HETEROLOGOUS EXPRESSION AND ENZYME ACTIVITY OF CHITIN DEACETYLASES FROM DIFFERENT ORGANISMS ON CRYSTALLINE CHITIN AND CHITOSAN

Research Project 2

Juan Pablo Cortés Tique

S3293785

MSc. Biomolecular Sciences

Supervisor:

Prof. Dr. Gert-Jan Euverink

ABSTRACT

Heterologous expression of four chitin deacetylases (CD) from different microorganisms: *Mucor rouxii* (MRCD)*, Bacillus cereus* (BC1960)*, Streptomyces lividans* (AXE) and *Arthrobacter* sp (*Ar*CE4), was attempted in order to test their enzyme activity in partially deproteinized and demineralized shrimp powder and chitosan. In addition, cultures from a waste water treatment plant were made and isolated in trace elements and chitin or chitosan as sole carbon source in the media with the aim of checking the ability to grown in such conditions. The SDS-PAGE results showed that there was no expression of MRCD, AXE and for the waste water treatment plant samples (CDCT and WACT) while the obtained protein concentration from expression cultures were 15.3 µM for BC1960 and 71.6 µM for *Ar*CE4. No acetic acid released was detected as consequence of chitin deacetylation activity.

INTRODUCTION

Chitosan is referred to a wide variety of polymers with different grades of acetylation in their N-glucosamine units randomly distributed along the chain; this acetylation is specifically found as a non-peptide bond in the amino group of monomers' carbon number 2. Chitosan possess different electrochemical properties than most of the naturally found polysaccharides. Furthermore, it can behave as a soluble compound in aqueous solutions depending on the degree of acetylation and the pH of the environment. This phenomenon is due to its positively-charged feature which differs from the common neutral or negatively-charged natural polymers (1). In addition, soluble chitosan has been used to synthesize biodegradable, non-toxic biomaterials for human, gels, biomedical elements. Moreover, these materials also have proven to possess antimicrobial properties on strains of *S. aureus* and *E. coli* on several materials, besides its antitumoral, antioxidant, antifungal on *Candida albicans* and immunostimulatory effects (2, 3, 4, 5). Therefore, chitosan is a very interesting molecule in the pharmaceutical industry as the versatility of its applications.

Chitin, from which chitosan is obtained, is the second most abundant polymer in nature, present as major constituent of exoskeleton in insects, cell wall in fungi, and also in shrimp shells. It consists in N-acetylglucosamine units linked by β-(1->4) bonds and can be found as α and β isomorphs which differ from one another in the orientation of the reducing end terminus. In α- chitin, the end reducing terminus are antiparallel orientated while in β-chitin the chains are arranged in parallel as shown in figure 1 (1). In addition, it has been proposed that chitin molecules

produced by *Saccharomyces cerevisiae* containa range of 120-150 monomer units and presents a molecular weight from around 24 kDa to 34.5 kDa (6).

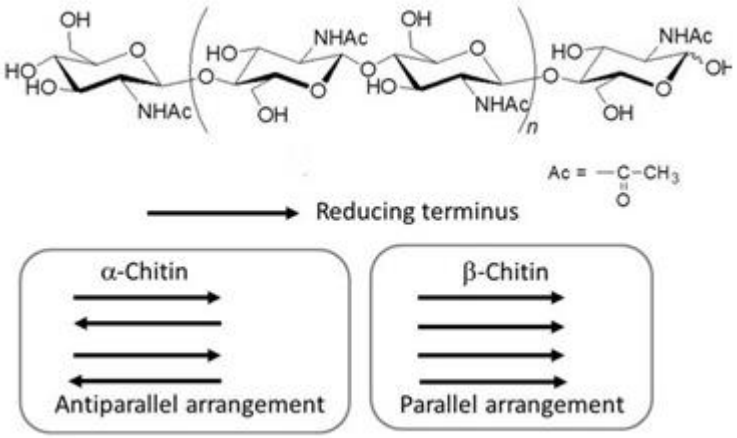


Figure 1. α and β chitin according to the end reducing terminus arrangement. n= 60 – 85

Even though chitin and chitosan share similar structures, chitin is insoluble in aqueous solutions due to the fact that all the monomer units are acetylated, which makes difficult its usage on medicine. Therefore, in order to be able to use it, conversion of chitin into chitosan is necessary in order to supply the market with a valuable product making that process of high interest to perform research on because of the potential applications of chitosan (7).

Currently, production of chitosan is achieved mainly by thermochemical methods using potassium hydroxide or sodium hydroxide at 100°C in order to eliminate the acetyl groups from chitin after elimination of proteins and minerals using acidic and alkaline treatment under high temperatures as well (8,9). This method has its drawbacks due to the environmental pollution, high energy consumption and the low quality of the product obtained as the pattern of deacetylation is randomly distributed along the chains which makes the final chitosan product partially soluble that is not suitable for further applications (10)

Alternatively, enzymatic methods for deacetylation of chitin, using chitin deacetylases (CD), have been achieved despite they are more expensive than full thermochemical process and yet some of these steps must be performed before enzymatic catalysis in order to solubilize chitin as the removing acetyl groups from crystalline chitin is not performed naturally by microorganisms and their CD. This is due to the configuration of chitin that does not allow the penetration of external molecules into the acetyl groups in the monomers. Therefore, it has been shown that crystalline chitin only can be deacetylated in less than 10 % of the total

monomer units, which means that some physical and/or chemicals methods are still needed. This enzymatic-thermochemical procedure allows the acetyl groups to expose to surrounding molecules like CD that are able to catalyse reactions on the non-peptide bond. CD were first described in fungi, specifically in *Mucor rouxii*, playing different roles in the metabolism. In *M. rouxii*, there are extracellular CD as well as intracellular depending on if infectious processes or cell wall biosynthesis are taking place (2,10). Moreover, CD have been also discovered in other species of fungi like *Aspergillus nidulans*, *Colletotrichum lindemuthianum* and *Absidia coerulea*; and bacteria such as some species of the genera *Bacillus*, *Streptomyces*, *Rhizobium, Arthrobacter* and *Vibrio* in which CD have been involved in nodulation factor biosynthesis and degradation of chitin from marine sources (1,11).

Currently, CD are classified as members of the carbohydrate esterase family number 4 (CE4) according to CAZy classification system, which is composed mainly by CD, peptidoglycan, chitooligosaccharides and acetyl xylan deacetylases. These proteins also vary in size from 12 to 150 kDa, are very stable between 30 to 60 °C and have pH optima between 4.5 and 12. Besides the usual presence of a carbohydrate domain which facilitates the substrate recognition and binding to the active site, CD also share homologous aminoacid sequences in specific motifs that in general are better known as the NoDB domain due to the similarities with the nodulation factor produced by rhizobia in which they play a role performing deacetylation of chitooligosaccharides (11). As shown in figure 2, this domain consists of five motifs, from which two molecules of Histidine and one Aspartate residues coordinate the metal ion stabilization, usually bivalent cationic, while another Histidine molecule plus a Leucine residue performs the accommodation of the acetyl group (11,12,13).

Figure 2. Alignment of aminoacid sequences from different strains of Aspergillus nidulans CDs (AnCDA, EAA65017, ACF22099), Streptomyces lividans (SlCE4), Streptococcus pneumoniae (SpPgdA), Colletotrichum lindemuthianum (ClCDA)

and Vibrio cholerae (VcCDA). Highlighted yellow residues represent conserved aminoacids in the motifs in the five motifs (12).

The heterologous expression of proteins and enzymes from different organisms is a recurrent strategy in order to facilitate protein production for further studies on their catalytic mechanism, enzymatic activity, roles in metabolism, etc. However, heterologous expression of CD has not been describe yet nor a strain that is capable to produce CD for chitosan production at industrial scale, in fact, only several strains of natural CD producers has been tested for the production of this enzyme, like *Colletotrichum lindemuthianum* 56676, *Penicillium monoverticillium* CFR2 and *Fusarium oxysporum* CFR8 (14,15). In contrast, chitooligosaccharides deacetylases from organisms like *Puccinia graminis* f. sp. *tritici* and *Rhizobium* sp. have been successfully expressed in a heterologous host, specifically *E. coli* (16,17)

Therefore, driven by the desire of producing heterologous CD from different organisms at industrial scale in the future, I aimed to design, express, purify and test enzymatic activity of CD that can catalyse reactions on crystalline chitin using *E*. *coli* as host.

