Enzyme activity screening of different Ceratocystis paradoxa strains

Barišić, Magdalena

Master's thesis / Diplomski rad

2023

Degree Grantor / Ustanova koja je dodijelila akademski / stručni stupanj: **University of Zagreb, Faculty of Food Technology and Biotechnology / Sveučilište u Zagrebu, Prehrambeno-biotehnološki fakultet**

Permanent link / Trajna poveznica: https://urn.nsk.hr/urn:nbn:hr:159:418978

UNIVERSITY OF ZAGREB
FACULTY OF FOOD TECHNOLOGY AND BIOTECHNOLOGY

GRADUATE THESIS

Zagreb, May 2023

Magdalena Barišić

ENZYME ACTIVITY SCREENING OF DIFFERENT *Ceratocystis paradoxa* STRAINS

This study was carried out at Werner Siemens-Chair of Synthetic Biotechnology (WSSB) at the Department Chemistry of Technical University of Munich under supervision of Thomas Brück, Prof. Dr. and Farah Quora, Dr. with the assistance of Melania Pilz, M. Sc.

Research project: EcoWash Cycle, Dr. Farah Quora

Technische Universität München

ACKNOWLEDGMENTS

Firstly, I would like to express my deepest gratitude to Thomas Brück, Prof. Dr. for enabling this remarkable opportunity to work at the Werner Siemens-Chair of Synthetic Biotechnology, TUM and for providing everything needed for the research. I am forever grateful to Farah Quora, Dr. for selfless and precious help. Additionally, I would like to thank Melania Pilz, M. Sc. for sharing time and her abundant knowledge, valuable guidelines, and kindness whenever it was needed. She left a wonderful mark on my research experience. I would like to thank Antonija Trontel, PhD, Assistant professor, for being a support and for many good advice of hers and great help. Lastly, I would like to say my truest thank you to everyone in the research group on WSSB.

Također željela bih se zahvaliti svim mojim prijateljima koji su bili tu i pružali neizmjernu podršku i razumijevanje tijekom svih godina studiranja te tako učinili ove studentske dane posebnima, a nastavljaju ih činiti i dalje. Na kraju hvala mojim roditeljima i bratu koji su bili tu kad je bilo najteže i proživljali samnom lijepe i manje lijepe trenutke, bili mi oslonac i najveći motivator. Bez svih vas zajedno, put bi bio teži!

BASIC DOCUMENTATION CARD

Graduate Thesis

University of Zagreb
Faculty of Food Technology and Biotechnology
Department of Biochemical Engineering
Laboratory for Biochemical Engineering, Industrial Microbiology and Melting and Brewing Technology
Scientific area: Biotechnical Sciences
Scientific field: Biotechnology

Graduate university study programme: Bioprocess Engineering

ENZYME ACTIVITY SCREENING OF DIFFERENT *Ceratocystis paradoxa* STRAINS

Magdalena Barišić, univ. bacc. ing. biotechn., 0058210733

Abstract:

In this work, qualitative screening of amylolytic, lignocellulolytic and proteolytic enzyme activity was performed for four strains of filamentous fungus *Ceratocystis paradoxa* (strains DSM 63054, CBS 101054, CBS 374.83, CBS 453.66). Screening was performed using colorimetric agar plates filled with 24 different commercial-coloured polymers. After preliminary tests, cultivations of *C. paradoxa* strains on different media was performed to determine if these strains have considerable cellulase, xylanase, galactanase and/or amylolytic activity. Media used in these cultivations were standard yeast extract peptone dextrose medium (YPD), and media containing starch (i.e., potato dextrose medium and medium containing waste bread) or lignocellulosic raw materials (i.e., wheat bran). Assays were performed in culture supernatants spectrophotometrically using colored substrates (red starch, azo-cm-cellulose, azo-xylan, azo-galactan). Strain DSM 63054 had highest cellulase activity (1150 mU mL^{-1}) in medium containing wheat bran and xylanase activity (1400 mU mL^{-1}) determined in medium containing waste bread, amongst all tested strains. Highest galactanase activity was determined for strain CBS 101054 in medium containing wheat bran (2100 mU mL^{-1}), while highest amylolytic activity was determined for the same strain in medium containing waste bread (2239 mU mL^{-1}).

Keywords: *Ceratocystis paradoxa, amylase, xylanase, cellulase, galactanase*
Thesis contains: 43 pages, 19 figures, 6 tables, 35 references, 0 supplements
Original in: English
Graduate Thesis in printed and electronic (pdf format**) form is deposited in:** The Library of the Faculty of Food Technology and Biotechnology, Kačićeva 23, Zagreb.
Mentor: *Antonija Trontel, PhD, Assistant professor*
Co-mentor: *Thomas Brück, Full professor*
Technical support and assistance: *Melania Pilz, M. Sc.*

Reviewers:

1. Mario Novak, PhD, Assistant professor (president)
2. Antonija Trontel, PhD, Assistant professor (mentor)
3. Andreja Leboš Pavunc, PhD, Associate professor (member)
4. Jasna Novak, PhD, Full professor (substitute)

Thesis defended: May 8, 2023

TEMELJNA DOKUMENTACIJSKA KARTICA

Diplomski rad

Sveučilište u Zagrebu
Prehrambeno-biotehnološki fakultet
Zavod za biokemijsko inženjerstvo
Laboratorij za biokemijsko inženjerstvo, industrijsku mikrobiologiju i tehnologiju slada i piva
Znanstveno područje: Biotehničke znanosti
Znanstveno polje: Biotehnologija

Diplomski sveučilišni studij: Bioprocesno inženjerstvo

ODREĐIVANJE ENZIMSKE AKTIVNOSTI RAZLIČITIH SOJEVA PLIJESNI *Ceratocystis paradoxa*

Magdalena Barišić, univ. bacc. ing. biotechn., 0058210733

Sažetak:
U ovom radu provedeno je kvalitativno određivanje amilolitčke, celulolitičke i proteolitičke aktivnosti za četiri soja plijesni *Ceratocystis paradoxa* (DSM 63054, CBS 101054, CBS 374.83, CBS 453.66). Određivanje enzimske aktivnosti provedeno je korištenjem agar podloga u koijima se nalazi 24 različita komercijalna obojana polimera. Nakon preliminarnih testova, sojevi plijesni *C. paradoxa* uzgojeni su u različitim podlogama kako bi se utvrdilo imaju li ovi sojevi značajnu celulaznu, ksilanaznu, galaktanaznu i/ili amilaznu aktivnost. Podloge korištene za uzgoj ovih plijesni su podloga koja se sastoji od kvaščevog ekstrakta, peptona i glukoze (YPD) i podloge koje sadrže škrob (tj. podloga s krumpirom i glukozom te podloga s otpadnim kruhom) ili lignocelulozne sirovine (pšenične mekinje). Određivanje enzimske aktivnosti provedeno je u supernatantima spektrofotometrijski s različitim supstratima (crveni škrob, azo-cm-celuloza, azo-ksilan i azo-galaktan). Najviša celulazna (1150 mU mL^{-1}) aktivnost u podlozi sa pšeničnim mekinjama te ksilanazna (1400 mU mL^{-1}) u podlozi sa otpadnim kruhom, određena je za soj DSM 63054, u usporedbi s drugim testiranim sojevima. Najviša galaktanazna aktivnost određena je za soj CBS 101054 u podlozi s pšeničnim mekinjama (2100 mU mL^{-1}), te je za isti soj određena i maksimalna amilazna aktivnost u podlozi s otpadnim kruhom (2239 mU mL^{-1}).

Ključne riječi: *Ceratocystis paradoxa, amilaze, ksilanaze, celulaze, galaktanaze*

Rad sadrži: 43 stranica, 19 slika, 6 tablica, 35 literaturnih navoda, 0 priloga
Jezik izvornika: hrvatski
Rad je u tiskanom i elektroničkom (pdf format) obliku pohranjen u: Knjižnica Prehrambeno-biotehnološkog fakulteta, Kačićeva 23, Zagreb
Mentor: doc. dr. sc. Antonija Trontel
Komentor: prof. dr. sc. Thomas Brück
Pomoć pri izradi: Melania Pilz, M. Sc.
Stručno povjerenstvo za ocjenu i obranu:
1. doc. dr. sc. Mario Novak (predsjednik)
2. doc. dr. sc. Antonija Trontel (mentor)
3. izv. prof. dr. sc. Andreja Leboš Pavunc (član)
4. prof. dr. sc. Jasna Novak (zamjenski član)

Datum obrane: 8. svibanj 2023.

Contents

1.INTRODUCTION

Nowadays there is a necessity in development of biotechnological processes which do not compete with the food and feed production. The usage of waste lignocellulosic biomass as a feedstock in different bioprocesses, could be economically and environmentally viable solution. The main problem arises in efficient and "cheap" conversion of lignocellulose to simple carbohydrates that can be used by different microorganisms. This can be achieved by the synergistic action of lignocellulolytic enzymes. Filamentous fungi are known to be able to degrade lignocellulolytic materials by the action of lignocellulolytic enzymes. For efficient degradation of lignocellulose, a number of cellulose (endoglucanases, exoglucanases and β-galactosidase), hemicellulose (e.g., xylanase, mannanase, arabinnase) and lignin-degrading enzymes (e.g., lignin peroxidases, manganese peroxidases, and laccases) are needed (Marđetko et al., 2021).

Ceratocystis paradoxa (Anamorph: *Thielaviopsis paradoxa*) is a filamentous fungus that is primarily known as a plant pathogen, but it has also been studied for its potential for industrial applications, such as the production of lipids, pigments, and enzymes. Research by Barros et al. (2010) investigated the enzyme activity of *C. paradoxa*, specifically the production of cellulolytic enzymes, xylanases and β-glucosidases. The authors found that *C. paradoxa* could be a consistent xylanase producer, making it a potential source for industrial applications in the pulp and paper, textile, and food industries. Another study has also explored the potential of *C. paradoxa* to perform steroid biotransformations (Peart et al., 2011).

The aim of the present work was to study the production of cellulolytic enzymes, xylanases, cellulase, amylase and galactanase by the fungi *C. paradoxa* in submerged fermentation and to investigate the impact of six different types of nutrient medium on enzyme yields. For the detection and quantification of enzymes Bradford assay and enzymatic assays were used. Bradford Assay was used for the quantification of protein concentration in a sample while for the determination of the enzyme activity, single assays were implemented for four different enzymes (cellulase, xylanase, amylase, galactanase).

2.LITERATURE REVIEW

2.1. FILAMENTOUS FUNGI

The Ascomycota is the largest phylum of fungi encompassing more than 33,000 named species and a vast number of undescribed fungi. The phylum includes yeasts and filamentous fungi, fungi that partner with algae and cyanobacteria to form lichen symbioses, mycorrhizal species, saprotrophs, and pathogens of plants and animals. Ascomycetes are utilised in industrial applications and also in food production and flavouring. Many species are known only as asexual fungi (anamorphs) that produce asexual spores (conidia) on stalks called conidiophores, but sexual phases (teleomorphs) have been identified in the life cycles of most ascomycetes that have been studied in detail. The majority of species within the Ascomycota are filamentous fungi. For a long time, filamentous fungi have been used for the industrial production of commercially relevant products, including enzymes, antibiotics, feed products, and many more (Ferreira et al., 2016). One major characteristic contributing to classifying fungi as filamentous fungi is the ability to possess hyphae. Filamentous fungi are the preferred source of industrial enzymes because of their excellent capacity for extracellular protein production. Fungi have two types of degradation systems: intracellular, together with the outer cell envelope layer, and extracellular, important for polysaccharide degradation. Furthermore, the extracellular enzymatic system includes two types of enzymes: hydrolytic, responsible for polysaccharide degradation; and oxidative (ligninolytic), which degrades lignin and opens phenyl rings. Fungi possess very efficient enzymatic system which ables them to degrade lignocellulosic materials. A number of filamentous fungi are capable of degrading biomass through the production of enzymes like cellulases (exoglucanases and endoglucanases), β-glucosidase, xylanases and others (Andlar et al., 2018).

2.1.1. *Ceratocystis paradoxa*

Ceratocystis paradoxa, part of the phylum Ascomycota, which is also known by plant pathologists as its common anamorph (imperfect state) *Thielaviopsis paradoxa* (De Seyn), is parasitic on a range of economic and food crops and is the cause of black rot of pineapple (Figure 1). Infection occurs through wounds produced when planting materials are removed from the mother plant. Allowing wounds to heal and suberise (cure) by storing on mother plants in the field during dry weather provides good control of the disease. *C. paradoxa* can survive

in the soil as chlamydospores, in decaying pineapple trash and if uncured or untreated material is planted in infested soils losses can be extremely high (Rohrbach and Johnson, 2003).

Figure 1. Pineapple crown affected by butt rot disease (Reproduced from Green et.al, 2015)

2.2. LIGNOCELLULOSIC BIOMASS

Lignocellulosic biomass (LB) is considered as a sustainable and alternative source of fuels, chemicals and materials. Valorisation of LB is expected to favour the transition from a fossil to a renewable carbon-based economy (so-called bioeconomy), thus limiting green-house gas emission and climate change, which have become strong policy priorities of the United Nations in the last years (UN). Lignocellulosic materials are divided into three main components: cellulose, hemicellulose, and lignin, where cellulose and hemicellulose together present around 70 % of all biomass. Both cellulose and hemicellulose are closely connected to component lignin through covalent and hydrogen bonds, which make its structure more robust and treatment resistant (Ufodike et al., 2020).

2.2.1. Cellulose

Cellulose is a linear component that consists of long-chain glucose monomer *β*-D-glucopyranose linked with *β*-(1,4)-glycosidic bonds, which can also reach more than one thousand units of glucose in length, with cellobiose being its repeating unit. Cellulosic chains are composed of around 1000 D-glucose units, which are arranged in microfibrils. Those fibrils form a lignocellulosic matrix with hydrogen linkages, which makes it very resistant, strong, and compact in its structure (Zoghlam and Paës, 2019). Cellulose consists of intermolecular and intramolecular hydrogen bonds plus van der Waals forces, which makes its structure crystalline. Where weaker bonds occur in the structure, the cellulose structure is amorphous because bonds are broken and the ordered arrangement is lost (Shen and Gnanakaran, 2009).

