enzymes might have been digested in the crude extract, thus underestimating the real enzymatic activities of the hybrid strains. The high concentration of reducing sugars in the crude extract of cultivations for the hybrid strains, including the cross Led-BP-1 x Led-T2-1, is correlated with the high *β*-glucosidase activities expected for fungi in general (Mfombep et al. 2013). This fact can be related to the premature production of sporophores, which was observed for the hybrid strains (Figure 2). The first harvest of mushrooms for the dikaryotic strains occurred after 10 days of primordia induction and the harvest extended up to the 16th day; for the hybrid strains, however, the first harvest occurred at the 8th day after the primordia induction, and harvest was extended up to the 10th day (Figure 2).

The hybrid strains that showed the best of extracellular enzymatic activities had also higher mushrooms productivities (Table 1). A more efficient enzyme activity promotes better nutrition of the mycelium and thus higher yield in sporophores (Royse 1990; Philippoussis et al. 2002; Pereira et al. 2003), as is confirmed by the results of BE for hybrid and dikaryotic strains.

Kwon et al. (2008) reported the activity of extracellular enzymes in *L. edodes* (amylase, avicelase, β-glucosidase, CM-cellulase, pectinase, protease, and xylanase) and identified higher activities in the progeny of monokaryon hyphae compared with two dikaryotic strains. In another study, the same authors (Kwon et al. 2015) measured the activities of extracellular enzymes β-glucosidase, avicelase, amylase, and pectinase in the progeny of *Agaricus bisporus* strains, showing higher activities than two dikaryotic strains, although these progeny showed no increased carboxymethylcellulase activity in relation to the dikaryotic strains. The analysis of the results in the literature, and the ones obtained in our work, suggests that the crosses were not physiologically ready for the production of sporophores. We suggest that comparisons of extracellular enzyme activities, associated with the

evaluation of cultivation parameters, would be useful to better understand the aspects of yields of hybrid and dikaryotic strains.

The high unit weight values obtained for dikaryotic strain Led-08 were not inherited by the two hybrid strains (Table 1). Several authors suggest that this inheritability is poorly understood (Rodier et al. 2000; Larraya et al. 2003; Foulongne-Oriol M et al. 2012). This might be explained by the fact that the propagated DNA content in the spores is haploid (homokaryons), whereas the evaluation of many important agronomic traits are only possible for dikaryotic phases, after crossing with a compatible mating type gene (Foulongne-Oriol M et al. 2012). In *Agaricus bisporus*, for instance, Foulongne (2012) observed that the earliest hybrids tended to produce a higher number of smaller mushrooms. Similar relationships have been described for other edible mushrooms, such as *P. ostreatus* (Larraya et al. 2003), and in our own work using *L. edodes* (Chapter 4, article III).

Further studies should be performed to identify the ideal physiological stage of *L. edodes* culture for the production of sporophores.

4. References

Foulongne-Oriol M, Rodier A, Rousseau T, Savoie JM (2012). Quantitative Trait Locus Mapping of Yield-Related Components and Oligogenic Control of the Cap Color of the Button Mushroom, Agaricus bisporus. Appl Environ Microbiol,78(7):2422-2434

Gupta B, Reddy, B.P.N; Kotasthane, A.S (2011). Molecular characterization and mating type analysis of oyster mushroom (Pleurotus spp.) using single basidiospores for strain improvement. World J Microbiol Biotechnol, 27(1): 1-9

Kwon HW, Back IJ, Ko HG, You CH, Kim SH (2008). Extracellular enzyme activities of the monokaryotic strains generated from basidiospores of shiitake mushroom. Mycobiology, 36(1):74-76

Larraya LM, Alfonso M, Pisabarro AG, Ramirez L (2003). Mapping of genomic regions (quantitative trait loci) controlling production and quality in industrial cultures of the edible basidiomycete Pleurotus ostreatus. Appl. Environ. Microbiol. 69: 3617–3625

Mfombep PM, Senwo1 ZN, Isikhuemhen OS (2013). Enzymatic activities and kinetic properties of β-glucosidase from selected white rot fungi. Adv in Biol Chem (3): 198-207

Pereira Júnior JA, Correia MJ, Oliveira NT (2003) Cellulase activity of a Lentinula edodes (Berk.) Pegl. strain grown in media containing carboximetilcellulose or microcrystalline cellulose. Braz Arch Biol Technol 46(3): 333–337

Philippoussis A, Diamantopoulou P, Zervakis G (2002) Monitoring of mycelium growth and fructification of Lentinula edodes on several agricultural residues. In: Sanchez JE, Huerta G, Montiel E (eds) Mushroom biology and mushroom products. UAEM, Cuernavaca, pp 279–287

Rodier A, Devesse C, Rousseau T, Védie R, Imbernon M, Olivier JM (2000). Breeding brown hybrids of button mushroom (Agaricus bisporus) from a factorial cross.Mushroom Sci. (15): 289–297

Royse DJ, Bahler BD, Bahler CC (1990). Enhanced yield of shiitake by saccharide amendment of the synthetic substrate. Appl. Environ. Microbiol. 56(2): 479-482

Table 1 - Yield of sporophores and unit mass of hybrid strains cultivated in carbon/nitrogen ratio 57.62/1 for sporophores formation in 130 days.

Dikaryotic parental strain	BE ($kg.kg^{-1}$ of substrate)	Unit weight (g)
Led-08	0.359	37.39
Led-T1	0.438	11.80
Led-T2	0.495	10.50
Led-BP	0.236	14.00
Hybrid strain	**BE ($kg.kg^{-1}$ of substrate)**	**Unit weight (g)**
Led-08 - 1 x Led-BP -2	0.196	11.35
Led-08 - 1 x Led-T2 -1	0.173	12.81
Led-BP -1 x Led- T2 -1	0.046	10.33