MATERIALS AND METHODS

Chitin Deproteination and Demineralization

50 g of shrimp powder were soaked with 200 mL of 1M HCl in order to remove the $CaCO_3$ until bubbles stopped appearing in the solution and then filtered with a sieve of 0.3 mm of pore diameter. The remaining material was boiled into 75 mL of 0.1 % SDS, 0.1 % NaOH solution in order to dissolve proteins. The solids in solution were allowed to precipitate, then washed by centrifugation three times at 5000 rpm for 10 minutes with dd water, and finally heated up to 100 °C in the oven overnight for then weight in order to calculate the yield: grams of remaining material obtained per gram of shrimp powder (g/g). Another procedure was carried out in an alternative way, performing the deproteination step first using 150 mL of the same SDS-NaOH solution. Demineralization was performed afterwards with HCl solution at the same concentration for further washing steps and finally dry out overnight. Chitosan used as substrate for enzymatic reactions was obtained from Sigma-Aldrich as commercial chitosan.

CD gene sequences, Alignments and Distance Tree analysis

Checking into the literature, four protein sequences from different microorganisms capable of producing CD were selected in order to express them in *E. coli* as

heterologous host. The criteria used for this selection was the ability to deacetylate crystalline chitin. Once the CD natural producers were selected, the sequences of these CD were used in protein-protein BLAST in the NCBI data base (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) with the aim to find proteins with similar domains and test them as potential CD. The distance tree analysis was obtained using the same informatic tool in the link and selecting the option in “other reports”.

Vectors and strain transformations

Once the protein sequences were chosen, plasmids were ordered to GenScript, inserted in a pET-30a vectors as shown in figure 3. The transformations were carried out using *E. coli* BL21 (DE3) by electroporation following the Biorad MicroPulser Electroporation Apparatus in order to prepare the cells; for this, a preinoculum of the strain was inoculated in 500 mL of L-broth and then incubated at 37 °C, 150 rpm until it reached OD_{600} value around 0.6. Afterwards, the cells were chilled on ice for 20 minutes and subsequently kept as close to 0 °C as possible using all reactants and containers previously chilled. The cells were harvested by centrifugation at 6000 rpm for 15 minutes at 4 °C. Then, the cells were resuspended in 500 mL 10 % glycerol solution. Centrifugation at same parameters as before was performed and subsequently the pellet was resuspended in 250 mL of 10 % glycerol solution. then, once again, the solution was centrifuged and the pellet resuspended in 20 mL of glycerol 10% and finally the same procedure was performed once last time and resuspended in 2 mL glycerol 10% solution. 50 µL of cells were mixed with 2 µL of each plasmid solution (0.2 mg/mL) from GenScript in electroporation cuvettes. The cuvettes were introduced into the Electroporator 2510 from Eppendorf and the pulse was set at 2500 V according to the equipment manual guide. Finally, 1 mL of LB medium were added to the cuvettes and incubated 1 hour at 37 °C and 150 rpm for further

dilution, plating in LB agar and incubation at 37 °C.

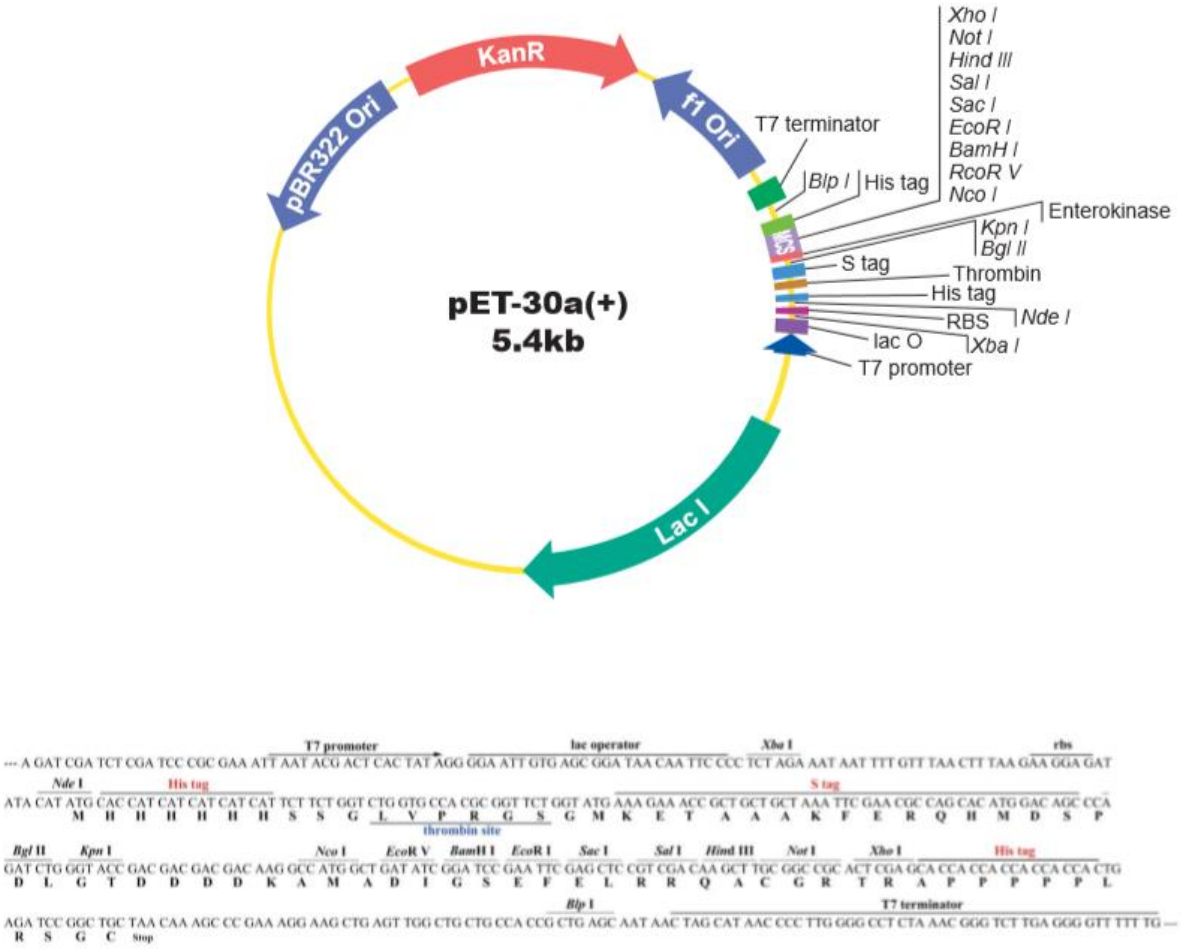


Figure 3. pET-30a(+) vector with size and all its restriction sites in which MRCD, BC1960 and AXE genes were cloned.

In addition, The *Ar*CE4 strain was a kind gift from Dr. Vincent Eijsink, which consists in a heterologous expression system in an *E.coli* host carrying a CD from a marine *Arthrobacter* species cloned in a pNIC-CH vector, presented in figure 4.