2.2.2. Hemicellulose

Hemicellulose is a heterogenous and amorphous structure of polymers that contains different monosaccharide subunits, which include D-glucose, D-mannose, D-galactose, D-xylose, and L-arabinose, as well as other sugar acids, such as D-galacturonic and D-glucuronic acids (Saha, 2003). The structure of hemicellulose is amorphous and is not physically strong, being easily hydrolysed by hemicellulose enzymes (Isikgor et al., 2015). If the hemicellulose is removed in the pretreatment process, that can lead to increased cellulose conversion due to the accessibility of enzymes to cellulose (Shen and Gnanakaran, 2009).

2.2.3. Lignin

Lignin is an aromatic polymer that is linked with covalent bonds to different xylans. It is a very complex heteropolymer of phenylpropanoied units that is composed of phenolic monomers, such as coniferyl, coumaryl, and sinapyl alcohol. Lignin contributes to the rigidity of the structure and its hydrophobicity (Agbor et al., 2012). Lignin, the linking part between cellulose and hemicellulose in the cell walls, obstructs cellulose conversion because of several factors, such as total lignin content and lignin structure. Lignin acts as a physical barrier and can limit the accessibility to polysaccharides (Santos et al., 2012). The highest levels of lignin are present in softwood, around 30–60 %, while grasses and other agricultural wastes contain only around 10–30 % of lignin (Zhang et al., 2022).

2.2.4. Pretreatment of lignocellulosic biomass

Pretreatment is the main step in removing and isolating hemicellulosic and cellulosic polysaccharides, which can be used to produce biopolymers and biochemical. There are different pretreatment methods used for the disruption of plant cell walls, such as physical, chemical, physicochemical, and biological pretreatment methods. The high chemical and structural complexity of LB at different scales is a strong limitation for the development of economically viable processes (Viikari et al., 2012). To overcome the resistance of plant cell wall to destruction, a physico-chemical pretreatment step is mandatory to "open" the network of polymers, so that enzymatic catalysts can access and convert polysaccharides into oligosaccharides and monosaccharides. Enzyme action is often hampered by structural features, which limit or prevent progression of the enzymes towards their substrate (Herbaut et al., 2018) and by chemical motifs (such as hydrophobic clusters made by lignin), which are known to bind enzymes more or less irreversibly, making them unavailable for their substrate (Zhang et al., 2012). In particular, lignin is very important factor who forms non-specific interactions with enzymes. As a result, the enzyme loading used in bioconversion of LB must be high enough to overcome these limitations, impacting the cost of the enzymatic hydrolysis step considered as a bottleneck to the establishment of cost-competitive biorefineries (Viikari et al., 2012). In nature, filamentous fungi are the most efficient decayers of LB with the secretion of a large array of complementary enzymes targeting the different components of plant cell walls (Gupta et al., 2016). Degradation of lignocellulosic biomass is described in Figure 2.

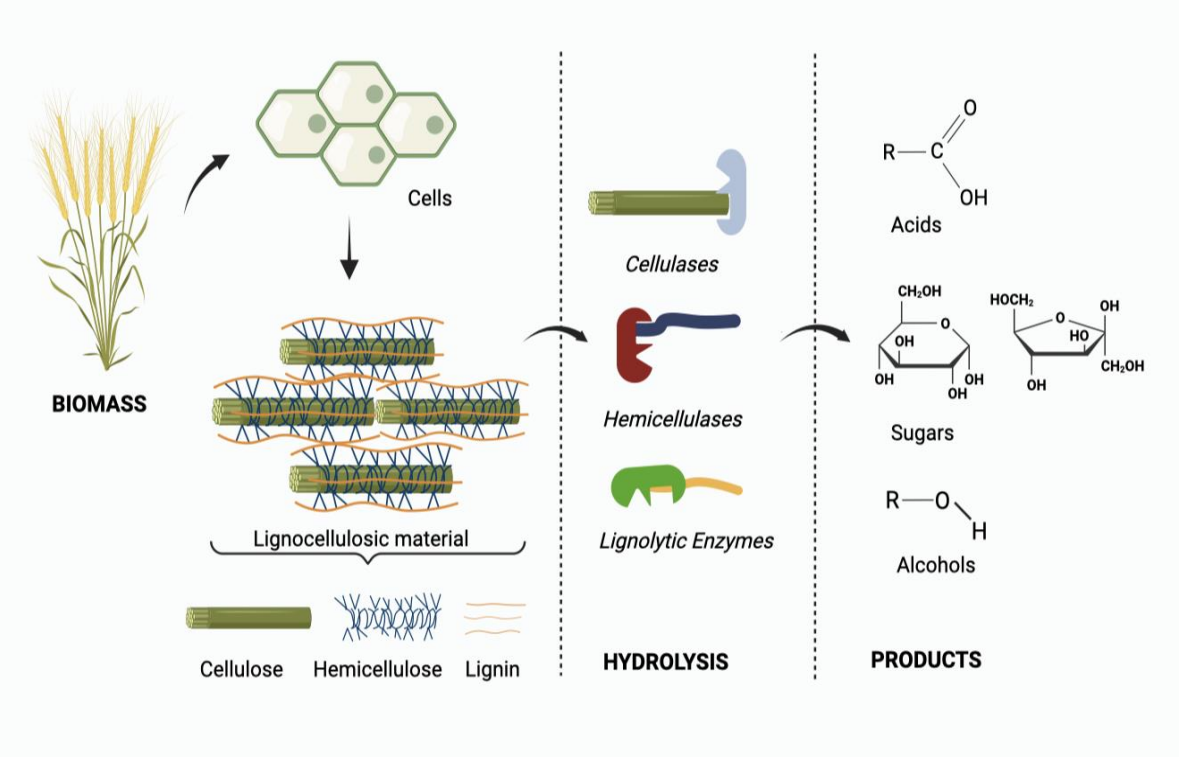


Figure 2. Degradation of lignocellulosic biomass

2.3. WHEAT BRAN

Wheat is the most important grain, which is in second place in terms of production in the world, right after corn. Wheat is a raw material for making processed food items such as semolina, all-purpose flour, pasta, noodles and many more. During such process wheat bran, an agricultural residue of the rolled milled wheat grain, is obtained as a by-product. Wheat grains consist of a series of anatomic parts: embryo, endosperm, containing starch grains for providing energy at germination; thick layer of aleurone cells, encircling an endosperm; and pericarp. Bran fractions consist of a pericarp, hyaline and aleurone layers. In the total mass of wheat grains a bran fraction is (14–16 %), embryo (2–3 %) and endosperm (mainly starch) (81–84 %) (Šramková et al., 2009). The traditional technology of wheat grains processing is based on separating an endosperm from the bran and embryo layers. Cells of the aleurone layer together with other pericarp layers and embryos are eliminated with forming a bran fraction. Wheat bran is separated from the other parts of the wheat kernel by milling, and the chemical composition of wheat bran predominantly comprises non-starch polysaccharides (approximately 38 %), starch (approximately 19 %), protein (approximately 18 %) and lignin (approximately 6 %), with the non-starch polysaccharides being approximately 70 % arabinoxylans, approximately

19 % cellulose and approximately 6 % *β*-(1,3)/*β*-(1,4)-glucan (Bergmans, 1996). It also contains significant amounts of phenolic acids such as ferulic and *p*-coumaric acids (Parker et al., 2005) which are esterified to arabinofuranosyl residues (Bunzel, 2004). Detailed microstructure of three layers and location of nutrients is described in Figure 3 and in Table 1.

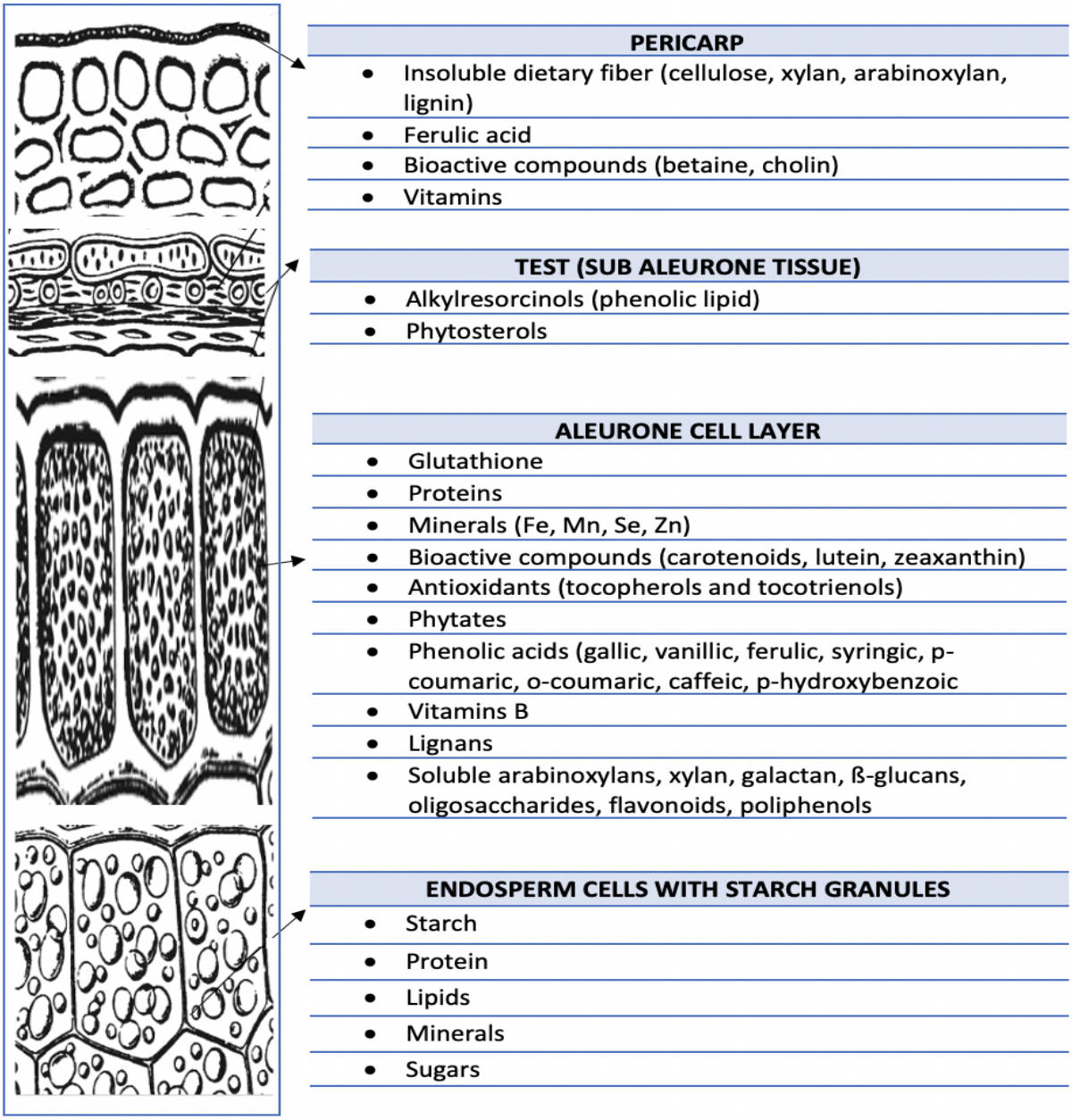


Figure 3. Microstructure of wheat bran (adapted from Kaprelyants et al., 2019)

Table 1. Composition of wheat bran (adopted from Palmarola-Adrados et al., 2005)

Components	g 100 g^{-1} Wheat bran
a) Non-starch polysaccharide	
Glucan	10.5
Xylan	18.3
Arabinan	10.1
Galactan	1.1
b) Starch	34.0
c) Klason lignin	5.0
d) Crude protein	13.5
Total	92.5

Unfortunately, wheat bran is referred to as 'waste', even though it has nutritional values and is perfectly reusable. These low cost and easily available agro-industrial wastes may serve as excellent substrates for fermentation to obtain various valuable secondary metabolites such as enzymes, organic acids, cellular proteins, and prebiotics of microbial origin (Arotupin et al., 2015). Among the agro-industrial substrates, WB is one of the most attractive alternatives to synthetic medium in fermentation processes (Pandey, 1992). The coarse variety of WB is an efficient substrate due to its heat dissipation, better air circulation, loose particle binding and efficient penetration by mycelia and it is cheaper than fine bran so it is a better prospect economically in fermentation industry (Malathi and Chakraborty, 1991). Almost every type of enzyme can be produced by fermentation of WB both by utilizing solid state fermentation (SSF) and submerged fermentation (SmF) systems. Some of them are: protease, α- amylase, lipase, xylanase, cellulase, pectinase, glucoamylase. Since the disobedient lignocellulosic structure often creates resistance, i.e., makes access difficult to enzymes in the process of saccharification, in bioconversion processes it is necessary to pretreat the lignocellulosic biomass to break the lignin sheath and facilitate access to the cellulose. There are many different approaches, among which biological pre-treatment using the enzymatic hydrolysis from microorganisms is particularly interesting. Therefore, it has gained great attention since their mild working conditions do not require high energy input and chemical additions. Wheat bran because of its nutritional content and large surface area is great carbon source which doesn't require any supplementary carbon or nitrogen source for production of lignocellulolytic enzymes. Moreover, no prior pretreatment is necessary for wheat bran for utilization in enzyme production (Thygesen et al., 2003).

3.EXPERIMENTAL PART

3.1. EXPERIMENTAL SETUP

Four isolates of the strain *Ceratocystic paradoxa* were investigated. They were received in form of freeze-dried spores and compared for enzyme secretion and activity. For the qualitative assay, enzyme activity was tested via the chair´s own colorimetric plate assay. The optimal inoculation concentration was determined via an experiment with increasing "Spore Forming Units" (SFU) number. The strains were cultivated on six different media [wheat bran 5 %, wheat bran 10 %, bread 5 %, bread 10 %, yeast extract peptone dextrose medium (YPD), potato dextrose medium (PD)]. Sampling was performed over 14 days. Based on the substrates used in qualitative plate assay, a quantitative photometric assay was preformed to see activity of the enzymes on the substrates.

3.2. WORKING MICROORGANISMS AND MEDIA

In this study, the following *C. paradoxa* fungi strains, provided by Westerdijk Fungal Biodiversity Institute and its CBS-KNAW Collection, were used as working microorganisms: CBS 101054, isolated from Rosa, located in De Zier, Netherlands; CBS 453.66, isolated from wood, located in Yunnan, China; CBS 374.83, isolated from Phoenix canariensis root, located in Maspalomas, Gran Canaria, Spain. Additionally, DSM 63054 strain, which was isolated for the first time in 1990 in an unknown country and on an unknown substrate was purchased from the DSMZ-German collection of Microorganisms and Cell cultures GmbH.