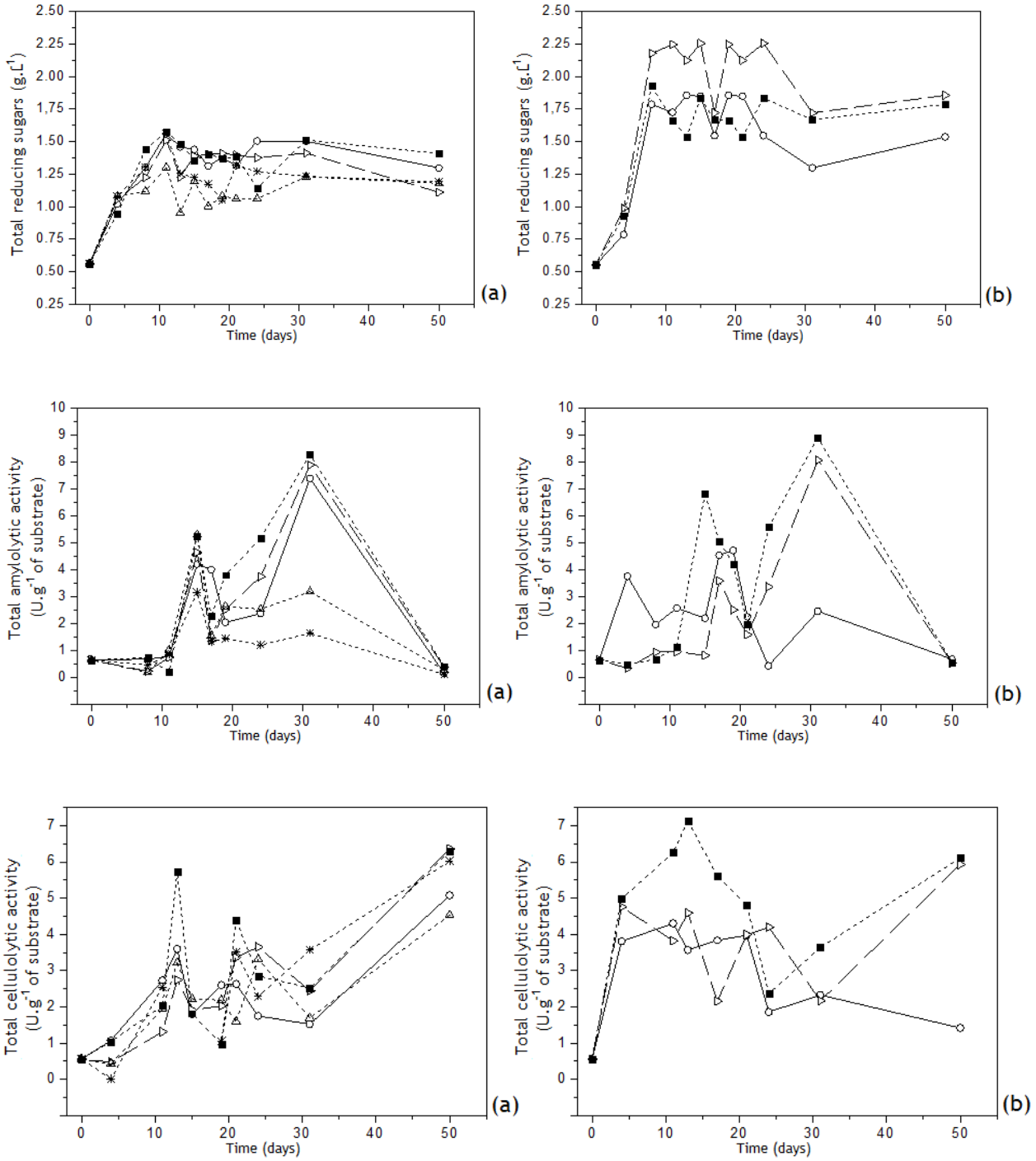

Figure 1 – Legend other page.

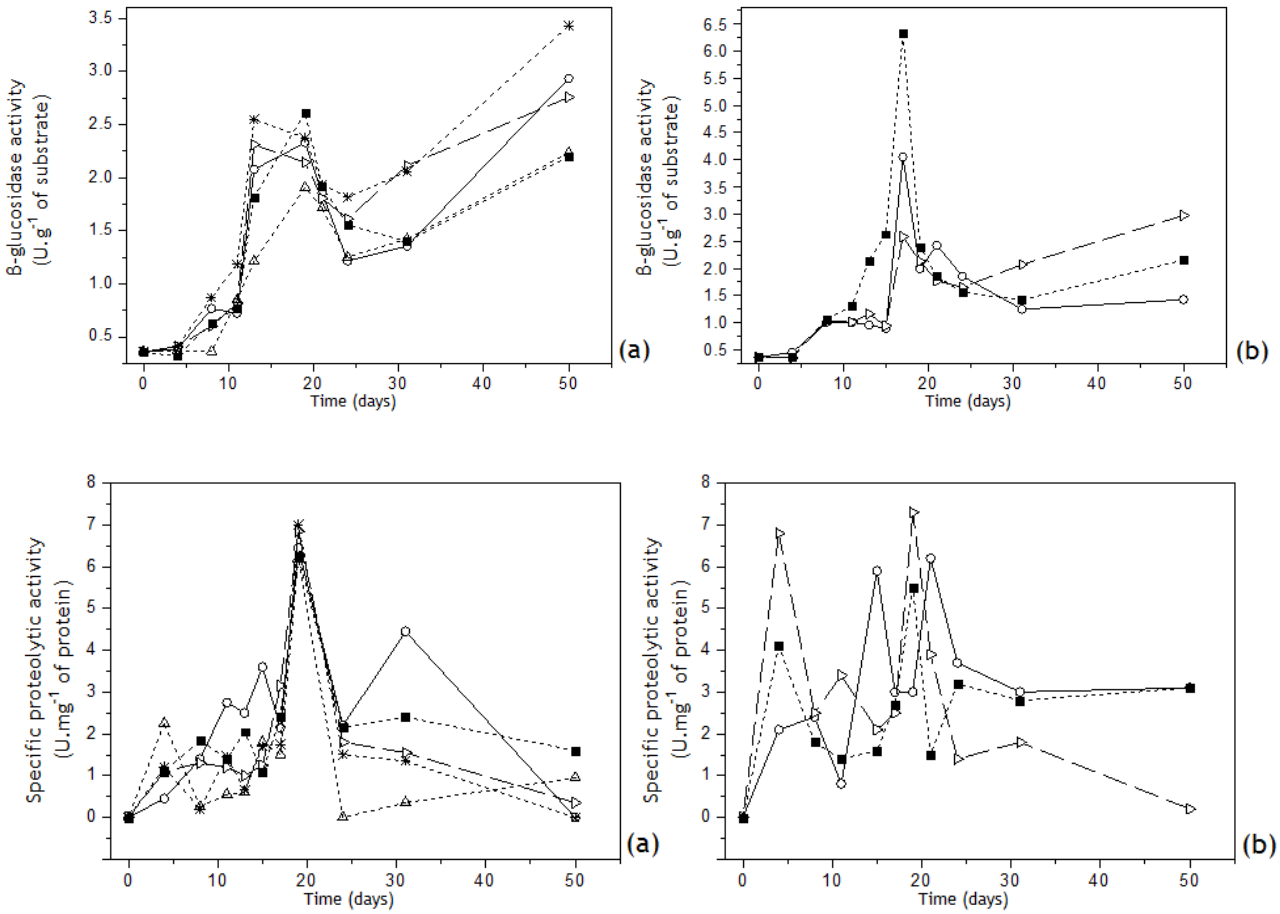

Figure 1- Enzyme assays of strains cultivated in test tubes containing 12 g of substrate at 25 ºC. Activities analyzed were total reducing sugar; total amylolytic activity; total cellulolytic activity; *β*-glucosidase activity; and specific proteolytic activity. (a): dikaryotic parental strains: (—○— - Led-08); (···▷·· - Led-T1); (··■·· - Led-T2) (··✳·· - Led-BP) (··△·· - Led-OTS); and (b): hybrid strains (—○— - Led – BP-1 x Led-T2-1); (···▷·· - Led-08-1 x Led-T2-1); (··■·· Led-08-1 x Led-BP-2).

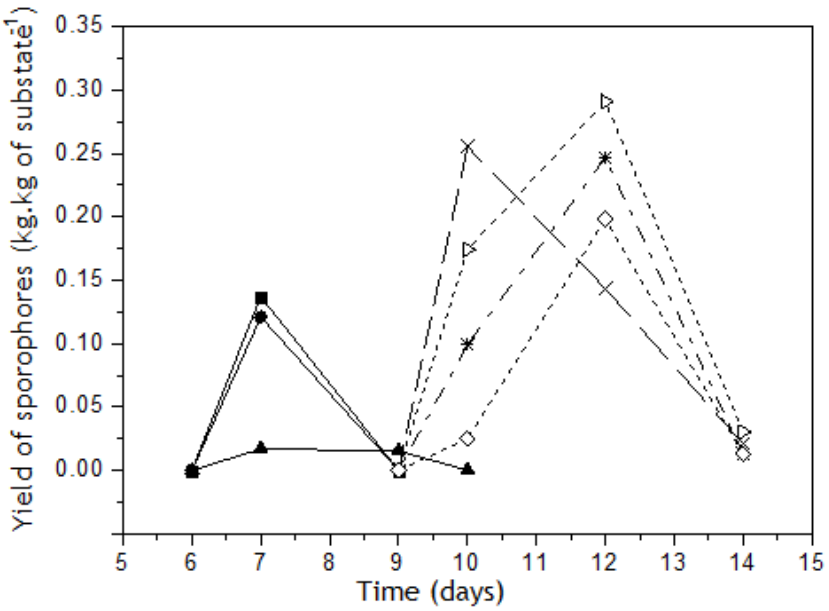

Figure 2. Yields of sporophores (kg.kg of substate^{-1}) during the first flush period (time in days after induction of the formation of pinning). Strains were grown in 57.62/1 carbon/nitrogen ratio for sporophores formation in 130 days: strains were: (··✳·· Led-08); (···×··· Led-T1); (···▷·· Led-T2); (··◇·· Led-BP); (—▲— Led – BP-1 x Led-T2-1); (—●— Led-08-1 x Led-T2-1); and (—■— Led-08-1 x Led-BP-2).