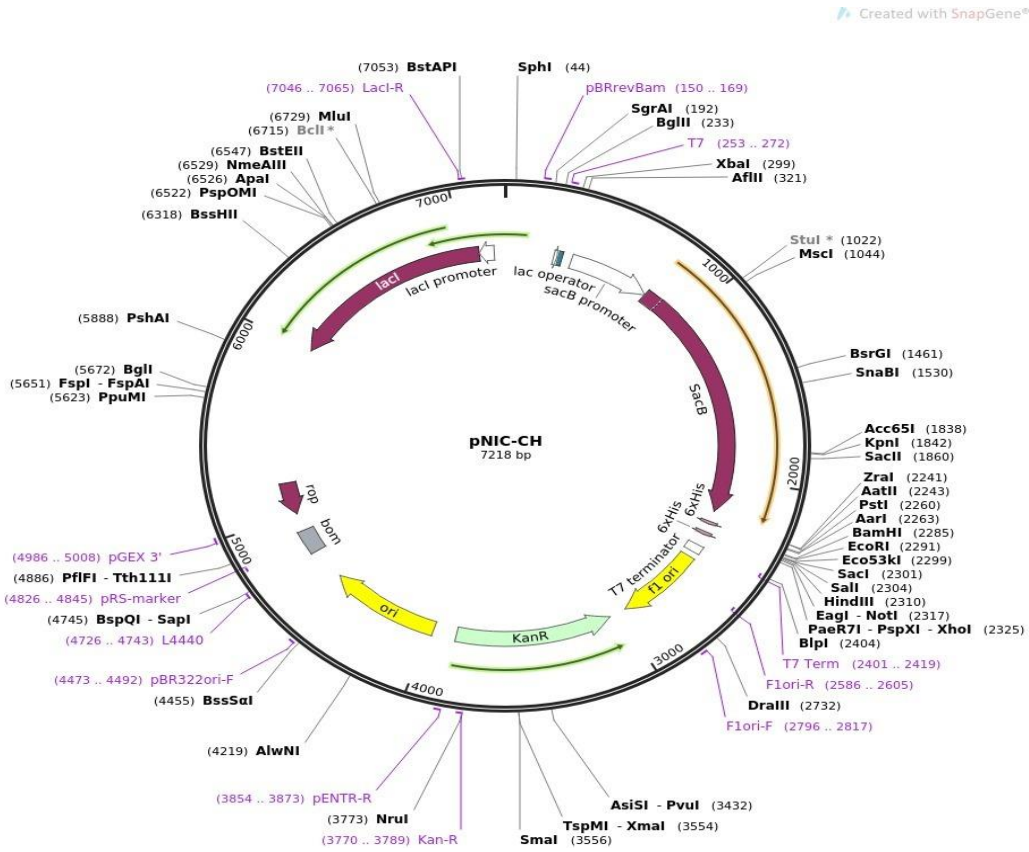


Figure 4. pNIC-CH plasmid which contains ArCE4A gen and is inserted in E.coli heterologous host

Growth cultures, Protein expression and Protein purification

20 mL of LB media with 50 µg/mL of kanamycin were inoculated with single colonies for each transformant and incubated overnight at 37 °C and 150 rpm. Thus, 2 mL of overnight culture were used to inoculate 200 mL of TB medium with kanamycin 50 µg/mL until the OD_{600} value reached 0.6. Afterwards, the culture was induced with IPTG to a final concentration of 0.2 mM and incubated overnight at 30 °C and 150 rpm in order to harvest the cells by centrifugation at 5000 rpm for 20 minutes. Subsequently, the supernatants were discarded and pellets resuspended in a sonication solution which was made of 10 mM imidazole, 20 mM tris-HCl and 150 mM NaCl at pH 8.0. for further sonication during 10 minutes of pulse, 1 second off, 1 second on at 28% amplitude. Next, 10 minutes of centrifugation of the supernatant were performed at 12000 rpm and 4°C for further filtration using a 0.45 µm membrane in order to obtain the cell free extract. Following this, the cell free extract was applied to a 1 mL Ni-sepharose column and left overnight while shaking at 4 °C for further collection of fractions by eluting the flow through and then washing the column with wash buffer which components were the same as sonication buffer but containing 20 mM imidazole instead of 10 and finally the

protein was eluted from the column using an elution buffer with the same components previously mentioned but with 300 mM imidazole.

Waste Water Treatment Plant Cultures

Samples from waste water treatment plant sludge were taken in order to test the deacetylase activity on chitin. These samples were grown for two months on media with 4 g/L of chitin or chitosan as sole carbon source, 4.65 g/L of K_2HPO_4 , 2 g/L of NaH_2PO_4 , 1 mL/L of Vishniac, 1 g/L of ammonium sulphate and 0.2 g/L of magnesium chloride at 30 °C and 150 rpm. Colony isolations were made on agar plates with the same composition of the media and incubated for 3 days at 37 °C. Later on, isolated colonies were inoculated in 25 mL of the same media previously mentioned and incubated at 37 °C and 150 rpm for 1 month, then they were scaled up to 250 mL with 1 % inoculum. After one month, the cultures were centrifuged at 8000 rpm for 20 minutes and the supernatant were used for enzyme activity.

Plasmid Digestion and Verification

5 mL of culture from each strain was used for plasmid isolation using miniprep kit from QIAgen for further DNA quantification and digestion using the following parameters: MRDA was treated with 1 µL XhoI and 1 µL NdeI restriction enzymes in 2X Tango buffer and incubated at 37 °C for 1 hour. The reaction was stopped by heating up the mixture to 80 °C for 10 minutes. For AxE and BC1960, 1 µL of plasmid was mixed with 1 µL of XmnI in 24 µL of 1X Tango buffer and then incubated at 37 °C during 15 minutes. Afterwards, 7 µL of Tango buffer were added plus 8 µL of NdeI and filled up to 40 µL with dd water. Finally the incubation was performed at 37 °C for 1 hour and inactivation 65 °C for 20 minutes. 1 µL of Smart Ladder from EuroGentec was mixed with 4 µL of TAE buffer with 1 µL gel loading dye 6X dye no SDS from New England BioLabs. The gel used was 0.8% agarose and run at 120 V for 1 hour and XhoI and NdeI restriction enzymes were obtained from Thermo Scientific while XmnI from minipcr New England BioLabs.

SDS-PAGE and Protein concentration

Protein electrophoresis was performed using 10 % of acrylamide gel, 160 V and 50mA for 45 minutes in buffer Tris-glycine-SDS 1X. 10 µL of samples were mixed with 10 µL of gel loading dye purple 6X no SDS from New England BioLabs and 30 µL of dd water for further incubation at 95 °C for 10 minutes. In order to reveal the gel, a comassie blue stain solution was added with the gel and kept overnight while gently shake for further revealing with decolouration solution made of 10% acetic acid and 20% methanol for 1 hour. Finally, the gel was washed with dd water. The

protein concentration was measured by Bradford method using Protein Assay Dye Reagent Concentrate from Biorad and following its instructions.

Enzyme activity

The activity assays were performed in triplicates, using the product of the standard method for deproteination and demineralization of shrimp powder. The reaction mixtures consisted in 5 mg/mL of chitin or chitosan with 10 µM $CoCl_2$, 300 nM enzyme and 50 mM Tris-HCl pH 8.0 in elution buffer for further incubation at 37 °C and 150 rpm for 4 days, and then quenched with acetonitrile to a final concentration of 50 %. For waste water treatment sludge, the reaction mixture consisted in 1 mL of supernatant containing 5 mg/mL of chitin and 10 µM of $CoCl_2$ and incubated at the same parameters previously mentioned. The mixtures were then spinned down at 12000 rpm for 10 minutes and the upper layer was filtered by a 0.2 µm pore membrane. Afterwards, the samples were run into Rezex ROA organic acid column from Phenomenex using a sulphuric acid 2.5 mM solution as mobile phase and a RI detector. The flow rate was set at 0.6 mL/min and the sample injection volume 10 µL with 30 minutes analysis.

RESULTS

Chitin Deproteination and Demineralization

The starting material consisted in 50 g of shrimp powder which were treated as previously mentioned in materials and methods. There were two different procedures: the first one in which demineralization was performed prior deproteination; and, the second one in which these steps were switched resulting in protein dissolving followed by demineralization. Results obtained after shrimp treatments were 29 g and 27 g for the standard and the alternative method respectively for the remaining material. According to this data, the yield for the standard method was 0.59 g/g while for the alternative was 0.54 g/g. Measurement the loosing of material in every step was not performed but 41% of the starting material was lost for the first procedure while for the second on was 46%. Therefore, the results showed that performing deproteination before demineralization increase the weight loosing and presumably, the purity of chitin.