The media composition used for the cultivations of fungi are given in Table 2 and showed in Figure 4. All media were autoclaved at 121 °C for 20 min and then 200 mL of sterile filtered glucose solution (150 g L^{-1}) was added. Potato extract and yeast extract were purchased from Foremedium (Norfolk, England). Bread and wheat bran were provided by BMB (Bayersicher Müllerbund).

Chemicals used during this research were purchased from Sigma-Aldrich (St. Louis, Missouri, USA) and were of analytical grade or the highest purity available.

Table 2. Media composition used for cultivation of fungi

Component (g L^{-1})	**Nutrient Media**					
	1	2	3	4	5	6
	PD medium	YPD	Bread 5 %	Bread 10 %	Wheat bran 5 %	Wheat bran 10 %
Potato extract	8	/	/	/	/	/
Glucose (Sterile filtrate)	3 %	3 %	/	/	/	/
Yeast extract	/	10	/	/	/	/
Peptone extract	/	20	/	/	/	/
Bread	/	/	50	100	/	/
Wheat bran	/	/	/	/	50	100

Figure 4. Media used for cultivation (from left to right; YPD, PD, bread 5 %, bread 10 %, wheat bran 10 %, wheat bran 5 %)

3.3. SPORE PRODUCTION AND HARVEST

The fungi were cultivated in Petri dishes (13.5 diameter) containing PD medium for 7 days at 28 °C. All strains were cultured starting from spore solutions in which a spatula was dipped and then applied to the culture medium to deposit the spores on the gel. Plates were sealed with parafilm to prevent drying out the agar. When the plates were colonized and covered by spores, the cultivation process was stopped.

Spore suspension from sporulating cultures were obtained by addition of Harvesting solution [100 mL of glycerol and 100 mL of 10x PBS buffer (phosphate-buffered saline, preparation; 1L; 80 g of NaCl, 2 g of KCl, 14.4 g of Na_2HPO_4, 2.4 g of KH_2PO_4, dissolved in 1 litre of water) autoclaved at 121 °C for 20 min] which was poured on the plates with a volume between 100 and 150 mL of Harvesting solution and the spores were gently scratched with a spatula. The spore suspension is taken up in a syringe and filtered through a sterile cotton cloth in a sterile syringe into prepared 50 mL falcon tubes. The falcons were preserved at -20 °C until further use.

3.4. SPORE TITER DETERMINATION

Dilution series were prepared by pipetting 100 μL spore suspension into Eppendorf tube containing 900 μL of PBS 1x buffer for 10^{-1} solution. Afterwards 100 μL of 10^{-1} solution was pipetted in a new Eppendorf tube containing 900 μL PBS 1x buffer for 10^{-2}, and so on until value of 10^{-6} was reached for a total of 4 tubes. For the plating 100 μL liquid spore solution was plated on a petri dish containing PDA+Triton X. Plates were sealed with parafilm and incubated at 28 °C until spores were detected, as soon as colonies were visible, they were counted on plates with naked eye. The spore solutions were then distributed into prepared Eppendorf tubes as defined inoculation stocks.

3.5. ENZYME PRODUCTION

Enzyme production was carried out in 400 mL culture flasks containing 300 mL of different carbon sources (potato media, YPD, bread, wheat bran). After sterilization, the culture media was inoculated with spore suspension, inoculation concentration was 10^4 SFU mL^{-1}. Triplicate cultures were incubated for 14 days in a rotary shaker (Eppendorf, model My Innova S44i) at 200 rpm and 28 °C. Samples of the culture supernatants, collected every day and centrifuged

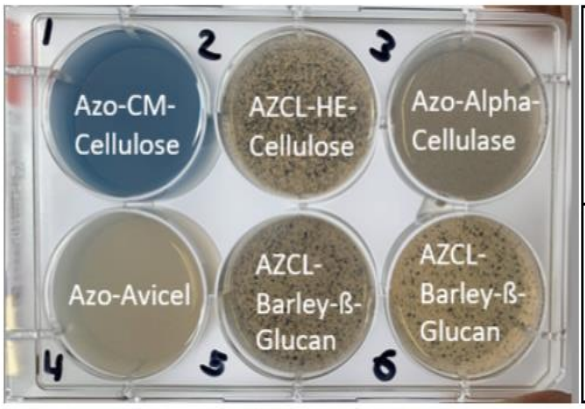
at 3000 rpm for 30 min at 10 °C and filtrated, were used for determination of enzyme activity.

3.6. ANALYTICAL METHODS

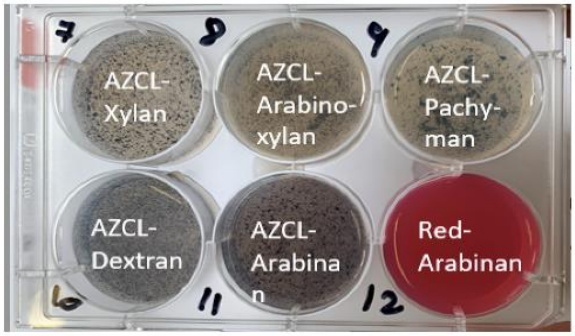
3.6.1. Enzyme activity-qualitative determination

The preliminary evaluation of enzymatic activity was determined using colorimetric, qualitative plate assay developed in TUM/WSSB. Agar plates were filled with 24 different commercial-coloured polymers for detection of enzyme activity as listed in Table 3. Shortly, inside a cavity on the 6 well plates supernatant from fungal cultivation was placed and plates were incubated at 28 °C. Subsequently, activities could be seen as halos surrounding the cavity, either less tinted then the original (soluble substrate) or darker hue of the halo (insoluble substrates).

Table 3. Detection of enzyme activity, qualitative plate assay



1 endo-1,4-ß-Glucanase	**2** endo-1,4-ß-Glucanase	**3** endo-1,4-ß-Glucanase
4 endo-1,4-ß-Glucanase	**5** Malt-ß-Glucanase & Licheninase	**6** endo-Cellulase



7 endo-1,4-ß-Xylanase	**8** endo-1,4-ß-Xylanase	**9** endo-1,3-ß-Glucanase
10 endo-1,6-α -Dextranse	**11** endo-1,5-α -Arabinase	**12** endo-1,5-α-Arabinase

13 α-Amylase	**14** α-Amylase	**15** Malt-limit-dextrinase & pullulanse
16 Malt-limit-dextrinase & pullulanase	**17** endo-Fructanase	**18** Chitosanase

19 Protease	**20** Protease	**21** endo-1,4-ß-Galactanase
22 Rhamnogalacturonan hydrolase/lyase	**23** endo-1,4-ß-Mannanase	**24** Lipase

3.6.2. Bradford Assay

The Bradford assay is colorimetric protein assay used for measuring the concentration of total protein in a sample. This assay is based on the shift of the dye, Coomassie blue (which exists in three forms: cationic, neutral, and anionic) absorption maximum from 470 nm to 595 nm after the protein binds in an acidic medium. In that case, the Coomassie blue binds protein in a sample, resulting in a change from its cationic(red) form to a protein bound anionic(blue) form which has a maximum absorbance at 590 nm. The amount of blue can then be measured using a spectrophotometer (EnSpire Multimode Plate Reader) to determine the concentration of protein in the sample (Figure 5). For the calibration curve, BSA was aliquoted in a concentration of 400 μg mL^{-1} and subsequently, the other concentrations (20-100 μg mL^{-1}) were prepared by dilution. The standard curve was pipetted in triplicates. Samples were also diluted, depending on expected protein levels (based on the blue colouring of the diluted sample). 50 μL of each sample was pippeted into 96 well plate and every sample was measured in triplicates. 200 μL of Bradford reagent (Roti-Quant solution) was added in each well using a multi pipette and then culture plate was incubated for 5 min at room temperature. The absorbance of the samples was measured at 595 nm. Protein concentrations were automatically calculated by the software.

Figure 5. EnSpire Multimode Plate Reader (Perkin Elmer, Waltham, USA)

3.6.3. Cellulase Activity Assay

This assay is used for determination of cellulase activity (endo-Cellulase) present in samples. As a substrate, Azo-CM-cellulose obtained from Megazyme was used. It is based on substrate being depolymerised by an endo-mechanism during the incubation of cellulase with dyed Azo-CM-cellulose, which results in production of low-molecular weight dyed fragments that remain in solution on addition of a precipitant solution to the reaction mixture.

For the substrate preparation, two grams of Azo-Cm-Celullose were added to 80 mL of boiling dd H_2O under vigorous stirring until the solution was homogenous and 5 mL of Sodium Acetate Buffer was added. The precipitant solution was prepared by adding 24 g of Sodium Acetate and 4.79 g of Zinc Acetate dihydrate in 150 mL of ddH_2O, pH was adjusted to 5.0 by adding few drops of 5M HCl and 800 mL of ethanol 95 % was added. For the assay, 200 µL of fungal supernatant was pre equilibrated at 40 °C for 5 minutes and then mixed with 200 µL of 2 % Azo-CM-Cellulose, vortexed and incubated at 40 °C for 10 minutes. The reaction was terminated by addition of 1.2 mL of precipitant solution. Then the solution was vortexed 10 seconds and equilibrated at room temperature for 10 minutes and vortexed again for 5 seconds. High molecular weight material was removed by centrifugation at 3000 rpm for 10 minutes at 4 °C. Supernatant was collected, and the absorbance measured at 590 nm using a Implen NanoPhototometer® (München, Germany).

Cellulase in the test solution was determined according to the standard curve. For the standard curve preparation, a solution of *endo*-cellulose (1200 U mL^{-1}; obtained from Megazyme) was diluted in Sodium Acetate buffer (pH 4.5,100 mM) to its final concentration 100 mU mL^{-1}. An example of standard curve for cellulase assay can be seen in Figure 6. One unit of enzymatic activity is determined as the amount of enzyme that releases 1 µmol of substance per minute under the specified assay conditions. Specific enzyme activity was expressed as enzyme units per mg of total protein.

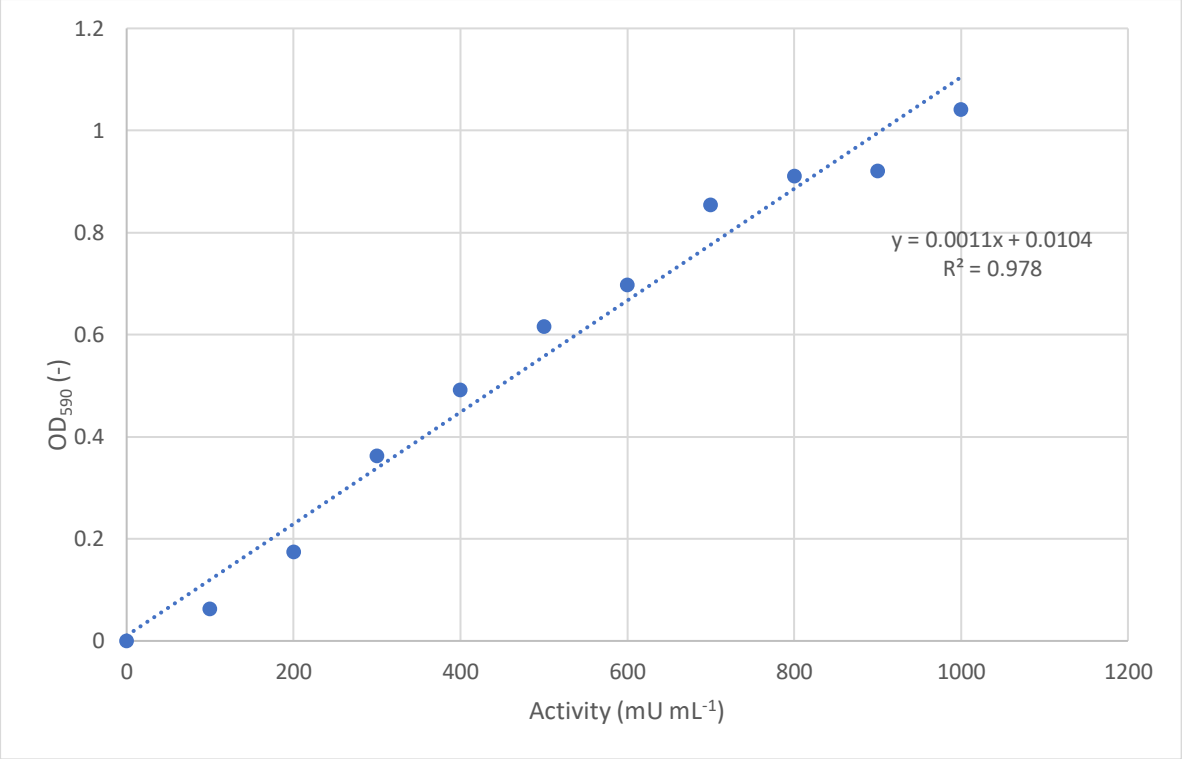


Figure 6. Standard curve for cellulase assay

3.6.4. Amylase Activity Assay

Amylase is responsible for hydrolysing starch. In our case as a substrate red starch was used, which in presence of amylase is hydrolysed to shorter polysaccharides, dextrins, maltose, and glucose. This assay is specific for *α*-amylase which is determined by reference to a standard curve through which absorbance at 510 nm is measured. For the standard curve preparation, a solution of 500 µL *alfa*-amylase (1000 U mL^{-1}; bought from Megazyme, Bray, Ireland) was diluted in 4500 µL of Buffer A (70 g NaOH, 134.4 g malic acid, 5.9 g $CaCl_2$ dissolved in 1 L of ddH_2O) to final concentration of 100 mU $µL^{-1}$ and used for preparation of standard curve. An example of standard curve for amylase assay can be seen in Figure 7. For the assay, 400 µL of fungal supernatant was pre equilibrated at 40 °C for 5 minutes and then mixed with 200 µL of 2 % red-starch solution and incubated for 10 minutes at 40 °C. The reaction was terminated by addition of 1.4 mL of ethanol 95 % and vortexed for 10 seconds. Again, it was equilibrated at room temperature for 10 minutes. High molecular weight material was removed by centrifugation at 3000 rpm for 10 minutes at 4 °C, supernatant was collected, and the absorbance measured at 510 nm using a Implen NanoPhototometer® (München, Germany).

One unit of enzymatic activity is determined as the amount of enzyme that releases 1 µmol of substance (reducing- sugar equivalents from the polymer) per minute under the specified assay conditions. Specific enzyme activity was expressed as enzyme units per mg of total protein.