5. Considerações finais

A eficiência produtiva de cogumelos em cultivo é dependente das linhagens propagadas e da formulação adequada dos substratos. Requer testes frequentes de viabilidade e de avaliação da produtividade das culturas-estoque, visto que ao serem repicadas continuadamente de forma vegetativa favorecerão a degeneração da reprodutibilidade das características fenotípicas existentes. A hibridação de hifas monocarióticas tem se demonstrado como a ferramenta mais eficiente de reverter esta instabilidade e propicia a geração de linhagens novas a serem avaliadas, pode contribuir para o aumento da produtividade dos cogumelos e é uma tecnologia promissora para fixar características de interesse na construção de novos genótipos. Conforme o Art. 10 da Instrução Normativa 37, (02/08/2011) – Ministério da Agricultura, Pecuária e Abastecimento, os inóculos adquiridos fora da unidade de produção deverão ter origem de produtor regularizado para tal fim, sendo proibido o uso de inóculo proveniente de material transgênico. Assim, o cruzamento de hifas monocarióticas é uma alternativa aceitável para o desenvolvimento de novas linhagens para cultivo de cogumelos comestíveis. Neste contexto, o artigo III, intitulado: The axenic cultivation of *lentinula edodes* (berk.) pegler: compost optimization and evaluation of dikaryotic strains**,** teve como objetivo avaliar a produtividade de cinco linhagens comerciais de L. edodes, considerando como variável de avaliação a eficiência biológica em cultivo axênico que foi correlacionada com a potencialidade de síntese de enzimas extracelulares (atividades amilolíticas, celulolíticas, proteolíticas e de β-glucosidase) responsáveis pela hidrólise do substrato em cada um dos genótipos testados. Os resultados demonstraram que as linhagens que apresentaram melhor potencial de produção de enzimas extracelulares promoveram maior rendimento em corpos de frutificação. Entretanto, conforme demonstrado no Apêndice intitulado EVALUATION OF HYBRID LENTINULA EDODES (BERK.) PEGLER STRAINS BY INTRASPECIFIC MONOSPORIC CROSSES, nas linhagens híbridas, obtidas através do cruzamento de hifas monocarióticas oriundas da germinação de esporos das linhagens parentais, a produção destas enzimas foi superior e o rendimento em cogumelos foi inferior em relação aos genótipos parentais e, além disto, apresentaram uma produção precoce de corpos de frutificação. Mesmo que em rendimentos inferiores, os resultados de produtividade em cogumelos entre as linhagens híbridas foram semelhantes às linhagens dicarióticas parentais quando se refere à comparação com a produção enzimática, demonstrando que os ensaios enzimáticos com melhor potencial representam genótipos superiores em produtividade. Recomenda-se que mais estudos sejam efetuados com repiques em estágios mais avançados de maturação da cultura de propagação para avaliação das linhagens híbridas de *L. edodes* obtidas neste estudo. Também foram estabelecidas, neste trabalho, condições ótimas de suplementação a base de farelo de trigo em serragem de *Eucalyptus saligna* e o melhor tempo de incubação do substrato para o cultivo desta espécie nas condições testadas, chegando a uma $R_{C/N}$ ótima de 57,62/1 superior ao que é apresentado na literatura de 40/1 (Chen, 2005) e um $I_{(t)}$ de 130 dias, utilizando uma proporção de spawn de 3,4% (fração volumétrica), reduzindo-se, deste modo, a demanda por suplementação com as matérias-primas avaliadas. Além do rendimento em cultivo também foram avaliadas as propriedades físico-químicas dos substratos que são apresentados no artigo I, intitulado: PHYSICO-CHEMICAL

PROPERTIES OF THE SUBSTRATES AND MYCELIAL GROWTH RATE DETERMINATION IN LENTINULA EDODES (BERK.) PEGLER DIKARYOTIC STRAINS, onde se concluiu que a biomassa fúngica de *L. edodes* obtém melhores condições de crescimento no intervalo de $R_{C/N}$ de 65,.90/1 e 45.,01/1, com uma densidade úmida variando de 308 para 318 $g.L^{-1}$ e densidade seca de 166 para 178 $g.L^{-1}$, quando se utiliza a combinação dos substratos avaliados.

O spawn é tradicionalmente produzido pela inoculação asséptica do micélio em grãos de cereais. Nesta configuração, sua produção demanda escalonamento contínuo de repicagens em função de possuir curto prazo de armazenamento sob refrigeração. Ao ser produzido em cultivo submerso, a vida útil das culturas de propagação sob armazenamento refrigerado pode ser prolongada em comparação ao estado sólido. Esta apresentação é uma alternativa promissora de gerenciamento das culturas, pois garante viabilidade e vigor do inóculo em maior amplitude de tempo e permite a mecanização do processo de inoculação em cultivo axênico. Assim, o trabalho apresentado como artigo II, intitulado: GROWTH KINETICS AND MODELING OF LENTINULA EDODES (BERK.) PEGLER CULTURES IN AN EXTERNAL LOOP AIRLIFT BIOREACTOR teve como objetivo de estudo desenvolver inoculantes líquidos para cultivo axênico da espécie *L. edodes* através da propagação da biomassa em um biorreator airlift de circulação externa, comparando condições de operação de aeração (0,16, 0,20 e 0,24 wmvvm) e de tamanhos de inóculo (1,0 $g.L^{-1}$ e 2,0 $g.L^{-1}$). Na melhor condição operacional (0,16 vvm e 1,0 $g.L^{-1}$ de i.s), foi desenvolvido um modelo matemático, utilizando o software EMSO para determinação dos coeficientes de produção de biomassa (X), formação de produto (CO_2), consumo de oxigênio dissolvido no meio de cultura (PO_2), formação de ácidos H+ pelo decréscimo do pH e consumo do substrato (Glicose). Os resultados deste trabalho demonstraram que a fisiologia respiratória de crescimento do fungo *L. edodes* no cultivo submerso foi afetado pelo pH (faixa de 2.96 – 3.0), e com acidificação do meio, o fungo pôde ser cultivado sob condições de baixo teor de oxigênio dissolvido, na faixa 23,9% de saturação. Sob condições de controle do pH em 5,0 (condição ideal para este fungo) houve um efeito prejudicial sobre o crescimento celular e o metabolismo quando o oxigénio dissolvido caiu para concentrações críticas, no caso de *L. edodes*, quando caiu abaixo dos 40 %, com consequente formação de um produto no instante 7,4 min (Figura 5) que não foi caracterizado neste trabalho e escurecimento do meio de cultura a partir deste instante do cultivo (Figura 6). A biomassa resultante dos cultivos na melhor condição operacional poderá ser utilizada como inóculo líquido para o cultivo da referida espécie, proporcionado a sua utilização em sistemas mecanizados de inoculação. Neste trabalho foram apresentadas soluções tecnológicas para o desenvolvimento deste produto e propostos processos passíveis de padronização para produção industrial e aumento de escala.

Figura 5 – Escurecimento do meio de cultura na cinética de crescimento *de Lentinula edodes* (Led-08) no cultivo em biorreator airlift de circulação externa. As condições foram: pH controlado a 5,0; temperatura de 25 ° C; e taxa de aeração de 0,20 vvm.