CD gene sequences and alignments

In order to express CD from different microorganisms in a heterologous host, four CD sequences were selected from literature: *Mucor rouxii* CD (MRCD), *Bacillus cereus* 1960 (BC1960), *Streptomyces lividans* acetyl xylan esterase (AXE) and *Arthrobacter* species chitin esterase (*Ar*CE4) as positive control. These sequences

were selected on the basis that one of them belong to one of the most studied CD which is MRCD, and the other two belong to bacteria which have been proven to be active on performing deacetylation of crystalline chitin or soluble forms of chitin while being included in the carbohydrate esterase family 4 such as is shown in table 1 which has been taken from a previous review (11) and finally the *Ar*CE4 was used as positive control.

Table 1. Citated carbohydrate esterase family 4 members, their natural host, substrates, cofactors and PDB code. This table is taken from (11) reference.

Subfamil [1]	Enzyme	Organism	PDB (Year)	Ref [2]	Polymer Substrates	COS Substrates [3]	Metal	PA [4] (on A_n)
Chitin DA	*Mr*CDA	*Mucor rouxii*	–		Chitin, chitosan	≥DP3	Zn^{2+}	D_n, $D_{n-1}A$
	*Cl*CDA	*Colletotrichum lindemuthianum*	2IW0 (2006)	[61]	Glycol-chitin	DP6>DP5>DP4>DP3>DP2	Co^{2+} Zn^{2+}	D_n
	*An*CDA	*Aspergillus nidulans*	2Y8U (2012)	[12]	Glycol-chitin, chitin, CM-chitin, acetylxylan	DP2>DP3>DP4>DP5	Co^{2+}	D_n
	*Pg*tCDA	*Puccinia graminis*	–	–	Glycol-chitin, colloidal chitin, chitosans	DP6>DP5>DP4	n.r. [5]	AAD_{n-2}
	*Pe*sCDA	*Pestalotiopsis* sp.	–	–	Colloidal chitin, chitosan DA10-60%	DP6>DP5>DP4	n.r.	$AAD_{n-3}A$
	*Pa*CDA	*Podospora anserina*	–	–	Glycol-chitin	>DP2	Zn^{2+}	D_n
	*Pc*CDA	*Pochonia chlamydosporia*	–	–	n.r.	DP5>DP4	n.r.	$ADDA_{n-3}$
	NodB	*Sinorhizobium meliloti*	–	–	COS	DP5>DP2 (DP4, DP3)	Mn^{2+} Mg^{2+}	DA_{n-1}
	*Vc*CDA (COD)	*Vibrio cholera*	4NY2 (2014)	[62]	COS	DP2>DP3>DP4>DP5>DP6	Zn^{2+}	ADA_{n-2}
	*Vp*CDA (COD)	*Vibrio parahaemolyticus*	3WX7 (2014)	[63]	COS	DP2, DP3	Zn^{2+}	n.r.
	*Ar*CE4	*Arthrobacter* sp.	5LFZ (2017)	[64]	Chitin, chitosan, acetylxylan	DP5>DP6>DP4>DP3>>DP2	Ni^{2+} (6)	A3D2
GlcNAc DA	*Spr*GdA	*Streptococcus pneumoniae*	2C1G (2005)	[65]	GlcNAc DA on peptido-glycan	$(GlcNAc)_3$	Zn^{2+}	ADA
	*Smu*PgdA	*Streptococcus mutants*	2W3Z (2008)	[66]	GlcNAc DA on peptidoglycan	DP6	Zn^{2+}	n.r.
	*Bc*PgdA (BC1960)	*Bacillus cereus*	4LIG (2014)	[67]	GlcNAc DA on peptido-glycan, glycol-chitin	DP6>DP5>DP4>>DP3>DP2	Co^{2+}	$D_{n-1}A$
	*Er*Pgd	*Enterbacterium rectale*	5JMU (2016)		GlcNAc deacetylase (annotated)		Zn^{2+}	
MurNAc DA	*Bs*PdaA	*Bacillus subtilis*	1W17 (2005)	[68]	MurNAc DA on peptido-glycan (Cwld digested)	No active on COS	Cd^{2+} (6)	
	*Ba*Pda (BA0424)	*Bacillus anthracis*	2J13 (2006)	[69]	MurNAc DA on peptido-glycan	n.r.	Zn^{2+}	
PPda (unk)	BC0361	*Bacillus cereus*	4HD5 (2012)	[70]	Substrate unknown Putative GlcNAc DA		Zn^{2+}	
	BA0330	*Bacillus anthracis*	4Y33 (2015)	[71]	Unknown. Not active on glycol-chitin, COS, pNPAc, synthetic munopeptide		Zn^{2+}	
	BA0150	*Bacillus anthracis*	4M1B (2014)	[72]	Presumably inactive (no metal coordination)		No metal	
	ECU11_0510	*Encephalitozoon cuniculi*	2VYO (2009)	[73]	Inactive (lack of Asp general base and His metal-binding)		No metal	
AXE	*Sl*AxeA	*Streptomyces lividans*	2CC0 (2006)	[74]	Acetylxylan, glycol-chitin, chitosan	DP2-DP4-DP6	Co^{2+}	DDD (A1D2)
	*Cl*AxeA	*Clostridium thermocellum*	2C71 (2006)	[74]	2-O-acetylxylan	No active on COS	Co^{2+}	
β-1,6GlcNAc DA	*Er*PgaB	*Escherichia coli*	3VUS (2012)	[75,76]	Poly-β-1,6-GlcNAc-de-N-acetylase	β-1,6-GlcNAc oligomers	Co^{2+} Ni^{2+} Zn^{2+}	
	*Ad*PgaB	*Ammonifex degensii*	4WCJ (2014)	[77]	Poly-β-1,6-GlcNAc-de-N-acetylase	β-1,6-GlcNAc oligomers	Ni^{2+} Co^{2+} Zn^{2+}	
	*Bb*PgaB	*Bordetella bronchiseptica*	5BU6 (2015)	[45]	Poly-β-1,6-GlcNAc-de-N-acetylase	β-1,6-GlcNAc oligomers	Ni^{2+} Co^{2+}	
	*Aa*PgaB	*Aggregatibacter actinomycetemcomitans*	4U10 (2015)		Poly-β-1,6-GlcNAc-de-N-acetylase	β-1,6-GlcNAc oligomers	Zn^{2+}	

[1] Chitin DA: chitin deacetylase; GlcNAc DA: peptidoglycan N-acetylglucosamine deacetylase; MurNAc DA: peptidoglycan N-acetylmuramic deacetylase; PPda (unk): putative polysaccharide deacetylase (unknown); AXE: acetylxylan esterase; β-1,6-GlcNAc DA: poly-β-1,6-*N*-acetylglucosamine deacetylase. [2] 3D structure publication. [3] Activity on chitooligo-sacchirides (β-1,4-linked GlcNAc oligomers) as a function of the degree of polymerization (DP). [4] Pattern of acetylation (PA). Structure of the main final deacetylated product. A: GlcNAc, D: $GlcNH_2$. Other patterns of acetylation with specific substrates are given in the text. [5] n.r.: not reported. [6] No evidence for native metal, but indicated the metal from purification/crystallization experiments.

The sequences from the chosen microorganisms are listed in table 2 and do not contain any signal peptide nor His-tail except for *Ar*CE4.

Table 2. Selected CD sequences from different microorganisms, their Uniprot code, molecular weight, length in amount of aminoacids and location regarding cell.