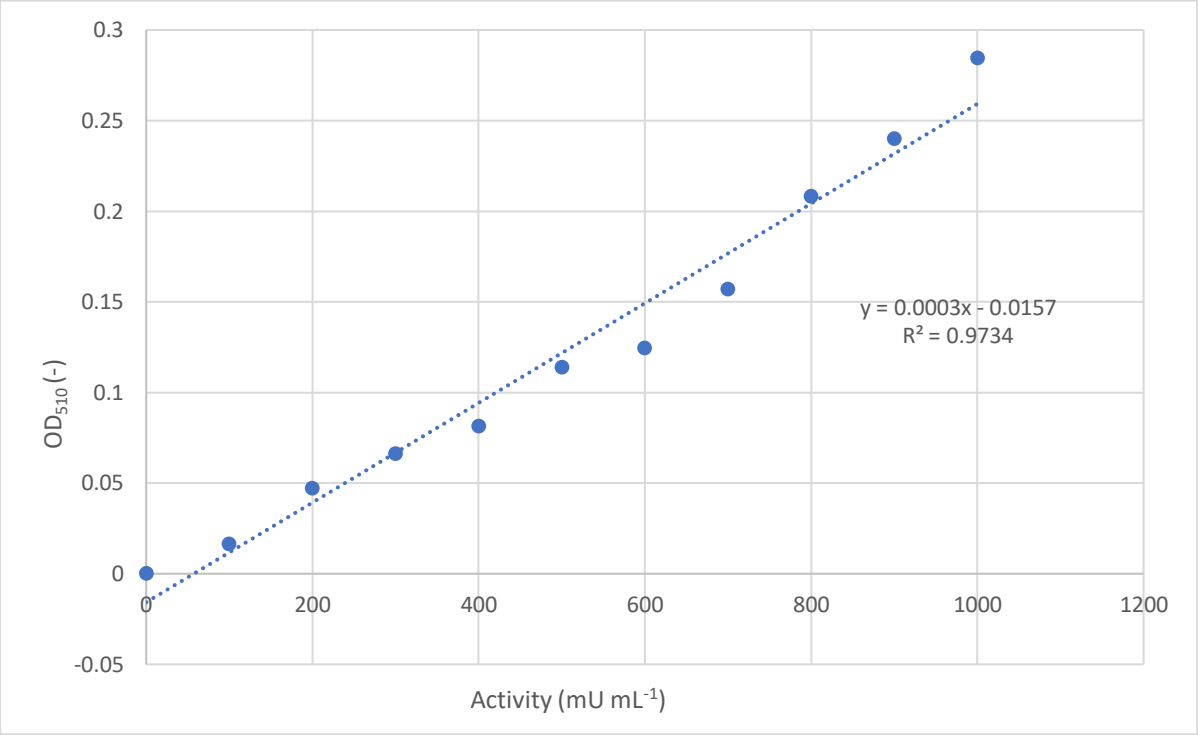


Figure 7. Standard curve for amylase assay

3.6.5. Xylanase Activity Assay

This assay is used for determination of endo-1,4-ß-D-xylanase activity present in samples. As a substrate, Azo-Xylan obtained from Megazyme (Bray, Ireland) was used. It is based on substrate being depolymerised by an endo-mechanism during the incubation of *endo*-xylanase with Azo- Xylan, which results in production of low-molecular weight dyed fragments that remain in solution on addition of ethanol to the reaction mixture.

For the substrate preparation, one gram of Azo-Xylan was added to 80 mL boiling dd H_2O under vigorous stirring until the solution was homogenous and volume was adjusted to 100 mL with ddH_2O.

For the assay, 200 µL of fungal supernatant was pre equilibrated at 40 °C for 5 minutes and then mixed with 200 µL of substrate solution, vortexed and incubated at 40 °C for 10 minutes.

The reaction was terminated by addition of 1.2 mL of ethanol 95 %. Then the solution was vortexed 10 seconds and equilibrated at room temperature for 5 minutes and vortexed again for 5 seconds. High molecular weight material was removed by centrifugation at 3000 rpm for 10 minutes at 4 °C. Supernatant was collected, and the absorbance measured at 590 nm using a Implen NanoPhototometer® (München, Germany). Xylanase in the test solution was determined according to the standard curve.

For the standard curve preparation, a solution of *beta*-xylanase M4 (1000 U mL^{-1}; obtained from Megazyme, Bray, Ireland) was diluted in Sodium Acetate buffer (pH 4.5, 100 mM) to its final concentration 30 mU μL^{-1}. An example of standard curve for xylanase assay can be seen in Figure 8. One unit of enzymatic activity is determined as the amount of enzyme that releases 1 µmol of substance per minute under the specified assay conditions. Specific enzyme activity was expressed as enzyme units per mg of total protein.

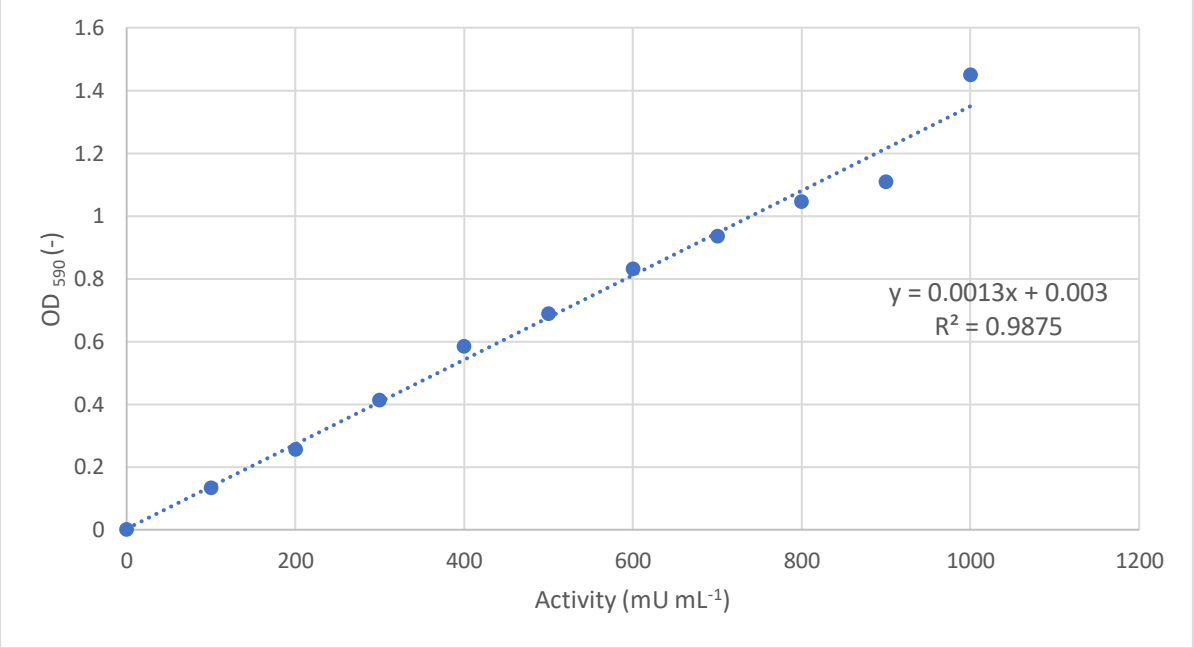


Figure 8. Standard curve for xylanase assay

3.6.6. Galactanase Activity Assay

This assay is used for determination of endo-1,4-ß-D-galactanase activity present in samples. As a substrate, Azo-Galactan obtained from Megazyme (Bray, Ireland) was used. It is based

on substrate being depolymerised by an endo-mechanism during the incubation of *endo*-galactanase with Azo-Galactan, which results in production of low-molecular weight dyed fragments that remain in solution on addition of ethanol to the reaction mixture.
For the substrate preparation, two grams of Azo-Galactan was added to 80 mL boiling ddH_2O under vigorous stirring until the solution was homogenous. The solution was cooled down to room temperature and 5 mL of Sodium Acetate buffer (pH 4, 2M) was added and volume was adjusted to 100 mL.

For the assay, 200 µL of fungal supernatant was pre equilibrated at 40 °C for 5 minutes and then mixed with 200 µL of substrate solution, vortexed and incubated at 40 °C for 10 minutes. The reaction was terminated by addition of 1.2 mL of ethanol 95 %. Then the solution was vortexed 10 seconds and equilibrated at room temperature for 5 minutes and vortexed again for 5 seconds. High molecular weight material was removed by centrifugation at 3000 rpm for 10 minutes at 4 °C. Supernatant was collected, and the absorbance measured at 590 nm using a Implen Nano Phototometer® (München, Germany).

Galactanase in the test solution was determined according to the standard curve. For the standard curve preparation, a solution of *endo-β*-D-galactanase (1300 U mL^{-1}; obtained from Megazyme) was diluted in Sodium Acetate buffer+BSA to its final concentration 100 mU $µL^{-1}$. An example of standard curve for Galactanase assay can be seen in Figure 9. One unit of enzymatic activity is determined as the amount of enzyme that releases 1 µmol of substance per minute under the specified assay conditions. Specific enzyme activity was expressed as enzyme units per mg of total protein.

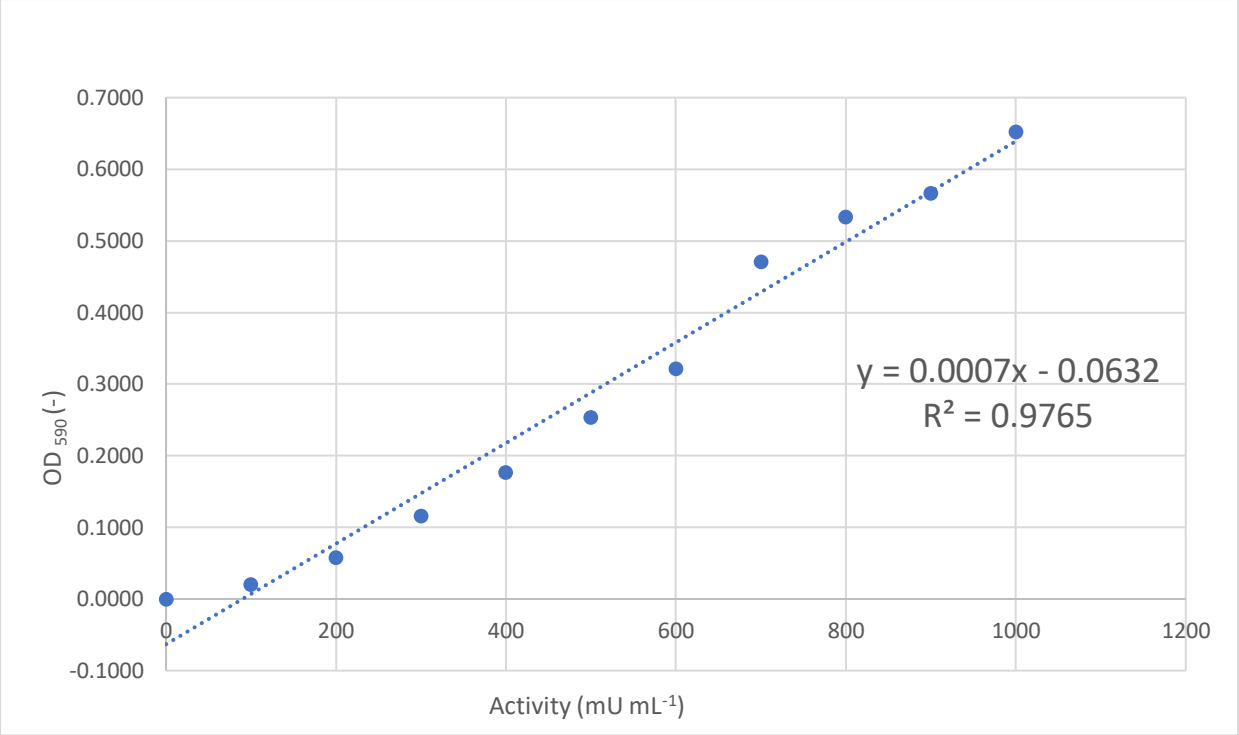


Figure 9. Standard curve for galactanase assay

4.RESULTS AND DISCUSSION

4.1. CULTURE CHARACTERISTICS AND MORPHOLOGY

It is known that when populations are geographically separated, they will eventually start to differ from each other, both in appearance and genetically. This happens through natural selection or genetic drift, both of which lead to an increase in biodiversity. Fungi are particularly interesting because over the years they have developed various ways of adapting to the environment, they may adopt different lifestyles and adapt to harsh environments (Naranjo-Ortiz and Gabaldon, 2019). On Figure 10 is the example how the cultures from same species have different morphology because they are isolated from different locations and from different hosts around the world. *C. paradoxa* CBS 374.83, isolated in Gran Canaria (Spain), spores are more elongated than *C. paradoxa* DSM 63054 (country of origin unknown) but they are the same color. Moreover, *C. paradoxa* CBS 101054, isolated in Rosa, (de Zier, Netherlands) has completely different type of spores and *C. paradoxa* CBS 453.66, isolated in Wood (Yunnan, China) has black spores.

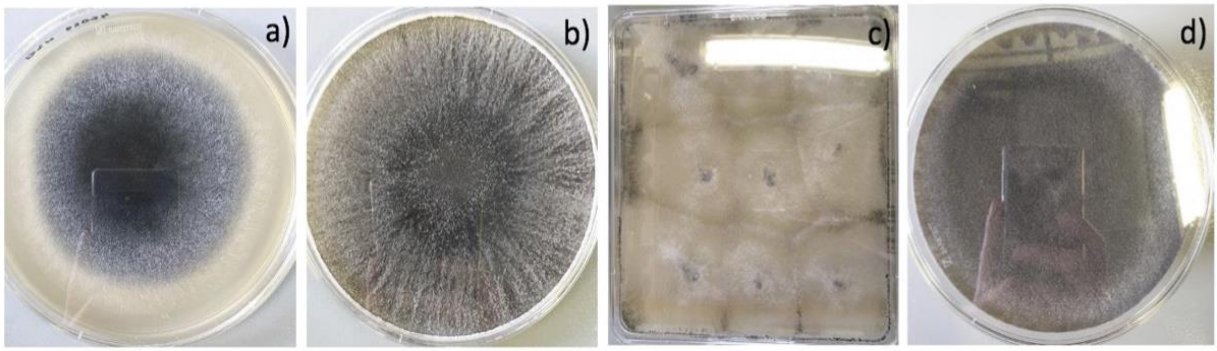


Figure 10. Image of mycelium growth of all 4 strains after formation of spores on solid potato medium: a) *C. paradoxa* DSM 63054 b) *C. paradoxa* CBS 374.83 c) *C. paradoxa* CBS 101054 d) *C. paradoxa* CBS 453.66

Furthermore, it was determined that some of the strains have special aroma, gel and pigments which is shown in Table 4. It is already known that filamentous fungi can produce an extraordinary range of pigments that include several chemical classes such as carotenoids, melanins, flavins and many more so *C. paradoxa* could have potential industrial application in this area as well, which would contribute to its value.