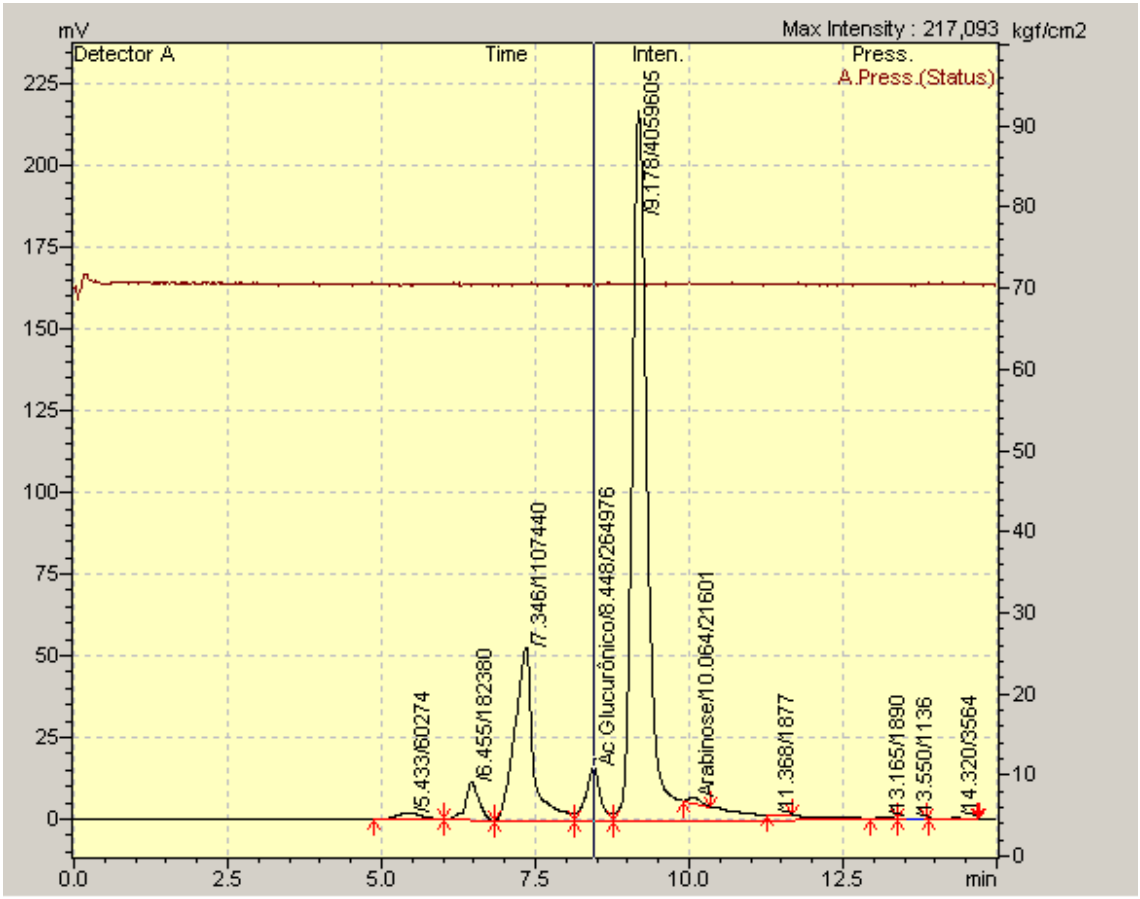

Figura 6 – Cromatograma do perfil do extrato bruto do cultivo no biorreator airlift de circulação externa com um detector de índice de refração e uma coluna Bio-Rad HPX-87H (300 mm x 7,8 milímetros) com 5 mM de ácido sulfúrico como eluente a 45 ° C, taxa de 0,6 mL.min-1 e volumes de amostra de 20 µ L. As condições foram: pH controlado a 5,0; temperatura de 25 ° C; e taxa de aeração de 0,20 vvm.

6. Perspectivas

A partir dos resultados obtidos neste trabalho, podem-se sugerir as seguintes linhas de investigação:

- Estudar a interação de compatibilidade entre os genes de acasalamento (mating-types) dos cruzamentos monospóricos obtidos.

- Avaliar as culturas híbridas sob diferentes estágios de propagação vegetativo do micélio, bem como sob diferentes estágios de maturação do corpo de frutificação.

- Estudar a influência do controle do pH em sistemas de cultivo submerso.

- Modelar o crescimento de *L. edodes* com outros açúcares de baixo peso molecular servindo como substrato.

- Estudar o escalonamento (”scale-up”) do bioprocesso proposto utilizando-se biorreatores airlift.

- Aplicar meios de cultura baratos, utilizando subprodutos agroindustriais nestes bioprocessos.

7. Referências bibliográficas

Alves F (2010). Modelagem e simulação de biorreator operando com fungos Trametes versicolor para produção de enzima lacase. Dissertação de Mestrado, Escola de Engenharia Mauá do Centro Universitário do Instituto Mauá de Tecnologia, São Caetano do Sul, SP, 80p

Badino AC Jr, Facciotti MC, Schmidell, W (2001). Volumetric oxygen transfer coefficients (kLa) in batch cultivations involving non-Newtonian broths. Biochem. Eng. J., (8):111-119

Beyer, DM. (2003). Basic Procedures for Agaricus Mushroom Growing, 16p

Bisko NA, Bilay VT, Babitskaya VG, Scherba VV, Mitropolskaya NY, Puchkova TA (2004). Biologically active substances from mycelia of Ganoderma lucidum, and Lentinula edodes. In: Romaine, Keil, Rinker, and Royse, Eds: Mushroom Science: Science and Cultivation of Edible and Medicinal Fungi. University Park, PA: The Pennsylvania State University Press. pp. 619-623

Borchers AT, Stern JS, Hackman RM, Keen CL, Gershwin ME (1999). Mushroom, tumors, and imumnity. PSEBM, 221(4):281-93

Breene WM (1990). Nutritional and medicinal value of speciality mushrooms. J Food Project, (53):883-94

Buléon A (1998). Starch granules: structure and biosintesis. Internacional journal of Biological Macromolecules, (23): 85-112

Buswell JA, Miles PG (1993). Genetics and breeding of edible mushrooms. Philadelphia: Gordon and Breach Scientific, p. 297-324

Buswell JA, Chang ST (1993). Edible mushrooms: attributes and applications. Amsterdam: Gordon and Breach Science Publishers, (15): 306-318

Buswell JA, Cai YJ, Chang ST (1993). Fungal and substrate associated factors affecting the ability of individual mushroom species to utilise different lignocellulosic growth substrates, In Mushroom Biology and Mushroom Products, eds Chang, S.T., Buswell, J.A. & Chiu, SW. pp. 141-150

Chang S, Miles PG (1989). Edible Mushrooms and their Cultivation. CRC Press Inc., Boca Raton, Florida. 345 p

Chang ST, Miles PG (2004). Mushrooms: Cultivation, Nutritional Value, Medicinal Effect and Environmental Impact. 2nd ed. CRC Press 480p

Chang ST (2008). Training Manual on Mushroom Cultivation Technology. Asian and Pacific Centre for Agricultural Engineering and Machinery (APCAEM) A-7/F, China International Science and Technology Convention Centre no. 12, Yumin Road, Chaoyang District, Beijing 100029, P.R. China, 65p

Chen AW (2001). Cultivation of Lentinula edodes on synthetic logs. Mushroom Growers' Newsletter 10(4): 3-9.

Chen AW (2004). Growing shiitake mushrooms. In: Mushroom Growers' Handbook 1: Oyster Mushroom Cultivation. Seoul, Korea: MushWorld. pp. 248-261.

Chen AW (2005). Mushroom Growers' Handbook 2: Shiitake Cultivation. Seoul, Korea: MushWorld.

Chisti MY, Moo-Young M (1987). Airlift reactors: characteristics, applications and design considerations. Chem Eng Commun (60): 195-242

Chisti MY (1989) Air-Lift Bioreactors, Elsevier, New York, 345p

Demiate MI, Shibata RKC (2003) Cultivo e análise da composição química do cogumelo do sol (Agaricus blazei Murril). Publ. UEPG Ci. Biol. Saúde, Ponta Grossa, v. 9, n. 2, p. 21-32

Ferreira ME, Grattapaglia D (1996). Polimorfismo de DNA amplificados ao acaso (RAPD). In:_. Introdução ao uso de marcadores moleculares em análise genética. Brasília: LUMMA, cap. 6, p. 38-51

Furlan JP (2011). Desenvolvimento de cinco linhagens de Agaricus bisporus lange (imbach) ("champignon de paris") em diferentes formulações de composto e meios de cultura. Dissertação de Mestrado, Faculdade de Ciências Agronômicas da Unesp, Botucatu, São Paulo, 77p

Gupta B, Reddy, B.P.N; Kotasthane, A.S (2011). Molecular characterization and mating type analysis of oyster mushroom (Pleurotus spp.) using single basidiospores for strain improvement. World J Microbiol Biotechnol, 27(1): 1-9

Gupta R, Mohapatra H, Goswami VK, Chauhan B (2003). Microbial α-Amylases: Biotechnological Perspective. Process Biochemistry 38(11): 1599-1616

Guzmán G, Mata G, Salmones D, Soto-Velazco C, Guzmán-Dávalos L (1993). El cultivo de los hongos comestibles. Instituto Politécnico Nacional. México. 245 pp

Herrera T, Ulloa M (1998). El reino de los hongos: Micología básica y aplicada. Fondo de cultura económica – UNAM. México. 552 pp

Hibbett DS et al.. (2007). "A higher level phylogenetic classification of the Fungi". Mycology Reserch 111 (5): 509–547

Kawai G, Kobayashi H, Fukushima Y, Ohsaki K (1996) Effect of liquid mycelial culture used as a spawn on sawdust cultivation of shiitake (Lentinula edodes). Mycoscience (37): 201-207

Kim S, Dale BE (2004). Global potential bioethanol production from wasted crops and crop residues. Biomass and Bioenergy (26):361-375

Kirk T, Farrell R (1987). Enzimatic "combustion": the microbial degradation of lignin. Annual Review of Microbiology (41):465-505