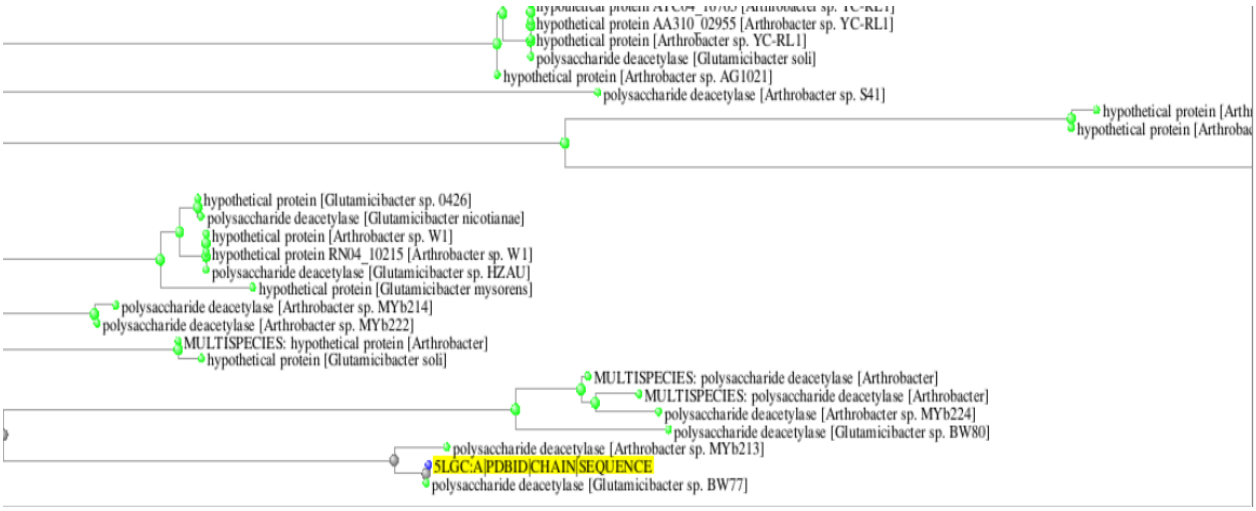
Data Base Code	Microorganism	MW / Length / Location	Sequence
5LGC	*Arthrobacter sp.*	25 kDa / 223 / intracellular	AGQPEPVATPPAVDCATTKCVALTFDDGPGEYTNRLLDELSEQHTP ATFFVLGKNVKKYPKTLKRMVDEGHQIG SHTFDHKDITKLTAEGIEHEVQWTDEAIEQAAGVKPQILRPPYGAHGA VYDRLIPYPLVLWDVDTLDWKHHDPQKTVRIA LEEAKPGSIILMHDIHESSVKAVPQLVSKLHDAGYTLVTVDQLFAGTD FKPAKAYDHRFKTNP
4L1G	*Bacillus cereus*	30 kDa / 275 / intracellular	MFAPYQWGLERDVSYAYMPYNSFYYGDYINSLPYAYIPQNYEVQMK ADDRGSWTPFSWVEKYAYAFSGPYNKAEVALTFDDGPDLEFTPKIL DKLKQHNVKATFFLLGENAEKFPNIVKRIANEGHVIGNHTYSHPNLAK VNEDEYRNQIIKTEEILNRL AGYAPKFIRPXYGEILENQLKWATEQNFMIVQWSVDTVDWKGVSAD TITNNVLGNSFPGSVILQHSTPGGHLQGSVDALDKIIPQLKTKGARFV TLPSMFQTSKERK
Q54413	*Streptomyces lividans*	34 kDa / 335 / extracellular	AACNGYVGLTFDDGPSGSTQSLLNALRQNGLRATMFNQGQYAAQN PSLVRAQVDAGMWVANHSYTHPHMTQLGQAQMDSEISRTQQAIAG AGGGTPKLFRPPYGETNATLRSVEAKYGLTEVIWDVDSQDWNNAST DAIVQAVSRLGNGQVILMHDWPANTLAAIPRIAQTLAGKGLCSGMISP QTGRAVAPDGSGGGGDGGGGGGGACTATLSAGQRWGDRYNLNV SVSGASDWTVTMNVPSPAKVLSTWNVNASYPS AQTLTAKSNGSGGNWGATIQANGNWTWPSVSCTAG
P50325	*Mucor rouxii*	46 kDa / 421 / extracellular	DTSANYWQSFTSQINPKNISIPSIEQTSSIDPTQECAYYTPDASLFTFN ASEWPSIWEVATTNGMNESAEFLSVYNSIDWTKAPNISVRTLDANGN LDTTGYNTATDPDCWWTATTCTSPKISDINDDISKCPEPETWGLTYD DGPNCSHNAFYDYLQEQKLKASMFYIGSNVVDWPYGAMRGVVDGH HIASHTWSHPQMTTKTNQEVLAEFYYTQKAIKLATGLTPRYWRPPYG DIDDRVRWIASQLGLTAVIWNLDTDDWSAGVTTTVEAVEQSYSDYIA MGTNGTFANSGNIVLTHEINTTMSLAVENLPKIISAYKQVIDVATCYNI SHPYFEDYEWTNVLNGTKSSATASGSATSASASGGATTAAAHIQAST SGAMSVLPNLALISAFIATLLF

Furthermore, these sequences were aligned to the NCBI data base using protein BLAST in order to find similar sequences or at least homologue sequences to some domains and motifs. The results showed that despite there were alignments with the conserved motifs which are involved in the catalytic activity, substrate binding or metal stabilization, the resulting sequences come from putative or hypothetical proteins. So that, these proteins have not been proven yet at laboratory scale to catalyse deacetylation of chitin or they do perform deacetylation of other molecules like histones in eukaryotes. In fact, that suggests there is still some investigation to be done in order to elucidate the capability of some of these enzymes to deacetylate chitin-like molecules. Also, most of the results from the

protein BLAST refer to predicted proteins which come from genomes in which NodB domain is present according to the DNA sequencing.

Moreover, when carrying out distance tree analysis, the presence of large amounts of putative and hypothetical proteins becomes clearer. For instance, distance tree analysis for *Ar*CE4 and BC1960 can be seen in figure 5. MCRD and AXE distance tree data are not shown, yet the results were similar presenting predicted proteins in the vast majority. In these figures, enzymes are grouped into family proteins and others are presented as singular proteins even thought when zooming in the distance tree, the members of these families turn out being hypothetical an putative proteins as well. Therefore, only the four initial enzyme sequences were selected for further heterologous expression.

A



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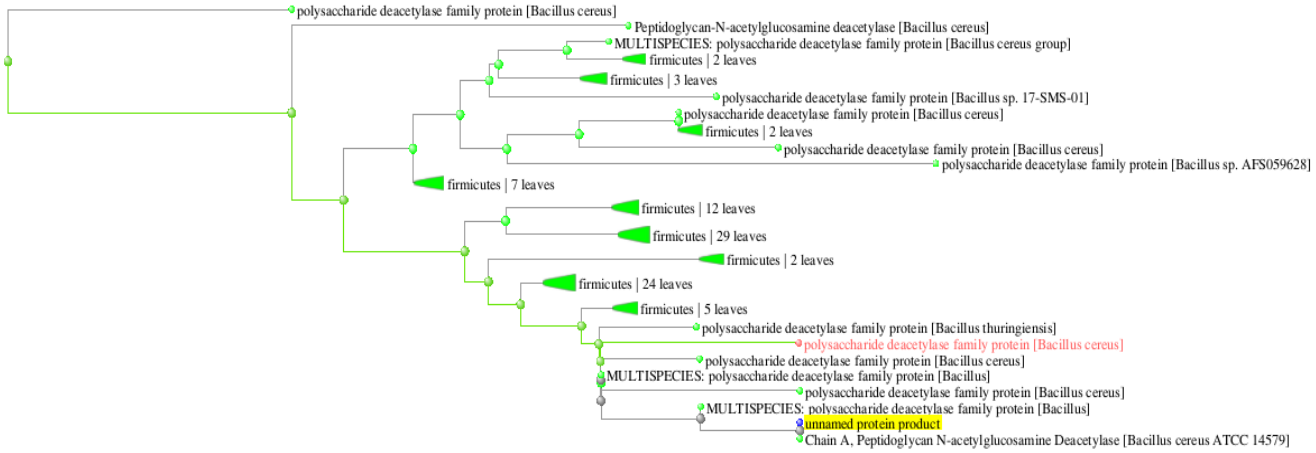


Figure 5. Distance tree analysis in which the most related sequences are shown to the reference. The yellow highlighted sequences correspond to the template

sequence, in this case A for ArCE4 and B for BC1960. The green lines and the name of the protein in red appears when a sequence is selected for further analysis. The length of the horizontal lines determine the difference between sequences, in other words, the longer the distance of horizontal lines between sequences, the more distinct they are.