Table 4. Summary of certain characteristics of the fungi determined during cultivation

STRAIN	enzymes	aroma	gel	pigments
Ceratocystis paradoxa CBS 374.83	✓	✓	✓	✓
Ceratocystis paradoxa DSM 63054	✓	✓	✓	✓
Ceratocystis paradoxa CBS 101054	✓	✓	-	✓
Ceratocystis paradoxa CBS 453.66	✓	✓	-	✓

As a result of spore titer determination described in section 3.4. it was observed that the highest concentration of spores was produced by the *Ceratocystis paradoxa* CBS 374.83 which is 1.04 $*10^7$ SFU mL^{-1}, while the *Ceratocystis paradoxa* CBS 101054 with 1.68 $*10^6$ SFU mL^{-1} resulted in lowest concentration of spores. To conclude, the best candidate after the plate assay for enzyme production are strains *Ceratocystis paradoxa* CBS 374.83 and *Ceratocystis paradoxa* DSM 63054 because of the highest spore production. Summary is shown in Table 5.

Table 5. Spore production from each strain expressed in SFU mL^{-1}

STRAIN	SFU mL^{-1}
Ceratocystis paradoxa CBS 374.83	$1,04 * 10^7$
Ceratocystis paradoxa DSM 63054	$2,97 * 10^6$
Ceratocystis paradoxa CBS 101054	$1,68 * 10^6$
Ceratocystis paradoxa CBS 453.66	$1,83 * 10^6$

4.2. EFFECTS OF MEDIA ON FUNGAL GROWTHS

Down below in Figures 11-14 are shown pictures of fungi cultures after 7 days of cultivation. The visual differences in fungal growth were the most immediately apparent result of varying the culture medium. All strains have quite similar morphology which can be described as mixture of smaller and larger pellets with filamentous mycelium. *C. paradoxa* CBS 374.83 and *C. paradoxa* DSM 63054 differ significantly in color when grown on PD from *C. paradoxa* CBS 101054 and *C. paradoxa* CBS 453.66. Similar observation was recorded on PD medium by Majumdar and Mandal (2018) while cultivating filamentous fungi *T. paradoxa*.

Figure 11. *C. paradoxa* CBS 374.83 cultivated in YPD, PD, bread 5 %, bread 10 %, wheat bran 5 % and wheat bran 10 % after 7 days, 28 °C, 200 rpm

Figure 12. *C. paradoxa* DSM 63054 cultivated in YPD, PD, bread 5 %, bread 10 %, wheat bran 5 % and wheat bran 10 % after 7 days, 28 °C, 200 rpm

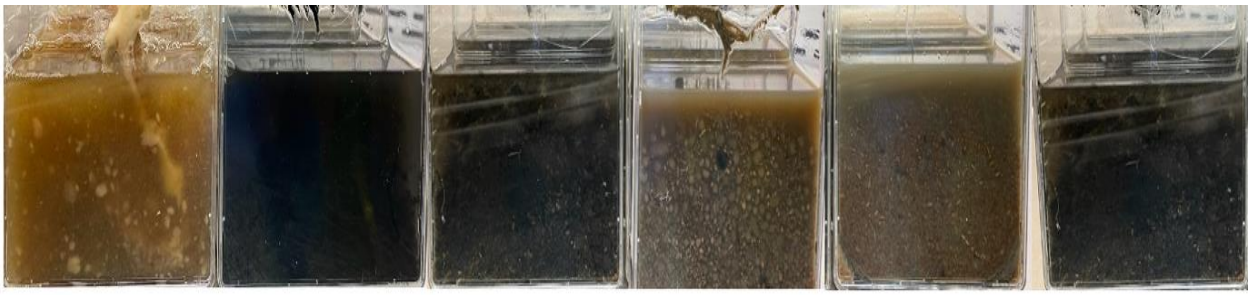


Figure 13. *C. paradoxa* CBS 101054 cultivated in YPD, PD, bread 5 %, bread 10 %, wheat bran 5 % and wheat bran 10 % after 7 days, 28 °C, 200 rpm

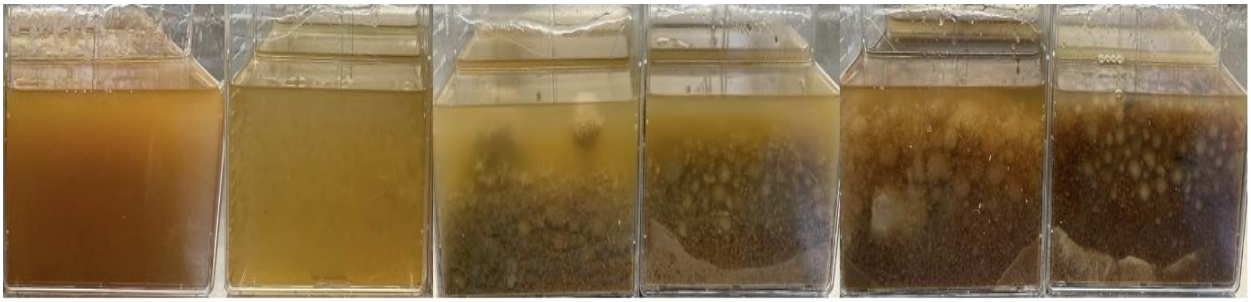


Figure 14. *C. paradoxa* CBS 453.66 cultivated in YPD, PD, bread 5 %, bread 10 %, wheat bran 5 % and wheat bran 10 % after 7 days, 28 °C, 200 rpm

4.3. CULTIVATION AND METABOLIC ABILITIES OF FUNGAL STRAINS

The enzyme activity of 4 different fungal strains; *Ceratocystic paradoxa* CBS 374.83, *Ceratocystic paradoxa* DSM 63054, *Ceratocystic paradoxa* CBS 101054 and *Ceratocystic paradoxa* CBS 453.66 was tested via the Chair´s own qualitative colorimetric plate assay as described in section 3.6. The fresh supernatant from the cultivation in PD medium was used to verify if there is some enzyme activity. A total of 24 substrates were tested. The results of the analysis in 24 different substrates are shown in Table 6, where for each of the 2 commercial-dyed polymer indicated in section 3.6., an activity was identified, and therefore the type of enzyme, which can be produced from each strain. Each tested substrate is degraded by a specific enzyme reported at the top of the table. Each colored box represents an activity detected: the color of the box is a primary and qualitative indication of the amount of activity. It was detected that all of the strains have ability to secrete enzymes able to degrade some kind of sugar polymer and all of the strains are able to synthetize the main and most common lytic enzymes for the most common sugar polymers in nature such as Cellulose, Starch, Xylan, Mannan and Arabinan. In this analysis for 8 substrates: Azo-Avicel, AZCL-Pachyman, AZCL-Dextran, AZCL-Pullulan, AZCL-Chitosan, Skim-milk platen, AZO-Casein and Olive oil Rhodamine B none of the strains have shown any activity, that is none of the corresponding enzymes can be synthetized by these fungi. Moreover, none of the *Ceratocystic paradoxa* strains is able to produce proteases in tested conditions.

Table 6. Qualitative determination of enzyme activities in liquid potato medium for four *C. paradoxa* isolates

			Ceratocystis paradoxa **CBS 374.83**	*Ceratocystis paradoxa* **DSM 63054**	*Ceratocystis paradoxa* **CBS 101054**	*Ceratocystis paradoxa* **CBS 453.66**
Number	**Enzyme**	**Substrate**				
1	endo-1,4-ß-Glucanase (Cellulase)	Azo-CM-Cellulose	**High**	++++	++	++
2		AZCL-HE-Cellulose	**High**	++	+	++
3		Azo-α-Cellulase	-	-	-	+
4		Azo-Avicel	-	-	-	-
5	Malt-ß-Glucanase (end-Cellulase) and Lichenase	AZCL-Barley-ß-Glucan	+	++++	-	+
6	Endo-Cellulase	AZCL-Xyloglucan	-	-	-	+
7	endo-1,4-ß-Xylanase	AZCL-Xylan	**High**	**High**	++	**High**
8		AZCL-Arabinoxylan	**High**	**High**		++++
9	endo-1,3-ß-Glucanase (Cellulase)	AZCL-Pachyman	-	-	-	-
10	endo-1,6-α-Dextranase	AZCL-Dextran	-	-	-	-
11	endo-1,5-α-Arabinanase	AZCL-Arabinan	**High**	**High**	++	++++
12		Red-Arabinan	**High**	**High**	++	+++
13	α-Amylase	Red CL-Amylose	**High**	**High**	++++	+++
14		Red Starch	**High**	**High**	++++	++
15	Malt limit-dextrinase and Pullulanase	AZCL-Pullulan	-	-	-	-
16		Red Pullulan	++++	++	+	+
17	endo-Fructanase	Azo-Fructan	-	++	+	+
18	Chitosanase	AZCL-Chitosan	-	-	-	-
19	Protease	Skim-milk platen	-	-	-	-
20	Protease	AZO-Casein	-	-	-	-
21	endo-1,4-ß-Galactanase	AZCL-Galactan	++++	**High**	++++	++
22	Rhamnogalacturonan hydrolase and lyase	AZCL-Rhamnogalacturonan I	++++	+	-	+
23	endo-1,4-ß-Mannanase	AZCL-Galactomannan	++++	+	+++	+
24	lipase	Olive oil Rhodamine B	-	-	-	-

- No activity	+ Activity	++ Good activity	+++ High activity	++++ Very high activity	**"High"** Unlimited activity

4.4. GROWTH CURVE ANALYSIS

Main aim during the growth curve analysis was to understand when each enzyme has the maximum activity during the 14 days growth period. For a description of the experiment, see the materials and methods section. The growth curve of *Ceratocystis paradoxa* can be divided into four distinct phases: lag phase, exponential phase, stationary phase, and death phase. The lag phase is the initial phase of the growth curve, during which the fungus adapts to the growth medium and prepares for cell division. During this phase, there is little or no increase in cell number, but the fungus may synthesize new proteins and enzymes necessary for growth. The exponential phase is the phase of rapid growth, during which the fungus multiplies at an increasing rate. This phase is characterized by a steep increase in cell number, as the fungus uses the nutrients available in the growth medium to synthesize new cellular components. The stationary phase is the phase of the growth curve during which the growth rate of the fungus slows down, and the number of cells in the culture stabilizes. During this phase, the growth rate of the fungus slows down due to the depletion of nutrients and the accumulation of toxic waste products. And at the end, death is the final phase of the growth curve, during which the number of viable cells in the culture decreases rapidly. This phase occurs when the environment becomes unfavourable for the fungus, either due to the depletion of nutrients or the accumulation of toxic waste products (Meletiadis et al., 2001).The shapes of the growth curves were different depending on the nutrient medium used and the species tested.

In the YPD media, *C. paradoxa* CBS 453.66 had the highest growth followed by *C. paradoxa* CBS 101054, *C. paradoxa* CBS 374.83 and *C. paradoxa* DSM 63054.While in PD medium *C. paradoxa* CBS 374.83 had the best growth followed by *C. paradoxa* DSM 63054, *C. paradoxa* CBS 101054 and *C. paradoxa* CBS 453.66. It can be seen that all strains grow better on YPD media which is commonly used for rapid fungal growth while it provides wide range of nutrients for fungal growth, including amino acids, vitamins, and trace elements (Fig 15.a).

PD medium (Fig 15.b), on the other hand, provides fewer nutrients but is still sufficient for the growth of many fungi. Also on media containing bread in concentration of 5 % all the strains have quite similar growth curve but *C. paradoxa* CBS 453.66 had the best growth with the maximum measured value on day 11 followed by *C. paradoxa* DSM 63054, *C. paradoxa* CBS 101054 and *C. paradoxa* CBS 374.83. In media which contains bread in concentration of 10 %, situation is not significantly different and *C. paradoxa* CBS 453.66 again showed the best growth followed by *C. paradoxa* CBS 101054, *C. paradoxa* 374.83 and *C. paradoxa* DSM

63054. The concentration of bread in media can have a significant impact on the growth of fungi. Generally, increasing the concentration of bread in the media will promote the growth of fungi up to a certain point, beyond which the growth may be inhibited. Bread is a good source of carbohydrates and some essential vitamins, which makes it a suitable substrate for fungal growth. However, at high concentrations, bread may provide too much carbohydrate and not enough other nutrients required for fungal growth, leading to reduced growth or even inhibition. Bread waste contains high concentration of starch (more than 70 % on dry matter) and proteins (up to 14 % on dry matter; Melikoglu and Webb, 2013) and treatment with amylases, amyloglucosidases and proteases easily lead to the release of compounds available for the microbial growth (Simpson et al., 2012).

Meanwhile, on media with wheat bran, good growth of all strains was observed. Although they all have very long lag phase, after slow initial growth, similar as in the other tested media, they start growing rapidly after 6 days. On media containing wheat bran in concentration of 5 % *C. paradoxa* 453.66 showed the best growth followed by *C. paradoxa* CBS 101054, *C. paradoxa* DSM 63054 and *C. paradoxa* CBS 374.83. Moreover, on wheat bran 10 % there is no significant difference between *C. paradoxa* CBS 101054 and CBS 453.66 but they are significantly higher than *C. paradoxa* CBS 374.83 and *C. paradoxa* CBS 63054. To sum up, wheat bran can be used as an efficient medium for cultivation. Growth of the fungi was monitored during cultivations by determining the concentration of proteins, since there was no possibility of taking a homogenous sample due to fungi growth morphology. The growth of *C. paradoxa* isolates is showed in Figure 15.