Koltin Y, Stamberg J, Lemke PA (1972). Genetic Structure and Evolution of the Incompatibility Factors in Higher Fungi. Bacteriological reviews. 36(2): 156-171

Koutinas AA, Wang R, Webb C (2003). Estimation of fungal growth in complex, heterogeneous culture. Biochemical Engineering Journal (14): 93–100

Kothe E (2001) Mating-type genes for basidiomycete strain improvement in mushroom farming. Appl Microbiol Biotechnol (56): 602– 612

Kothe E (2001) Sexual development in basidiomycetes. In: Osiewacz H (ed) Molecular biology of fungal development. Dekker, New York (in press): 245-273

Kuek C, Tommerup IC, Malajczuck, N (1992). Hydrogel bead inocula for the production of ectomycorrhizal eucalyptus for plantations. Mycol. Res., (96): 273-277

Li A, Begin M, Kokurewicz K, Bowden C, Horgen PA (1994). Inheritance of strain instability (sectoring) in the commercial button mushroom, Agaricus bisporus. Applied Environmental Microbiology. (60):2384–2388

Lynch M, Milligan BG (1994). Analysis of population genetic struture with RAPD markers. Molecular Ecology, (3): 9-19

Mattila P, Konko K, Eurola M, Pihlava JM, Astola J, Vahteristo L, Hietaniemi V, Kumpulainen J, Valtonen M, Piironen V (2001). Contends of vitamins, Mineral Elements, and Phenolic Compounds in Cultivated Mushrooms. J. Agric. Food Chem., v. 49 (5): 2343-2348

Mattila P, Suonpaa K, Piironen V (2000). Functional properties of edible mushrooms. Nutrition. v. 16 (8): 694-696

Minhoni MTA et al. (2005). Cultivo de Lentinula edodes (Berk.) Pegler – (Shiitake). 2. ed. Botucatu: FEPAF, 72 p

Mitchell, DA (1992). Biomass estimation in solidstate cultivation. Solid substrate cultivation. (4): 53-63

Molena O (1986). O moderno cultivo de cogumelos. São Paulo: Nobel, 170p

Moore-Landecker E (1996). Fundamentals of the fungi. 4a edición. Editorial Prentice Hall. USA. 574 pp.

Moraes LMP, SAID S, PIETRO R (2004). Amilases. Enzimas como agentes Biotecnológicos. Ribeirão Preto: Legis Summa, 412p

Moresi M (1981). Optimal design of airlift fementers. Biotechnol. Bioeng., (23):2537-2560

Nigam P, Singh D (1995). Enzyme and microbial systems involved in starch processing. Enzyme and Microbial Technology, (17): 770-778

Novaes MRCG, Fortes RC (2005). Efeitos antitumorais de cogumelos comestíveis da família agaricaceae. Rev Nutr Brasil, 4(4): 207-217

Novaes MRCG, Novaes LCG (2005). Fármaco-nutrientes em cogumelos comestíveis Agaricales e outros basidiomicetos. Rev Bras Nutr Clin. 20(3): 181-7

O'Shea SF, Chaure PT, Halsall JR, Olesnicky NS, Leibbrandt A, Connerton IF, Casselton LA (1998) A large heromone and receptor gene complex determines multiple B mating type zpecificities in Coprinus cinereus. Genetics (148): 1081–1090

Oei P (2003). Lentinula edodes (shiitake) cultivation on sterilized substrates, on wood logs. In: Mushroom Cultivation. 3rd ed. Leiden, the Netherlands: Backhuys. (1): 325-341

Oliveira LP (2004). Produção de inoculante, seleção e aplicação de fungos ectomicorrízicos em mudas de Pinus taeda L. Dissertação de Mestrado, Universidade Federal de Santa Catarina, Florianópolis, Brasil, 77p

Oliveira RS (2010). Inclusão do substrato de Pleurotus Ostreatus na Cinética de Fermentação in vitro do feno de braquiária. Dissertação de Mestrado, Universidade de Brasília, Distrito Federal, Brasil, 43p

Onken U, Träger M. (1990). A simplified model of hydrodynamics in airlift loop reactors. Bioprocess Engineering (5): 129-139

Onken U, Weiland P (1983). Airlift Fermenters: Construction, Behavior, and Uses, Adv. Biotechnol. Processes, 67-95

Pegler DN (1983). The genus Lentinula (Tricholomataceae tribe Collybieae. Sydowia, (36): 227-239

Pointing SB (2001). Feasibility of bioremediation by white-rot fungi. Applied Microbiology Biotechnology (57): 20-33

Raimbault M (1998). General and microbiological aspects of solid substrate fermentation. Journal of Biotechnology, Amsterdam, (1): 1-15

Rinker DL (1993). Commercial mushroom production. Ministry of Agriculture and Food, Ontario, publication, number 350, RV-10-93-8M. Queen's Printer for Ontario, Ontario, Canada.

Romaine CP, Schlagnhaufer B (1992). Characteristic of a hydrated alginate-based delivery system for cultivation of the button mushroom. Appl. and Environ. Microbiol. (58): 3060-3066

Rossi MJ (2006). Tecnologia para produção de inoculantes de fungos ectomicorrízicos utilizando cultivo submerso em biorreator airlift. Tese de Doutorado, Universidade Federal de Santa Catarina, Florianópolis, Brasil, 188p

Sanchez C (2004). Mini review: modern aspects of mushroom culture technology. Appl Microbiol Biot 64(1): 756-62

Silva EM, Machuca A, Milagres AMF (2005). Effect of cereal brans on Lentinula edodes growth and enzyme acticities during cultivation on forestry waste. Letters in Applied Microbiology, Oxford, (40) 283-288

Silveira M, Wisbeck E, Ninow J, Gern RMM, Furlan SA (2006). Pleurotus cultivation in submerged culture and nutritional value of fruit bodies cultivated in solid culture. In: Larroche C, Pandey A, Dussap CG (Orgs.). Current topics on bioprocesses in food industry (1):197-203

Singh SK, Doshi A, Yadav MC, Kamal S (2006) Molecular characterization of speciality mushroom of Western Rajasthan, India. Curr Sci (91):1225–1230

Smith J, Rowan NJ, Sullivan R (2002). Medicinal Mushrooms: Their therapeutic properties and current medical usage with special emphasis on cancer treatments. Cancer Research UK. University of Strathclyde

Sonnenberg ASM (2000). Genetics and breeding of Agaricus bisporus. In Van Griensven (ed.), Science and Cultivation of Edible Fungi. Balkema, Rotterdam. p. 25-39

Stamets P (1983). The mushroom cultivator. Agarikon, Olympia, WA, 415 p

Stamets P (2000). Growing gourmet and medicinal mushrooms. 3rd edition. Ten speed press, Berkeley, Toronto, Canada. 574 p

Stamets P (2005). Mycelium running: how mushrooms can help save the world. Hong Kong: Ten Speed, 356 p

Sun Y, Cheng J (2002). Hydrolysis of lignocellulosic materials for ethanol production: a review. Bioresouce Technology, (83): 1-11

Tarui HP (1997). Colonização de serragens e produção de basidiocarpos em diferentes espécies de eucalipto, pelo cogumelo comestível Lentinula edodes (Berk.) Pegler (shiitake). 44 p. Monografia (Curso)-Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista, Jaboticabal.

Urben AF et al (2001). Produção de cogumelos comestíveis por meio de tecnologia chinesa modificada. Brasília: EMBRAPA, Recursos Genéticos e Biotecnologia, 151 p

Valencia-del Toro G (2002). Estudios sobre la expresión del color de los esporóforos de Pleurotus spp. por apareamiento de neohaplontes compatibles y progenies monospóricas.Tesis de doctorado. UNAM. México, D.F.

Wang L, Ridgway D, Gu T, Moo-Young M (2005). Bioprocessing strategies to improve heterologous protein production in filamentous fungal fermentations. Biotechnology Advances, New York, 23(2): 115-129

Williams JGK, Kubelik AR, Livak K, Rafaslki JA, Tingey SV (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Research, 18 (65): 3-15

Wu JL (2000). Shiitake Production in China. Beijing, China: Chinese Agricultural Press.