Protein expression and Concentration

After transformation, expression and purification of the proteins using a heterologous host, the protein markers show bands for *Ar*CE4 and BC1960 which are approximately 25-30 kDa. Figure 6 presents different elution fractions from *Ar*CE4 purification in which can be seen that some protein is eluting after several elution steps and even might be that some enzyme is eluting from the CFE and FT fractions which are not shown in figure 6.

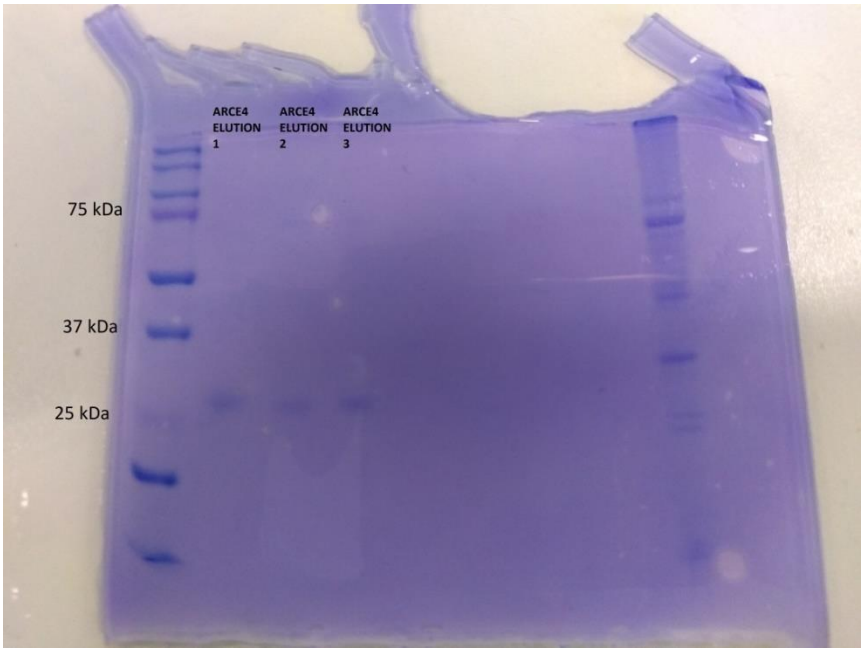
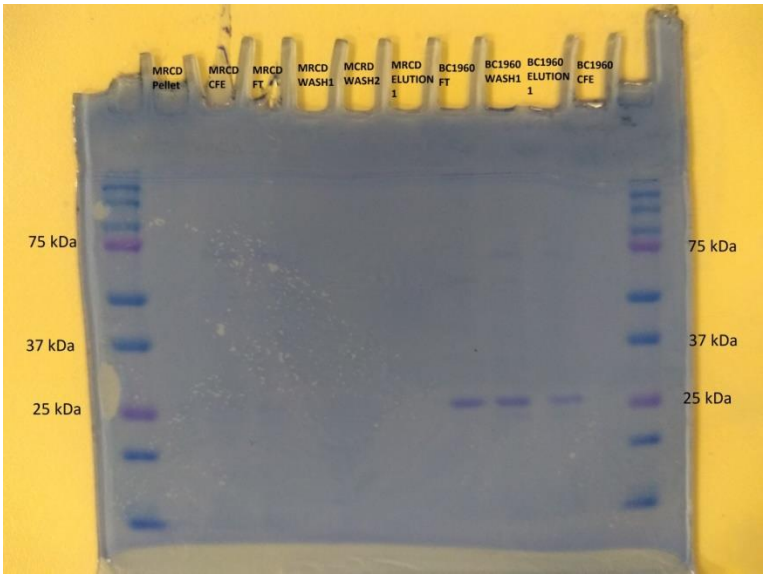


Figure 6. Elution fractions 1, 2 and 3 as sequential steps in the elution process of the enzyme purification for ArCE4 (~25 kDa). Lane 1: standard molecular weight marker; lane 2: ArCE4 elution number 1; lane 3: ArCE4 elution number 2; lane 4: ArCE4 elution number 3.

Figure 7 shows purification steps for BC1960, MRCD and AXE in which is clearly seen the only fractions that contain the protein of interest are FT, wash 1 and elution 1 from the BC1960's purification process as the molecular weight is consistent with what is expected, around 30 kDA. For AXE's CFE and FT, it can be seen that there are bands with a little bit less than 70 kDa in size and also the concentration is too low in these fractions. Supernatants from CDCT and WACT

were directly run in the after culture centrifugation in order to test the presence of extracellular proteins. The gel shows that there was no protein present.

A



B

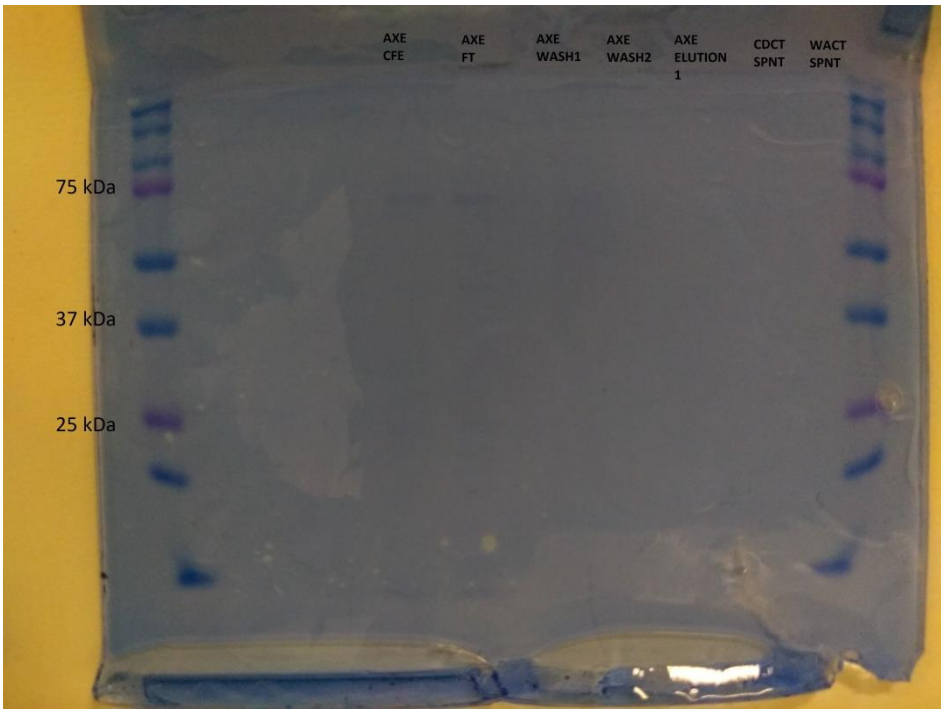


Figure 7. Different purification fractions from BC1960, MRCD, CDCT and WACT. (A) MRCD and BC1960 fractions and (B) AXE fractions, CDCT and WACT supernatants.

Bradford method was carried out for protein concentration, and the results obtained were 15.3 µM for BC1960 and 71.6 µM for *Ar*CE4. For the rest of the proteins, there was some protein content (data no shown) but as the gels showed, there was no apparent band so those results were discarded. The standard deviation of the different replicants was less than 0.05 and R^2=0.98.

Waste Water Treatment Plant Cultures

After incubation of the waste water treatment plant cultures in plates, several colonies were obtained with different features. Only two colonies were tested for deacetylation of chitin. One of them was named WACT which is a yellow sticky colony while CDCT is a more solid and grey colony that forms a ring-like structure in the centre and the rest of the colony diffuses in the plate as presented in figure 8. There were also some dark brown small colonies which were not tested.