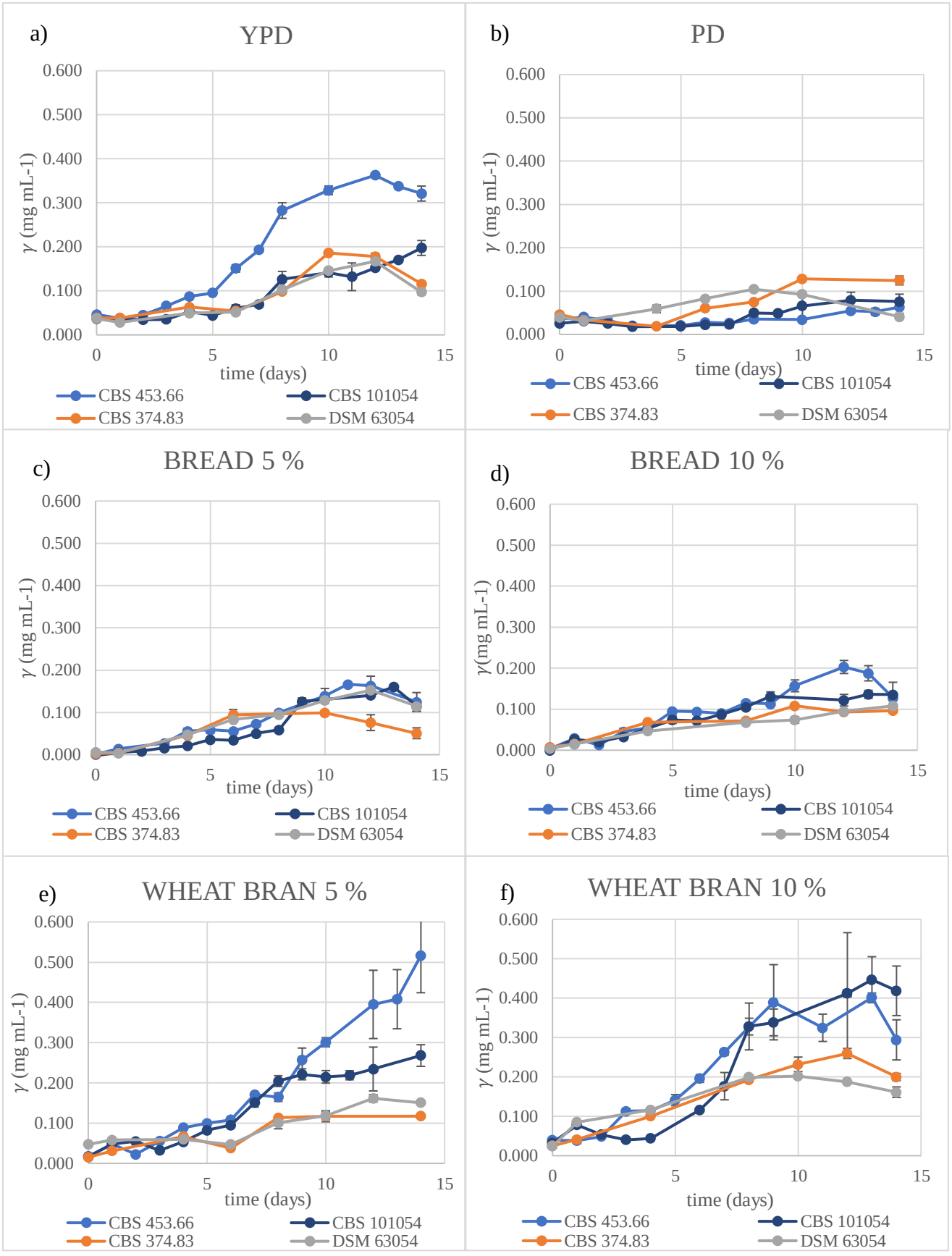


Figure 15. Growth of *C. paradoxa* isolates on six different media: a) YPD; b) PD; c) bread 5 %; d) bread 10 %; e) wheat bran 5 %; f) wheat bran 10 %

4.5. *C. paradoxa* ENZYME PRODUCTION

Main goal during this experiment was to see which strain has the highest and broadest spectrum of enzyme activities. All 4 strains were compared and tested for cellulase, xylanase, amylase and galactanase activity in six different media. In the following sections, results for each enzyme production are presented separately.

4.5.1. *C. paradoxa* cellulase activity

Figure 16 present the cellulase production of *C. paradoxa* isolates cultivated in six different media (YPD, PD, bread 5 %, bread 10 %, wheat bran 5 %, wheat bran 10 %).

From the graphs it can be seen that all strains have very slow initial growth and enzyme activity starts rising after 4 days, meaning that in the first days the fungus needs to adapt to its new environment and establish a metabolic pathway for enzyme production. In other words, certain precursor molecules, such as amino acids or nucleotides, that are necessary for enzyme production, need to be synthesized.

From the result obtained in Figure 16, it is evident that best celullase producer on YPD media are DSM 63054 and CBS 101054. CBS 101054 reaches its maximum value on day 6 with an activity of 500 mU mL^{-1} ± 50 mU mL^{-1} while on the other hand DSM 63054 has slower initial growth and the cellulose activity reaches highest value after 14 days. CBS 374.83 and CBS 453.66 have similar shape of the curves and the cellulase production is quite lower with highest value reached at day 14 with an activity of 250 mU mL^{-1} ± 50 mU mL^{-1}.

On PD medium there is evidently much higher celullase expression among all strains. Maximum activities measured are: 1300 mU mL^{-1} ± 50 mU mL^{-1} for DSM 63054 at day 14; 1400 mU mL^{-1} ± 50 mU mL^{-1} for CBS 374.83 at day 12; 1100 mU mL^{-1} ± 50 mU mL^{-1} for CBS 101054 and 1000 mU mL^{-1} ± 50 mU mL^{-1} for CBS 453.66. It is easy to notice that CBS 374.3 and CBS 101054 show similar behaviour, a slow growth in activity in the first days followed by a much-improved activity in the second part of the curve. This behavior could mean that the two strains regulate cellulase expression in a very similar way.

On the media containing bread in concentration of 5 % the maximum observed cellulase activities were: DSM 63054 with 1750 ± 50 mU mL^{-1}at day 14, CBS 374.83 with 500 mU mL^{-1} at day 14, CBS 101054 with 450 mU mL^{-1} and CBS 453.66 with 400 mU mL^{-1}. On the other

hand, in more concentrated media, containing bread in concentration of 10 % there is much lower cellulase activity. The maximum observed cellulase activities were reached at day 14 for all strains, the values are: DSM 63054 with 500 mU mL^{-1}; CBS 101054 with 400 mU mL^{-1}; CBS 374.83 with 250 mU mL^{-1} and CBS 453.66 with 100 ± 50 mU mL^{-1}.

On the media containing wheat bran in concentration of 5 % the maximum observed cellulase activities were: DSM 63054 with 1150 ± 50 mU mL^{-1} reached at day 8, CBS 374.83 with 900 mU mL^{-1} at day 12, CBS 453.66 with 600 mU mL^{-1} at day 12 and CBS 101054 with 400 mU mL^{-1} ± 50 mU mL^{-1} reached at day 14. On more concentrated media, wheat bran 10 % the cellulase expression is similar among all strains, and peak enzyme activities are reached at the last day. From the graphs it can be seen that cellulase activity is definitely more favored in the the wheat bran 5% medium than wheat bran 10 % medium. Barros et al. did a study where they used carboxymethylcellulose (CMC) test to determine the cellulolytic activites in the *C. paradoxa* culture supernatants and maximum enzyme concentrations were approximately 540 IU L^{-1} for CMCase on the wheat bran (Barros et al., 2010).

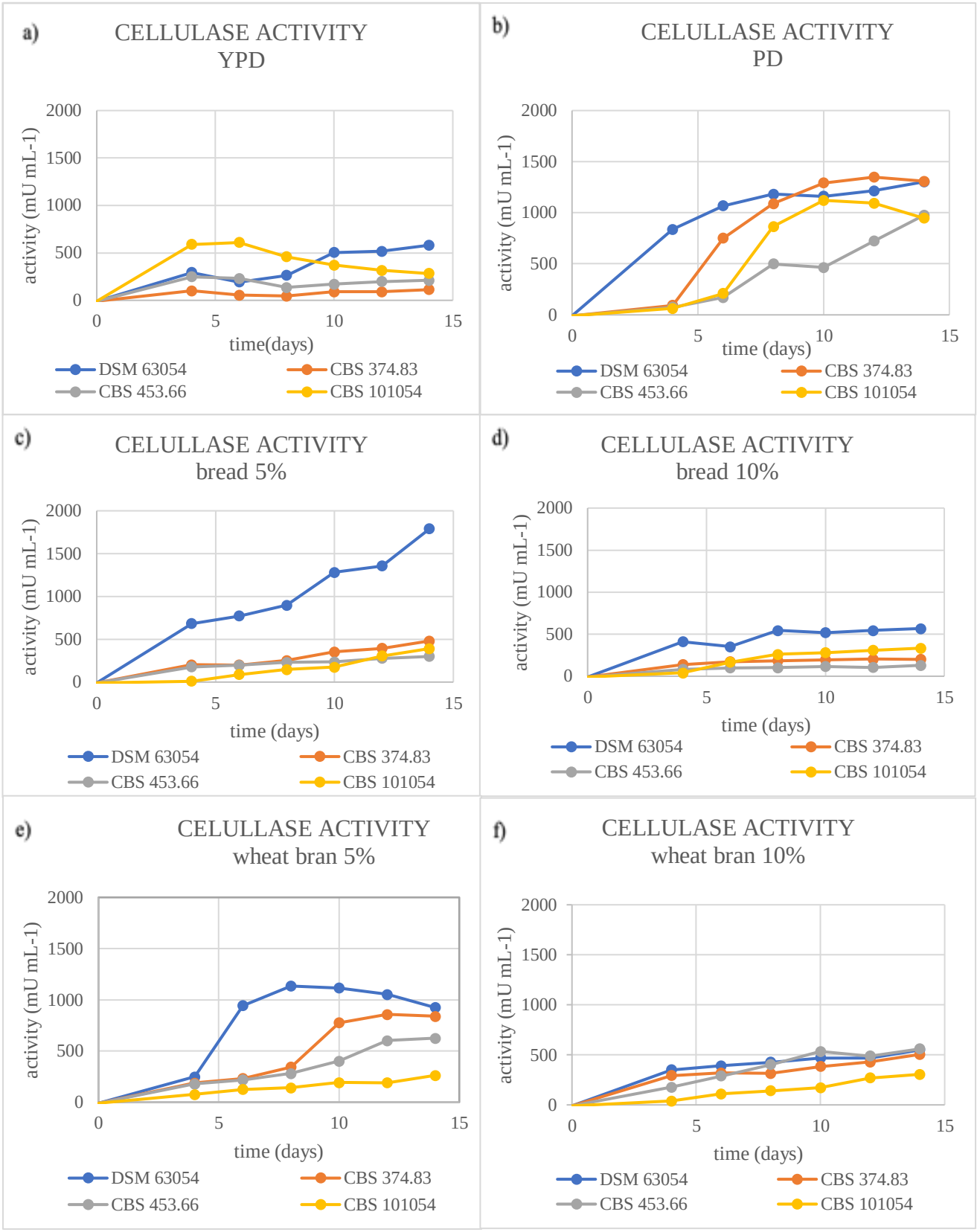


Figure 16. Cellulase production in the culture supernatants of *C. paradoxa* isolates using different carbon sources: a) YPD; b) PD; c) bread 5 %; d) bread 10 %; e) wheat bran 5 %; f) wheat bran 10 %

4.5.2. *C. paradoxa* xylanase activity

Figure 17 present the xylanase production of *C. paradoxa* isolates cultivated in six different media (YPD, PD, bread 5 %, bread 10 %, wheat bran 5 %, wheat bran 10 %).
The obtained results confirmed previously reported results saying that *C. paradoxa* turned up to be a consistent xylanase producer (Barros et al., 2010).

C. paradoxa DSM 63054 showed a consistent xylanase production for all tested carbon sources. Enzyme peak levels were 1400 mU mL^{-1} on YPD, 1400 mU mL^{-1} on PD, 1400 mU mL^{-1} on bread 5 %, 250 ± 50 mU mL^{-1} on bread 10 %, 300 ± 50 mU mL^{-1} on wheat bran 5 % and 100 ± 50 mU mL^{-1} on wheat bran 10 %. Xylanase production starts rising after day 4 for all media, except for wheat bran where there is almost no activity present until day 6 and in the end, it is almost insignificant. The reason to that can be some sort of regulatory mechanism that inhibits the expression of the enzyme or fungi uses energy to express other kinds of enzymes that are more needed. This enzyme shows the highest activity value in PD medium.

Peak enzyme activities for *C. paradoxa* CBS 101054 were observed at different cultivation times. Maximum activity on YPD was observed on the 4th day reaching 500 mU mL^{-1} and on PD on the day 10 reaching 1000 mU mL^{-1}. For bread 5 % and bread 10 %, the results for xylanase activities showed significant differences. Xylanase activity was favoured in bread 10 % medium, reaching 500 ± 50 mU mL^{-1} on day 4 while in bread 5% medium there is no activity at all. In wheat bran 5 % in the first 5 days there is almost no activity noticeable, and the activity reaches its maximum on day 8 with 100 mU ± 50 mU mL^{-1} and remains stable on these values while on wheat bran 10 % there is no activity at all. *C. paradoxa* 101054 is isolated from rose plants so it is possible that xylan could be a rare polymer in rose plants meaning that the fungus did not have the need to synthetize an active enzyme.

For *C. paradoxa* CBS 374.83 maximum activities measured are: 800 mU mL^{-1} at day 12 in YPD, 1550 ± 50 mU mL^{-1} at day 14 in PD, 700 ± 50 mU mL^{-1} at day 14 in bread 5 %, 450 ± 50 mU mL^{-1} at day 14 in bread 10 %, 500 ± 50 mU mL^{-1} in wheat bran 5 % at day 14 and 100 ± 50 mU mL^{-1} at day 14. This strain has the highest xylanase activity in wheat bran 5 % among all the strains.

C. paradoxa CBS 453.66 has turned up to be a worst xylanase producer. Maximum activities measured are 400 mU mL^{-1} at day 14 in YPD, 600 mU mL^{-1} at day 14 in PD, 150 ± 50 mU mL^{-1} at day 14 in bread 5 %, 250 ± 50 mU mL^{-1} at day 14 in bread 10 %, 100 ± 50 mU mL^{-1} in wheat bran 5 % at day 10 and 200 ± 50 mU mL^{-1} at day 6. In bread 5 % and bread 10 % there is almost no difference in activity, in both medium activity rise slowly and reaches its maximum on the last days of cultivation. On wheat bran 5 % there is no activity present during the whole 14 days period with exception on day 10, which is strange and is most likely a mistake.