Yang ST (2007). Bioprocessing for value-added products from renewable resources: New technologies and applications. Amsterdam: Elsevier, 684 p

Zhang YHP, Lynd LR (2004). Toward an aggregated undertanding of enzymatic hydrolysis of cellulose: non complexed cellulase systems. Biotechnology Bioengeniring, (88): 797-824

8. Anexos

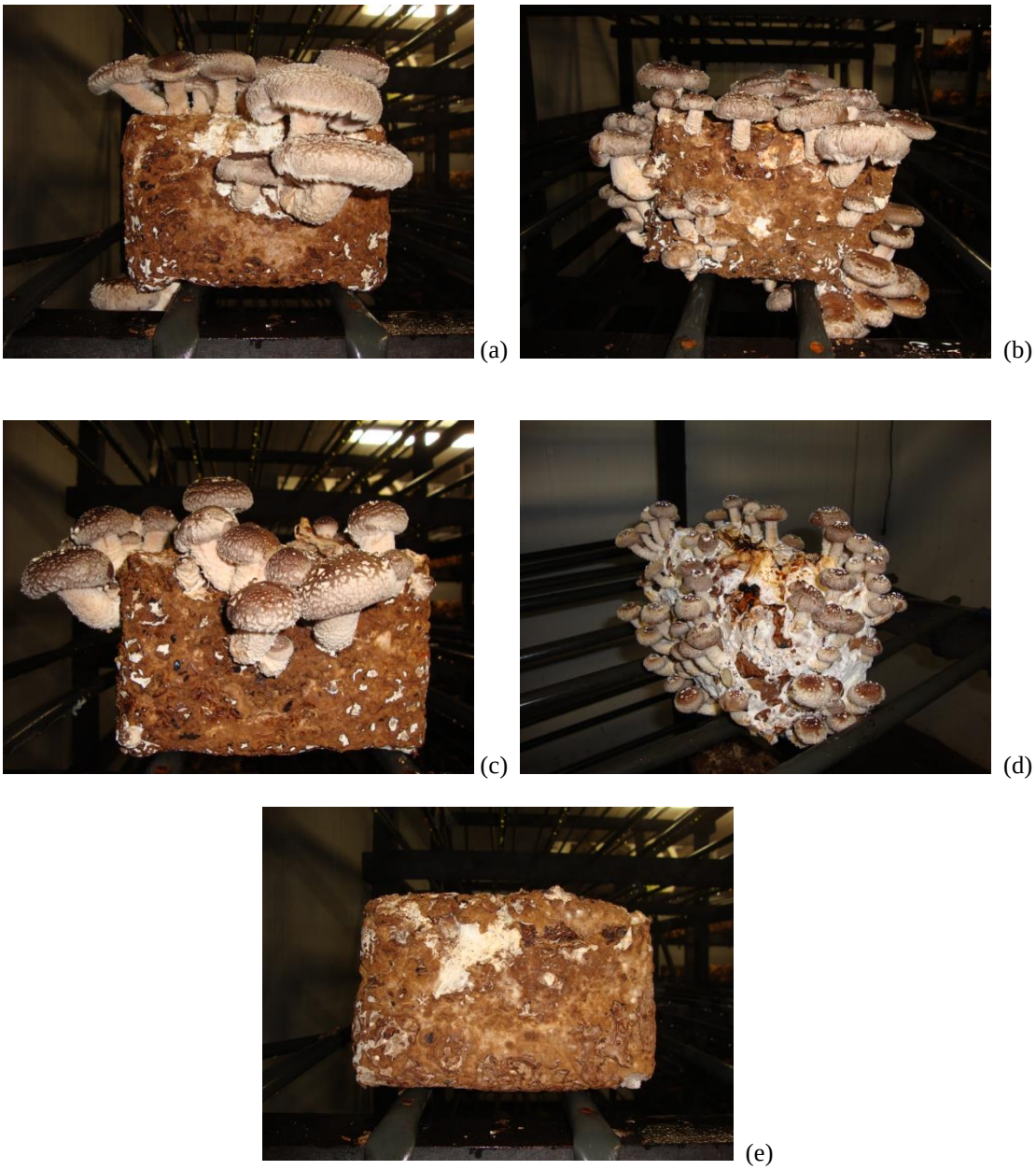

Figura 7 – Produção de *Lentinula edodes* em sistema axênico. Linhagens dicarióticas: (a) Led-08; (b) Led-T1; (c) Led-T2; (d) Led-BP; (e) Led-OTS.

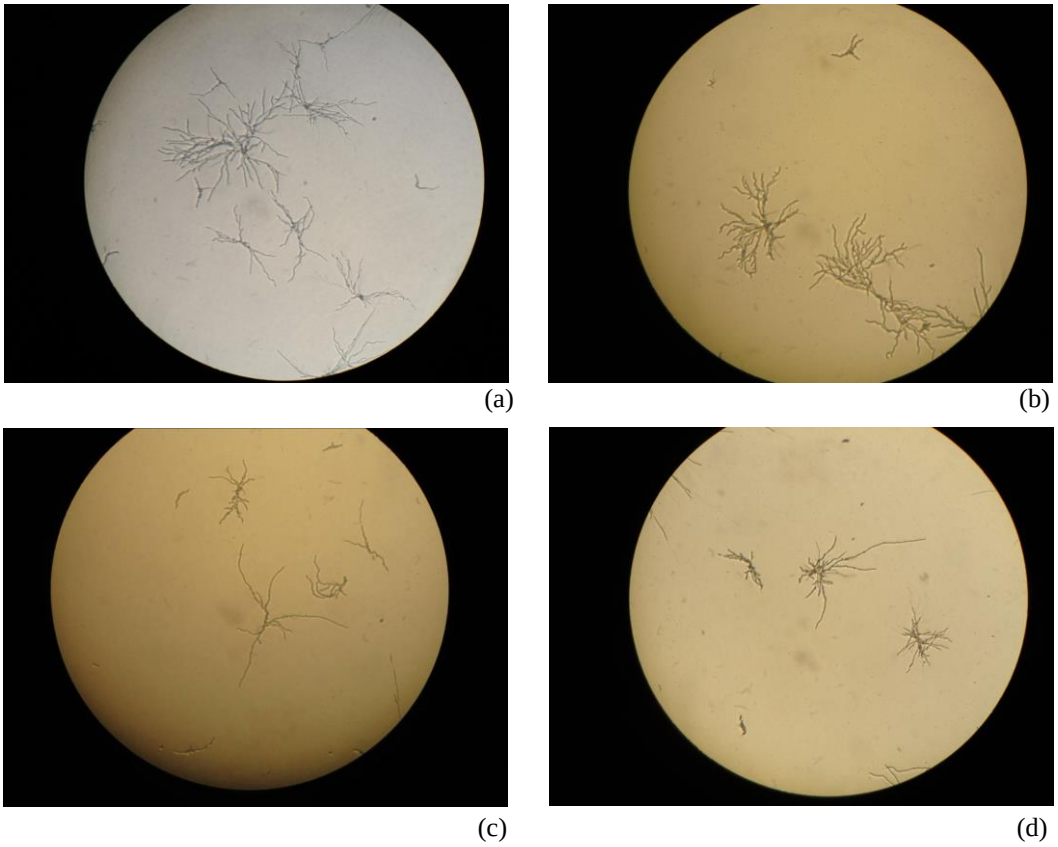

Figura 8 – Hifas monocarióticas. Linhagens dicarióticas: (a) Led-08; (b) Led-T1; (c) Led-T2; (d) Led-BP.

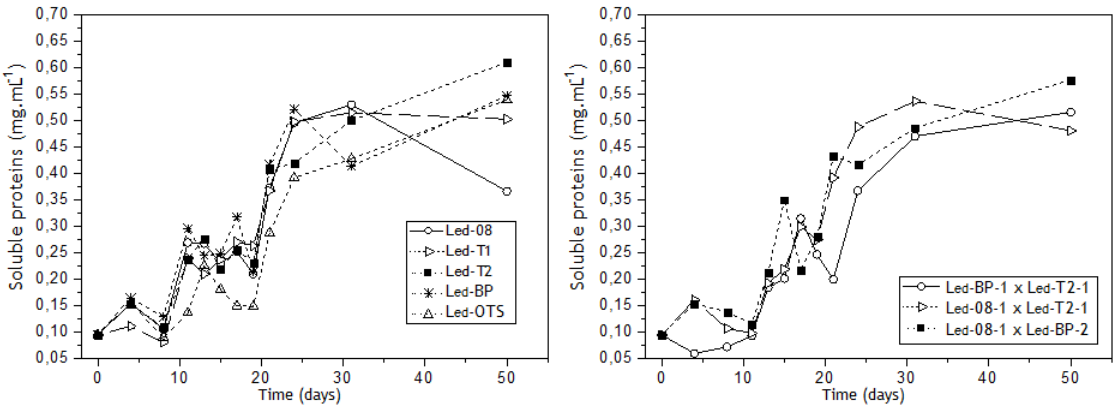

Figura 9 – Teor de proteínas solúveis do extrato bruto nos ensaios enzimáticos das linhagens dicarióticas e híbridas.