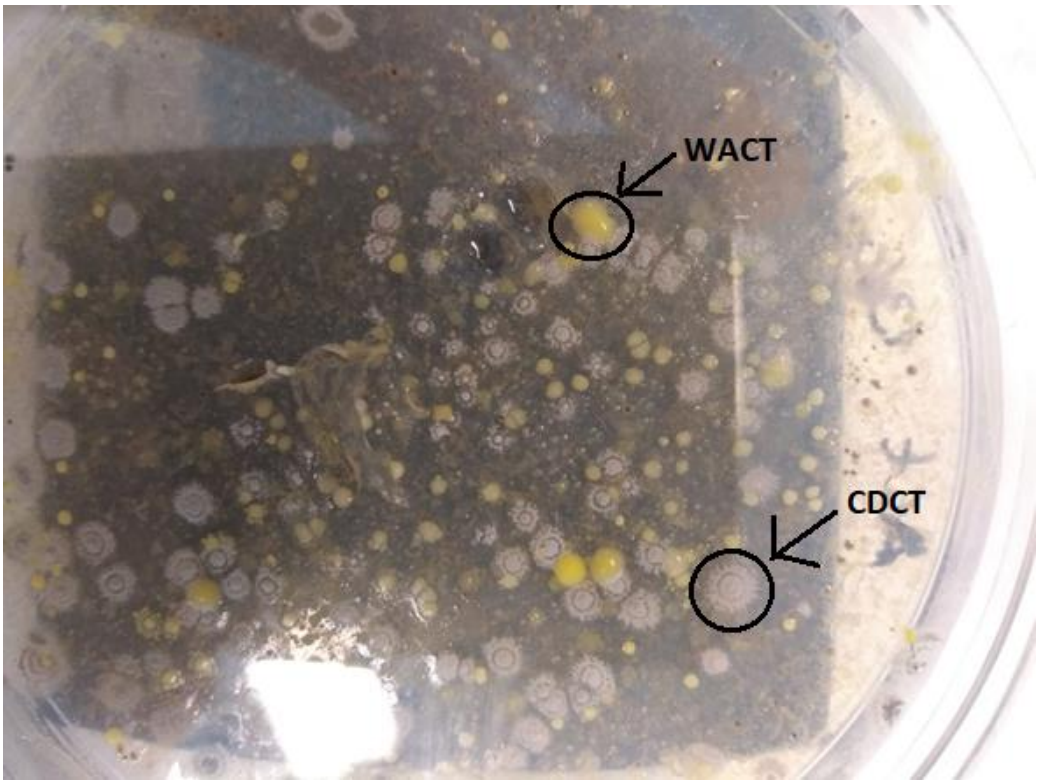


Figure 8. WACT and CDCT colonies grown in chitin and mineral plate media. These colonies were isolated from cultures from waste water treatment plant.

In contrast, chitosan plates inoculated with the same sample presented similar colonies. However, colonies from chitosan plates did not grow when inoculating chitosan media with only chitosan as sole carbon source (data not shown).

Therefore, colonies from chitosan plates were not taken into account for extracellular enzyme production.

Enzyme activity

Negative controls and reactions solutions presented particular colours as shown in figure 9. Especially negative controls showed a more intense violet colour while the reaction mixtures turned to violet more slightly than the stock cobalt solution. Chitin controls were the ones with most drastic changes in the colour as well as the precipitated material composed mainly by the substrates. Originally, the colour of the starter reaction mixtures and controls was slightly less colourful than the stock cobalt solution (data not shown).

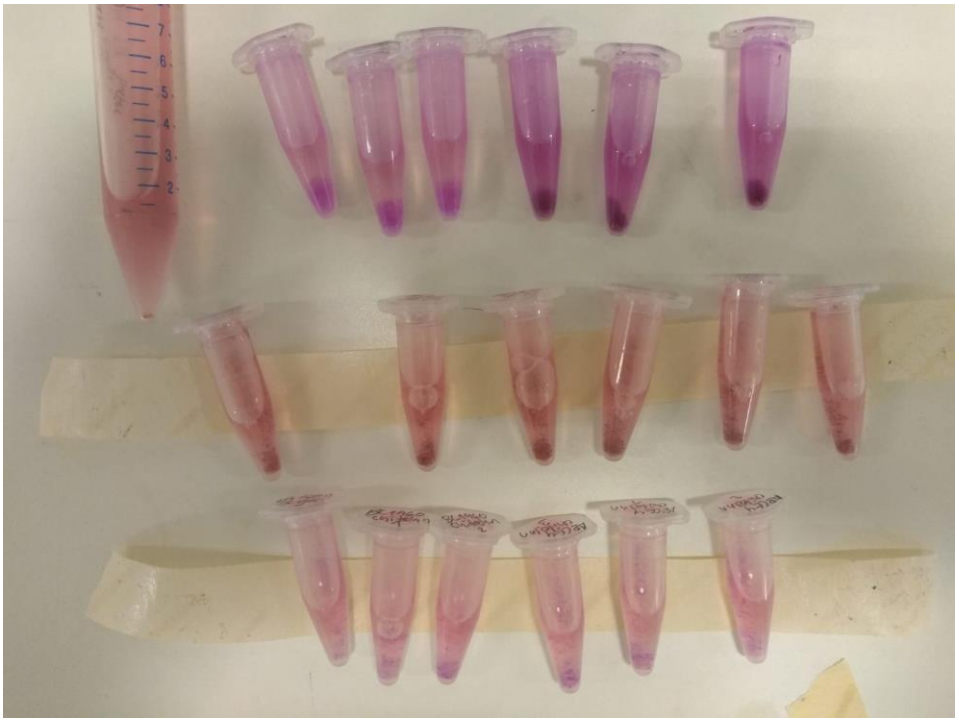
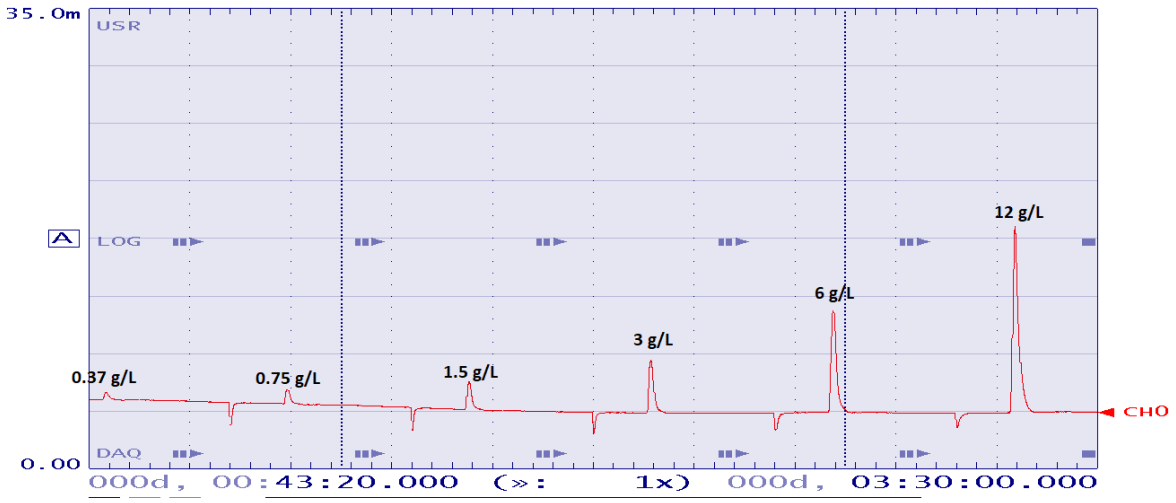


Figure 9. Negative controls and reaction mixtures after incubation. Top three eppendorf tubes in the left correspond to chitosan controls while the three in the right are chitin controls. The middle line of eppendorf tubes are enzyme reactions on chitin, being the three in the left for BC1960 and for ArCE4 the three in the right. similarly, the bottom tubes are arranged same as the middle tubes but reactions were performed on chitosan. The falcon tube in the top left is the stock cobalt solution 100 µM used for reactions.