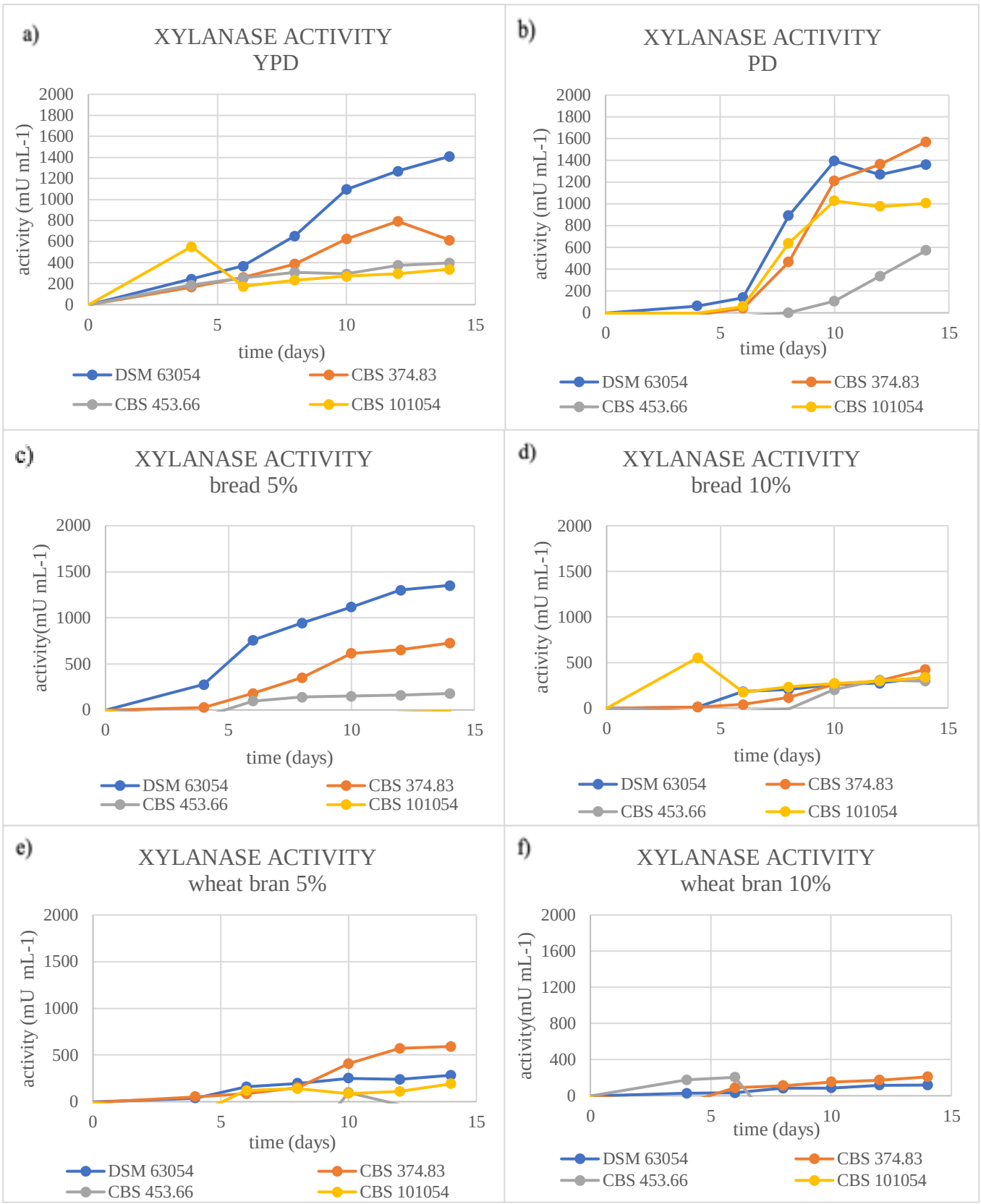


Figure 17. Xylanase production in the culture supernatants of *C. paradoxa* isolates using different carbon sources: a) YPD; b) PD; c) bread 5 %; d) bread 10 %; e) wheat bran 5 %; f) wheat bran 10 %

4.5.3. *C. paradoxa* amylase activity

Figure 18 present the amylase production of *C. paradoxa* isolates cultivated in six different media (YPD, PD, bread 5 %, bread 10 %, wheat bran 5 %, wheat bran 10 %).

C. paradoxa CBS 453.66 was able to produce amylase in all media evaluated. In YPD expression starts quite late at day 6 and then it reaches very high level of activity already at day 8 with values similar to the maximum reached at day 14 with a value of 800 ± 50 mU mL^{-1}. On PD activity is relatively low with a maximum of 400 ± 50 mU mL^{-1} at the day 14. On bread 5 % maximum activity of 750 ± 50 mU mL^{-1} is reached at day 10 and then it starts to slowly decrease, while on bread 10 % the maximum activity is already reached at day 6 and peak values were approximately double of those observed in bread 5 % medium. In both wheat bran 5 % and 10 % medium activity is almost the same, reaching its maximum at day 8 with 400 ± 50 mU mL^{-1}. *C. paradoxa* CBS 101054 showed a consistent amylase production for all tested carbon sources. In YPD medium activity is very low with a maximum of 250 mU mL^{-1} at day 10. In potato medium, as expected, the maximum value (750 ± 50 mU mL^{-1}) is higher and the expression is faster, due to the high amount of starch present in the potato extract. With bread medium the graph is completely different, amylase is super expressed early especially in bread 10 % with 1600 ± 50 mU mL^{-1} reached already at day 6. Regarding wheat bran medium, wheat bran 5 % has showed to be better than wheat bran 10 %. Overall, CBS 101054 is the best amylase producer in wheat bran 5 % among all *C. paradoxa* isolates. Peak enzyme activities for *C. paradoxa* DSM 63054 were observed at different cultivation times. On YPD there is no detectable activity before day 7 and it is very poorly expressed. In PD medium the activity is absent in the first 4 days and then rises suddenly reaching its maximum of 1000 mU mL^{-1}. In bread 5 %, expression starts early and slightly falls and rises again reaching its maximum of 2000 mU mL^{-1} at day 14, while on bread 10 % maximum is reached already at day 4. The amylase behaviour in wheat bran 5 % medium and wheat bran 10 % is almost the same reaching its maximum around day 7. Peak enzyme activities for *C. paradoxa* CBS 374.83 were 250 ± 50 mU mL^{-1} on YPD, 500 ± 50 mU mL^{-1} on PD, 750 ± 50 mU mL^{-1} on bread 5 %, 600 ± 50 mU mL^{-1} on bread 10 %, 500 ± 50 mU mL^{-1} on wheat bran 5 % and 600 ± 50 mU mL^{-1} on wheat bran 10 %. In another study (Abdullah et al., 2012) during the cultivation of *Aspergillus niger* on a wheat bran, amylase production gradually increased with time up to 72 hours, similar to our case (Abdullah et al., 2014). Amylase production starts rising after day 4 for all media, except for YPD where there is almost no activity present until day 6.

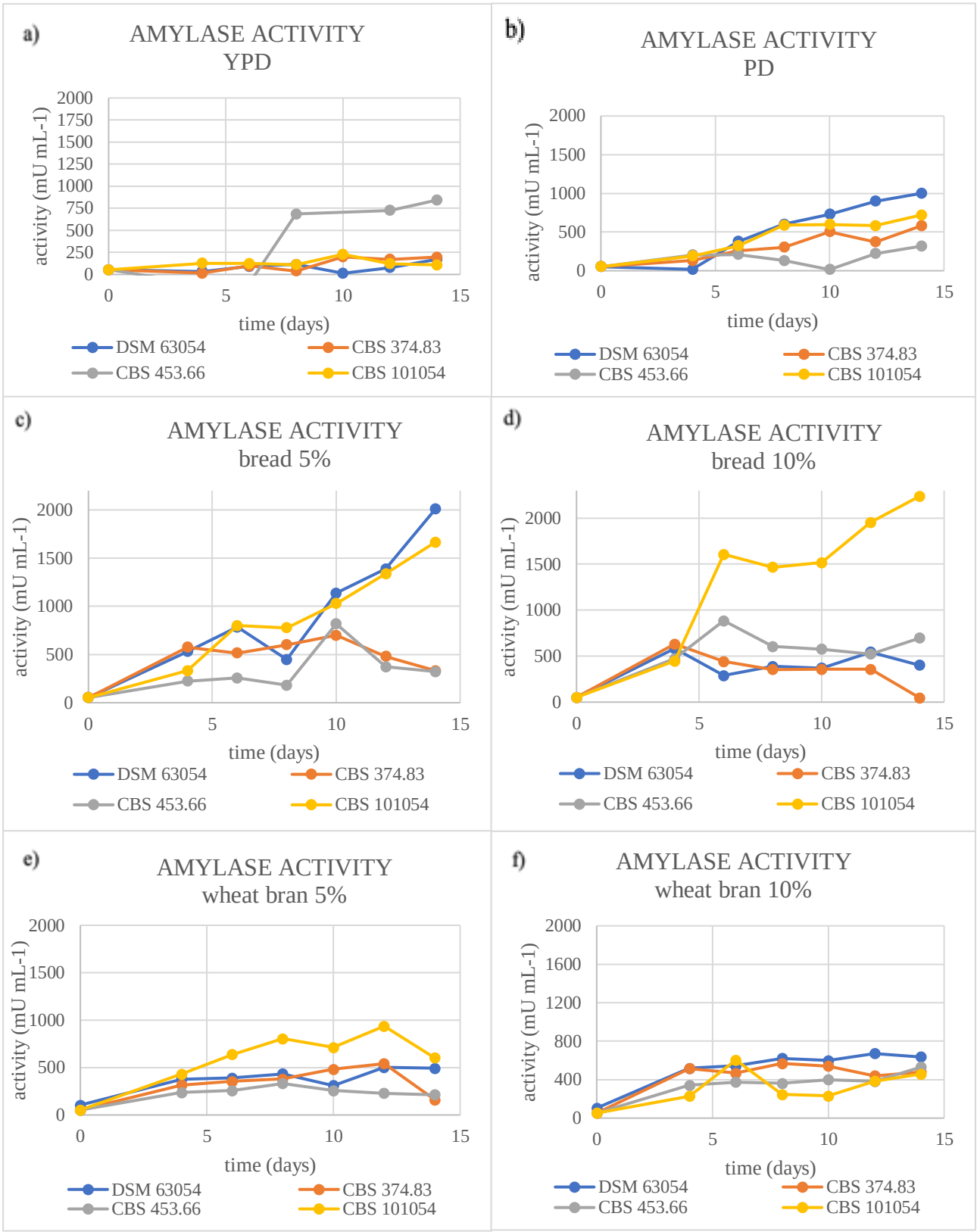


Figure 18. Amylase production in the culture supernatants of *C. paradoxa* isolates using different carbon sources: a) YPD; b) PD; c) bread 5 %; d) bread 10 %; e) wheat bran 5 %; f) wheat bran 10 %

4.5.3. *C. paradoxa* galactanase activity

Figure 19 present the galactanase production of *C. paradoxa* isolates cultivated in six different media (YPD, PD, bread 5 %, bread 10 %, wheat bran 5 %, wheat bran 10 %).

C. paradoxa CBS 101054 showed overall good galactanase production in all media. Activity starts rising after day 4 and keeps rising until it reaches its maximum at day 14, for all media. Maximum activities measured are 500 ± 50 mU mL^{-1} in YPD, 1600 ± 50 mU mL^{-1} in PD , 750 mU mL^{-1} in bread 5 %, 1250 ± 50 mU mL^{-1} in bread 10 % , 1100 ± 50 mU mL^{-1} in wheat bran 5 % and 2100 ± 50 mU mL^{-1} in wheat bran 10 %. It is interesting to notice that for both bread 10 % and wheat bran 10 % media comparing to less concentrated ones, galactanase activity almost doubles at the end probably due to greater substrate availability. From the graphs it can be seen that galactanase activity is the highest in the wheat bran 10 % medium, probably because of its composition.

C. paradoxa CBS 374.83 has turned up to be the lowest galactanase producer comparing to other *C. paradoxa* isolates. The number and the curves are similar in all media. The activity is very low in all media and rises only after day 10 to modest levels (maximum at 500 ± 50 mU mL^{-1} in wheat bran 10 %) this could mean that galactans are not present in high amount compared to other sugar polymers.

Peak enzyme activities for *C. paradoxa* DSM 63054 were observed at different cultivation times. Maximum activity was observed on YPD on the 14th day reaching 1100 mU mL^{-1} ± 50 mU mL^{-1}, same as for bread 5 %. On PD, bread 10 %, and wheat bran 10 % the activity curve is pretty much the same, the maximum point is reached after 8-10 days and it remains stable till the end. The galactanase behaviour in wheat bran 5 % medium is almost the same as for *C. paradoxa* CBS 374.83.

C. paradoxa CBS 453.66 was able to produce galactanase in all media evaluated. Enzyme peak levels were 250 ± 50 mU mL^{-1} on YPD, 400 ± 50 mU mL^{-1} on PD, 400 ± 50 mU mL^{-1} on bread 5 %, 1100 ± 50 mU mL^{-1} on bread 10 %, 400 ± 50 mU mL^{-1} on wheat bran 5 % and 1100 ± 50 mU mL^{-1} on wheat bran 10 %. This enzyme shows the highest activity value in bread 10 % medium. Unfortunately, there is not enough data on the galactanase activity of *C. paradoxa* for comparison, but in a previous study submitted by Andersen et al., where *Trichoderma reesei* was grown on semi solid wheat bran/ sphanagum peat medium, *T. reesei* had in general good mananase and cellulase activities but very low galactanase activity (Andersen et al., 2016).