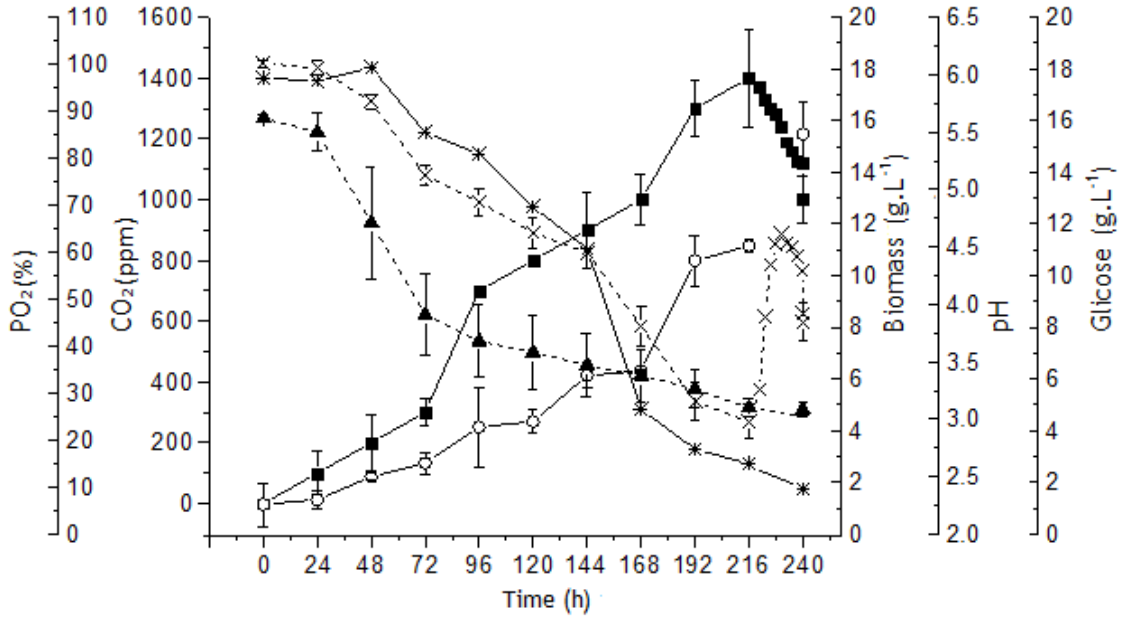

Figure 10 - Growth kinetics of *Lentinula edodes* (Led-08) cultivation in the ELAB. Conditions were: temperature of 25°C, air flow of 0.16 vvm, and initial biomass of 1.0 $g.L^{-1}$. Experimental data: (○) biomass; (■) metabolic production of CO_2; (▲) pH; (✕) dissolved oxygen; and (✳) glucose consumption.

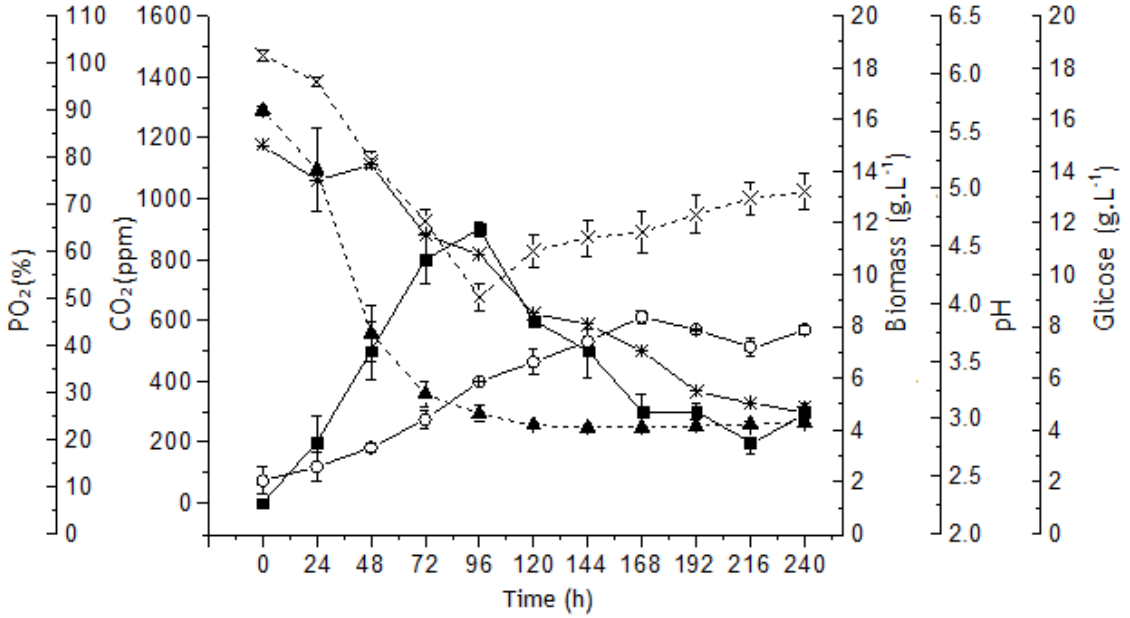

Figure 11 - Growth kinetics of *Lentinula edodes* (Led-08) cultivation in the ELAB. Conditions were: temperature of 25°C, air flow of 0.16 vvm, and initial biomass of 2.0 $g.L^{-1}$. Experimental data: (○) biomass; (■) metabolic production of CO_2; (▲) pH; (✕) dissolved oxygen; and (✳) glucose consumption.

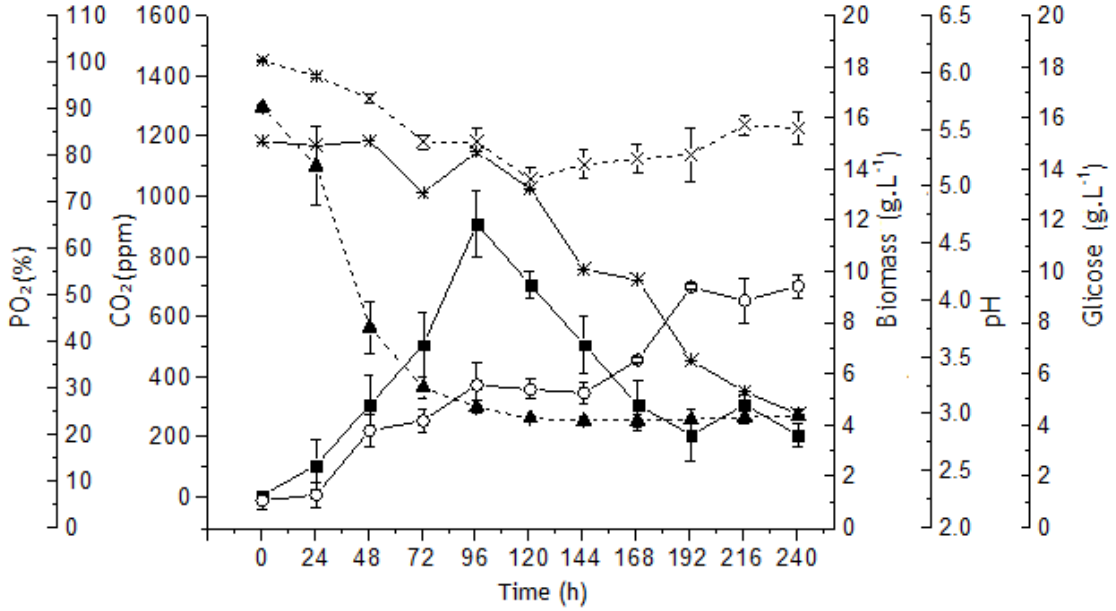

Figure 12 - Growth kinetics of *Lentinula edodes* (Led-08) cultivation in the ELAB. Conditions were: temperature of 25°C, air flow of 0.20 vvm, and initial biomass of 1.0 $g.L^{-1}$. Experimental data: (○) biomass; (■) metabolic production of CO_2; (▲) pH; (⧖) dissolved oxygen; and (✳) glucose consumption.

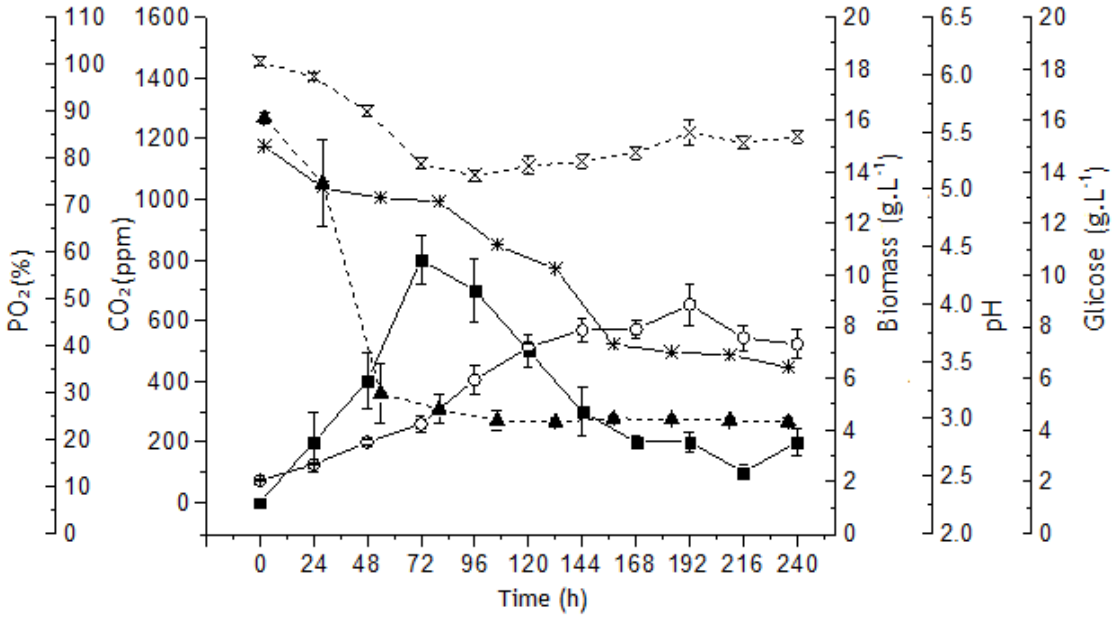

Figure 13 - Growth kinetics of *Lentinula edodes* (Led-08) cultivation in the ELAB. Conditions were: temperature of 25°C, air flow of 0.20 vvm, and initial biomass of 2.0 $g.L^{-1}$. Experimental data: (○) biomass; (■) metabolic production of CO_2; (▲) pH; (⧖) dissolved oxygen; and (✳) glucose consumption.

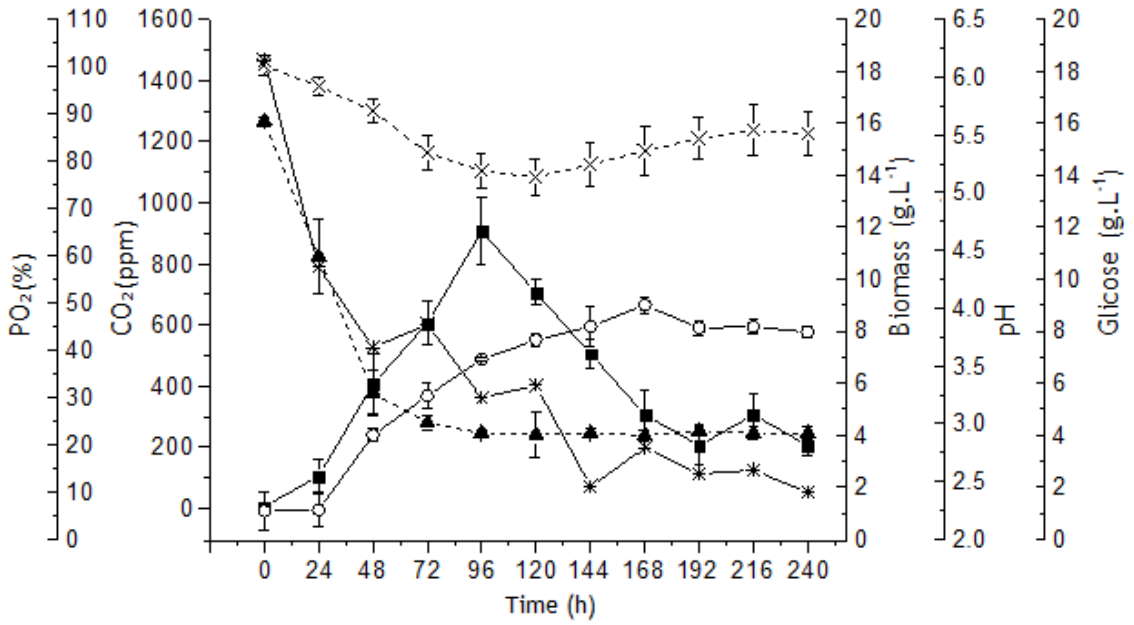

Figure 14 - Growth kinetics of *Lentinula edodes* (Led-08) cultivation in the ELAB. Conditions were: temperature of 25°C, air flow of 0.24 vvm, and initial biomass of 1.0 $g.L^{-1}$. Experimental data: (○) biomass; (■) metabolic production of CO_2; (▲) pH; (⧖) dissolved oxygen; and (✳) glucose consumption.

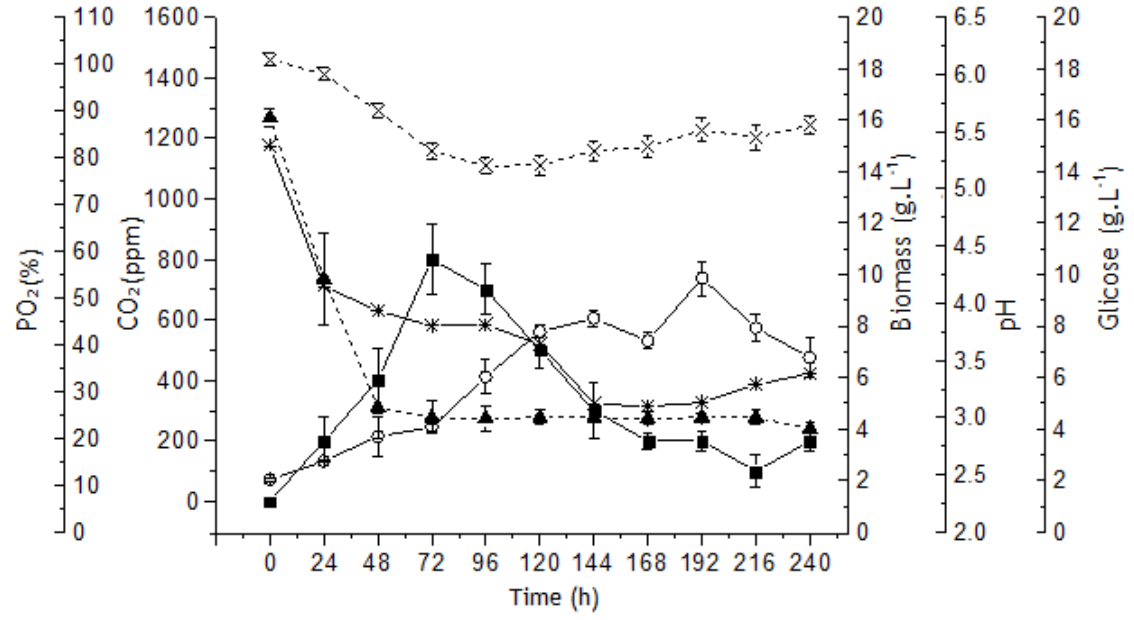

Figure 15 - Growth kinetics of *Lentinula edodes* (Led-08) cultivation in the ELAB. Conditions were: temperature of 25°C, air flow of 0.24 vvm, and initial biomass of 2.0 $g.L^{-1}$. Experimental data: (○) biomass; (■) metabolic production of CO_2; (▲) pH; (⧖) dissolved oxygen; and (✳) glucose consumption.