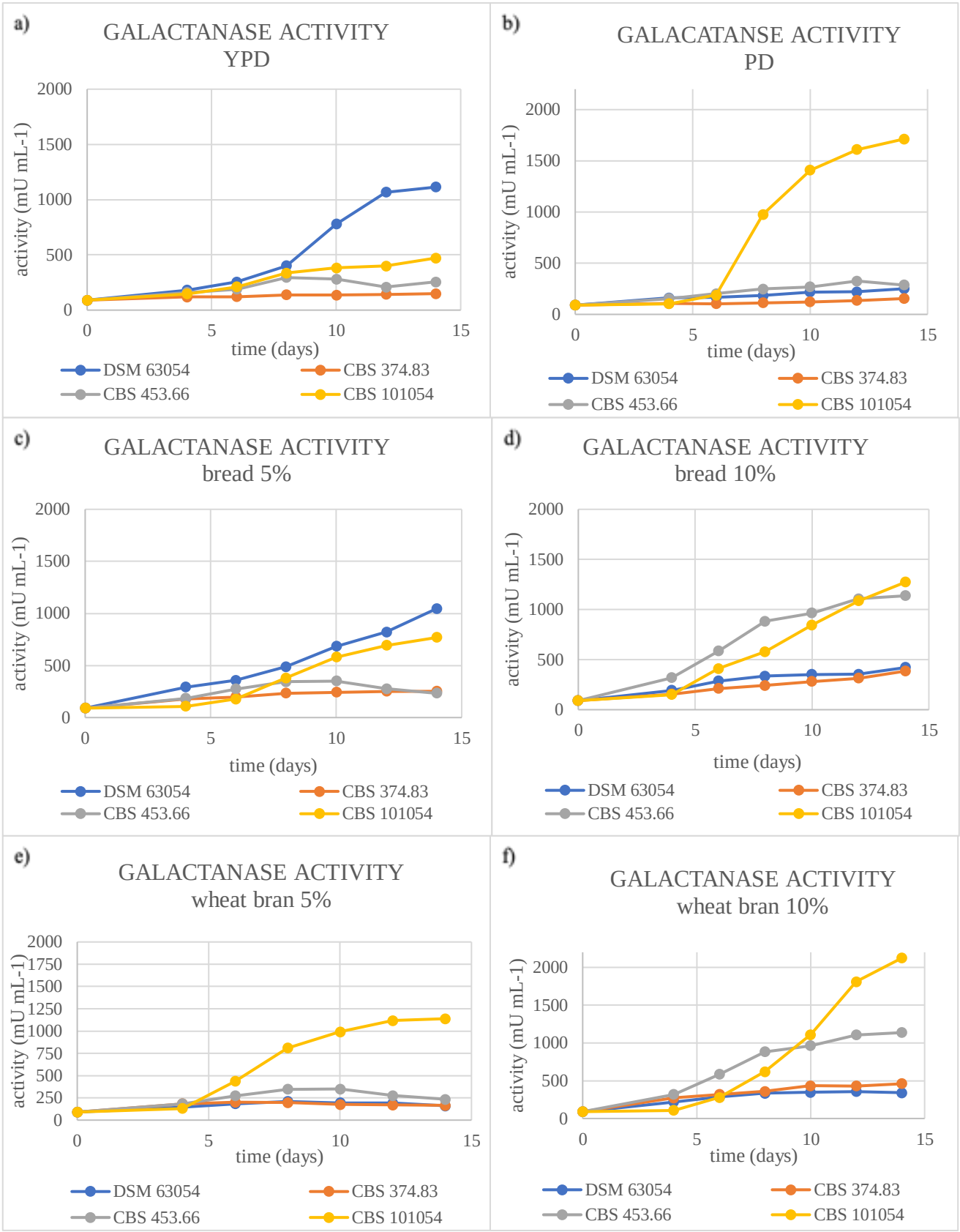


Figure 19. Galactanase production in the culture supernatants of *C. paradoxa* isolates using different carbon sources: a) YPD; b) PD; c) bread 5 %; d) bread 10 %; e) wheat bran 5 %; f) wheat bran 10 %

5. CONCLUSIONS

From the results presented in this work following conclusions can be made:

1. Qualitative screening of amylolytic, lignocellulolytic, proteolytic and lypolytic enzyme activity was performed for four strains of filamentous fungus *Ceratocystis paradoxa*, DSM 63054, CBS 101054, CBS 374.83 and CBS 453.66 by colorimetric test using agar plates. Amylolytic and lignocellulolytic activity was determined for all four tested strains. None of the tested strains had significant proteolytic and lipolytic activity.
2. *Ceratocystis paradoxa* strains DSM 63054, CBS 101054, CBS 374.83 and CBS 453.66 grow in form of pellets and produce amylolytic and lignocellulolytic enzymes in YPD, potato media, media with waste bread and media with wheat bran.
3. Amongst tested strains *C. paradoxa* DSM 63054 had highest cellulolytic activity ranging from 520 mU mL^{-1} for YPD medium to 1150 mU mL^{-1} for wheat bran containing medium.
4. Highest galactanase activity was determined for *C. paradoxa* strain CBS 101054 in medium containing wheat bran (2100 mU mL^{-1}). The same strain had highest amylolytic activity in medium containing waste bread (2239 mU mL^{-1}).
5. Inhibitory effect of high initial substrate load (w = 10 %) on growth and enzyme production was observed during cultivations in media with waste bread and in media with wheat bran for *C. paradoxa* strains DSM 63054, CBS 101054, CBS 374.83 and CBS 453.66. Only increase in galactanase activity was observed in 10 % wheat bran media compared to media containing 5 % substrate load for strains CBS 101054 and CBS 453.66.
6. Selection and further optimization of media and cultivation conditions used for production of lignocellulolytic and amylolytic enzymes is needed.

6. REFERENCES

Abdullah R, Shaheen N, Iqtedar M, Naz S, Iftikhar T (2014) Optimization of cultural conditions for the production of *alpha* amylase by *Aspergillus niger* (BTM-26) in solid state fermentation *Pak J Bot* **46**, 1071-107.

Agbor VB, Cicek N, Sparling R, Berlin A, Levin DB (2011) Biomass Pretreatment: Fundamentals toward Application. *Biotechnol Adv* **29**, 675–685. https://doi.org/10.1016/j.biotechadv.2011.05.005

Andersen B, Poulsen R, Hansen GH (2016) Cellulolytic and xylanolytic activities of common indoor fungi. *International Biodeterioration & Biodegradation* **107**, 111–116. https://doi.org/10.1016/j.ibiod.2015.11.012

Andlar M, Rezić T, Marđetko N, Kracher D, Ludwig R, Šantek B (2018) Lignocellulose degradation: An overview of fungi and fungal enzymes involved in lignocellulose degradation. *Eng Life Sci* **18**, 768-778. https://doi.org/10.1002%2Felsc.201800039

Arotupin DJ, Fabunmi TB, Gabriel-Ajobiewe RAO (2015) Enzyme activity of microorganisms associated with fermented husk and testa of cola acuminate. *Res J Microbiol* **10**, 466. http://dx.doi.org/10.3923/jm.2015.466.475

Barros RRO, Oliveira RA, Gottschalk LMF et al. (2010) Production of Cellulolytic Enzymes by Fungi *Acrophialophora nainiana* and *Ceratocystis paradoxa* using Different Carbon Sources. *Appl Biochem Biotechnol* **161**, 448–454. https://doi.org/10.1007/s12010-009-8894-3

Bergmans MEF, Beldman G, Gruppen H, Voragen AGJ (1996) Optimisation of the selective extraction of (glucurono) arabinoxylans from wheat bran: use of barium and calcium hydroxide solution at elevated temperatures. *J Cereal Sci* **23**, 235–45. https://doi.org/10.1006/JCRS.1996.0024

Bunzel M, Ralph J, Steinhart H (2004) Phenolic compounds as cross-links of plant derived polysaccharides. *Czech J Food Sci* **22**, 64–7. https://doi.org/10.17221/10613-CJFS

Ferreira JA, Mahboubi A, Lennartsson PR, Taherzadeh MJ (2016) Waste biorefineries

using filamentous ascomycetes fungi: present status and future prospects. *Bioresour Technol* **215**, 334-45. https://doi.org/10.1016/j.biortech.2016.03.018

Green J, Nelson S (2015) Heart and Root Rots of Pineapple, *Plant disease*, **106**, 1-7.

Gupta VK, Kubicek CP, Berrin JG, Wilson DW, Couturier M, Berlin A, Filho EXF, Ezeji T (2016) Fungal enzymes for bio-products from sustainable and waste biomass. *Trends Biochem Sci* **41**, 633–45. https://doi.org/10.1016/j.tibs.2016.04.006

Herbaut M, Zoghlami A, Habrant A, Falourd X, Foucat L, Chabbert B, Paës G (2018) Multimodal analysis of pretreated biomass species highlights generic markers of lignocellulose recalcitrance. *Biotechnol Biofuels* **11**, 52. https://doi.org/10.1186/s13068-018-1053-8

Isikgor FH, Becer CR (2015) Lignocellulosic Biomass: A Sustainable Platform for the Productio of Bio-Based Chemicals and Polymers. *Polym Chem* **6**, 4497–4559. http://dx.doi.org/10.1039/C5PY00263J

Kaprelyants L, Pozhitkova L, Buzhylov M (2019) Application of co-bioprocessing techniques (enzymatic hydrolysis and fermentation) for improving the nutritional value of wheat bran as food functional ingredients. *Eureka: life sciences* **5**, 31-45. http://dx.doi.org/10.21303/2504-5695.2019.00992

Majumdar N, Mandal NC (2018) Growth and Sporulation Physiology of Postharvest Pathogen *Thielaviopsis paradoxa (De Seynes)* Höhn. *Int J Curr Microbiol App Sci* **7**, 537-544. https://doi.org/10.20546/ijcmas.2018.707.066

Malathi S, Chakraborti R (1991) Productions of alkaline protease by a new *Aspergillus flavus* isolate under solid substrate fermentation conditions for use as a depilation agent. *Appl Environ Microbiol* **57**,712-716. https://doi.org/10.1128/aem.57.3.712-716.1991

Marđetko N, Trontel A, Novak M, Pavlečić M, Ljubas BD, Grubišić M, Tominac VP, Ludwig R, Šantek B (2021) Screening of Lignocellulolytic Enzyme Activities in Fungal Species and Sequential Solid-State and Submerged Cultivation for the Production of Enzyme Cocktails. *Polymers* **13**, 3736. https://doi.org/10.3390/polym13213736.

Meletiadis J, Meis JFGM, Mouton JW, Verweij PE (2001) Analysis of Growth Characteristics of Filamentous Fungi in Different Nutrient Media. *Journal of Clinical Microbiology* **39**, 478–484. https://doi.org/10.1128/jcm.39.2.478-484.

Melikoglu M, Webb C (2013) Use of waste bread to produce fermentation products. In: Kosseva M, Webb C (Eds.) Food Industry Wastes: Assessment and Recuperation of Commodities, pp. 63-76. https://doi.org/10.1016/B978-0-12-391921-2.00004-4

Naranjo-Ortiz MA, Gabaldón T (2019) Fungal evolution: major ecological adaptations and evolutionary transitions. *Biol Rev Camb Philos Soc* **94**, 1443-1476. https://doi.org/10.1111%2Fbrv.12510

Palmarola-Adrados B, Borska PC, Galbe M, Zacch G (2005) Ethanol production from non-starch carbohydrates of wheat bran. *Biores Technol* **96**, 843–850. https://doi.org/10.1016/j.biortech.2004.07.004

Pandey A (1992) Recent process developments in solid-state fermentation. *Process Biochem.* **27**, 109–117. https://doi.org/10.1016/0032-9592(92)80017-WParker ML, Ng A, Waldron KW (2005) The phenolic acid and polysaccharide composition of cell walls of bran layers of mature wheat (*Triticum aestivum* L. cv. Avalon) grains. *J Sci Food Agri* **85**, 2539–47. http://dx.doi.org/10.1002/jsfa.2304

Peart PC, McCook KP, Russell FA, Reynolds WF, Reese PB (2011) Hydroxylation of steroids by *Fusarium oxysporum*, *Exophiala jeanselmei* and *Ceratocystis paradoxa*. *Steroids* **76**, 1317–1330. https://doi.org/10.1016/j.steroids.2011.06.010

Rohrbach KG, Johnson M (2003) Pests, diseases and weeds. In: Bartholomew PDP, Rohrbach KG (Eds.) The Pineapple: Botany, Production and Uses. Cabi Publishing., Wallingford, Oxfordshire, UK, pp. 203–251.

Saha BC (2003) Hemicellulose Bioconversion. *J Ind Microbiol Biotechnol* **30**, 279–291. https://doi.org/10.1007/s10295-003-0049-x

Santos RB, Lee JM, Jameel H, Chang HM, Lucia LA (2012) Effects of Hardwood Structural and Chemical Characteristics on Enzymatic Hydrolysis for Biofuel

Production. *Bioresour Technol* **110**, 232–238. https://doi.org/10.1016/j.biortech.2012.01.085

Shen T, Gnanakaran S (2009) The stability of cellulose: a statistical perspective from a coarse-grained model of hydrogen-bond networks. *Biophys J* **96**, 3032-40. http://dx.doi.org/10.1016/j.bpj.2008.12.3953

Simpson JK, Nollet LM, Toldrá F, Benjakul F, Paliyath G Hui YH (2012) Food Biochemistry and Food Processing, 2nd Edn. Wiley-Blackwell Publishing, Oxford.

Šramková Z, Gregová E, Šturdík E (2009) Chemical composition and nutritional quality of wheat grain. *Acta Chimica Slovaca* **2**,115–138.

Thygesen A, Thomsen AB, Schmidt AS, Jørgensen H, Ahring BK, Olsson L (2003) Production of cellulose and hemicellulose-degrading enzymes by filamentous fungi cultivated on wet-oxidised wheat straw. *Enzyme Microb Tech* **32**, 606-15. http://dx.doi.org/10.1016/S0141-0229(03)00018-8

Ufodike CO, Eze VO, Ahmed MF, Oluwalowo A, Park JG, Okoli OI, Wang H (2020) Evaluation of the Inter-Particle Interference of Cellulose and Lignin in Lignocellulosic Materials. *Int J Bio Macromol* **147**, 762-767. https://doi.org/10.1016/j.ijbiomac.2020.01.234

Viikari L, Vehmaanperä J, Koivula A (2012) Lignocellulosic ethanol: from science to industry. *Biomass Bioenergy* **46**, 13–24. https://doi.org/10.1016/j.biombioe.2012.05.008

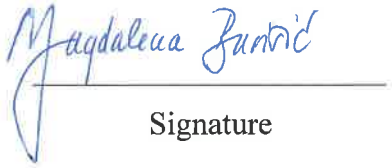
Zhang JH, Tang M, Viikari L (2012) Xylans inhibit enzymatic hydrolysis of lignocellulosic materials by cellulases. *Bioresour Technol* **121**, 8–12. https://doi.org/10.1016/j.biortech.2012.07.010

Zhang L, Larsson A, Moldin A, Edlund U (2022) Comparison of lignin distribution, structure, and morphology in wheat straw and wood. *Ind Crops Prod* **187**, 115432. https://doi.org/10.1016/j.indcrop.2022.115432

Zoghlami A, Paës G (2019) Lignocellulosic Biomass: Understanding Recalcitrance and Predicting Hydrolysis. *Front Chem* **18**, 7:874. https://doi.org/10.3389/fchem.2019.00

DECLARATION OF ORIGINALITY

I MAGDALENA BARIŠIĆ declare that this master's thesis is an original result of my own work and it has been generated by me using no other resources than the ones listed in it.



Signature