After the HPLC assays, chromatograms of standards showed that acetic acid elutes at around 17 minutes as presented in figure 10A. However, figure 10B revealed that there was no signal of acetic acid released in the enzymatic activity

samples as the long peaks correspond to imidazole, proved by running only elution buffer into the HPLC and the same large peak was shown at around 14 minutes of elution (data not shown).



B

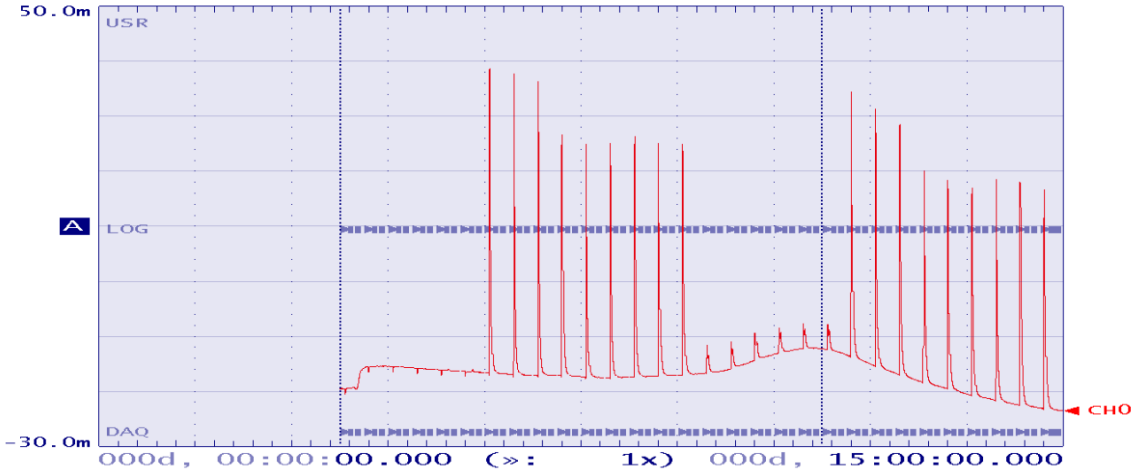


Figure 10. Chromatograms showing standard concentrations of acetic acid from 0.37 g/L to 12 g/L (A) and also from 0.025 g/L to 0.4 g/L followed by serial triplicates of enzymatic activity samples (B) in which large peaks correspond to imidazole in each reaction. The reaction mixtures were made in triplicates, beginning from the first three large peaks for negative controls for chitin, followed by BC1960, ArCE4, CDCT and WACT. Finally, triplicates of chitosan negative controls, BC1960 and ArCE4 with chitosan.

In addition, the bottom limit detection of the method with which the samples were run, was deducted by the figure 10B in which is shown that 0.05 g/L of acetic acid were detected but no concentrations below. Despite is not clearly seen in the

graph, zooming in the first 6 samples, that comprises concentrations between 0.02 and 0.4 g/L, can make 0.05 g/L peak visible. After the standards samples, enzymatic activity samples are presented in triplicates shown in a row, meaning that the first three large peaks correspond to replicates of chitin controls, followed by triplicates of BC1960, *Ar*CE4, CDCT and WACT with chitin and finally triplicates of chitosan controls, BC1960 and *Ar*CE4 with chitosan. AXE was not taken into consideration as the protein band presented in some of the fractions did not correspond to the expected protein size for the specific CD. No activity was either detected for deacetylation of chitosan using BC1960 nor *Ar*CE4.

Plasmid Digestion and Verification

In spite of the fact that plasmid digestions were carried out several times for the plasmids containing BC1960, MRCD and AXE genes, the results did not only show any band for the samples but also for the standard ladder. Figure 11 shows the results of the DNA electrophoresis in which is clearly seen that no bands are present for either the samples or the standards. Furthermore, expected results on how the electrophoresis should have been carried out successfully are presented in figure 11 where it can be seen the multiple bands produced by the digestion with restriction enzymes using the software SerialCloner 2.6.1

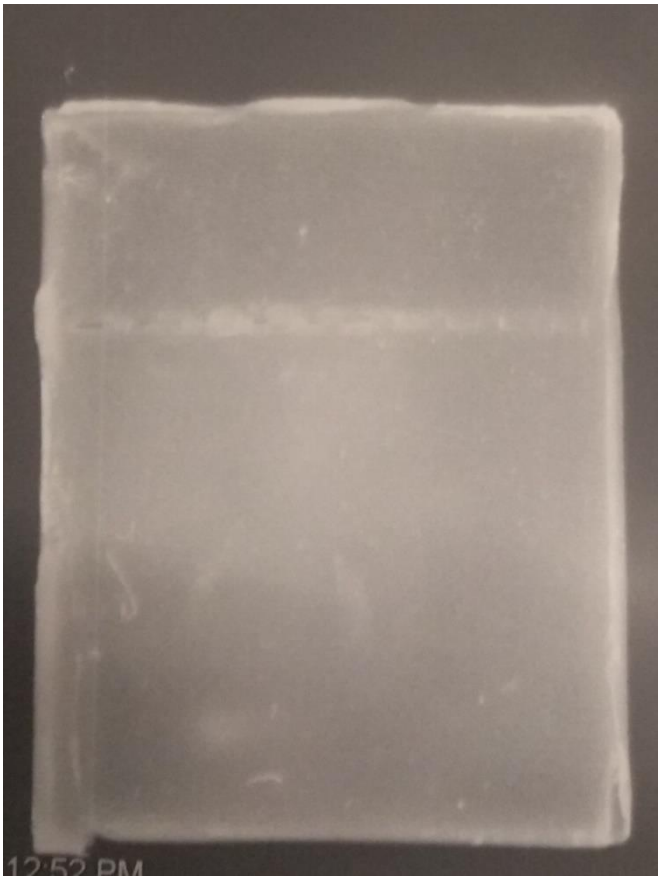


Figure 11. DNA electrophoresis of plasmid digestion for BC1960, MRCD and AXE

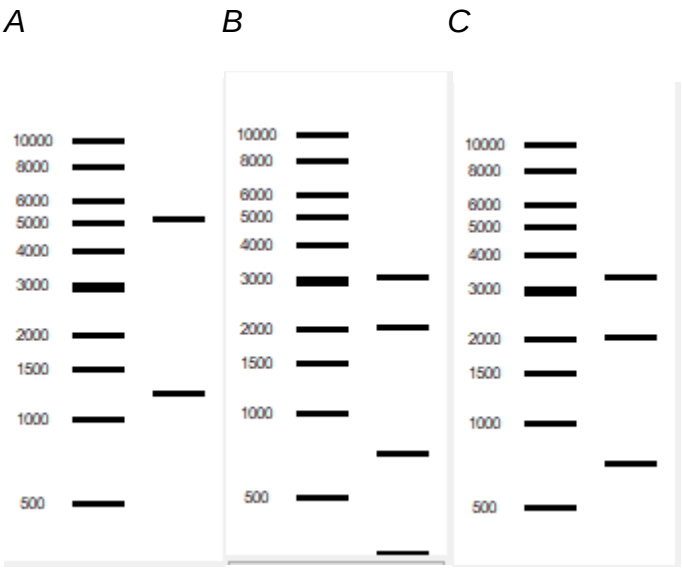


Figure 12. Predicted plasmid digestion for MRCD (A), BC1960 (B) and AXE (C) using SerialCLoner 2.6.1 software. AxE and BC1960 *plasmids were cut with XmnI and NdeI while MRDA was cut with XhoI and NdeI.*

According to figure 12, MRCD digestion should have shown two bands with sizes of around 5 kbp and 1.25 kbp, while BC1960 comprises sizes of approximately 3 kbp, 2 kbp, 800 bp and a minor band in the bottom of the gel. Finally, AXE digestion should have shown almost the same bands as BC1960 except for the smallest band which is not present. To prevent confusion if the minor band of BC1960 digestion is not present, an alternative to differentiate with AxE is using another restriction enzyme. BamHI is a proper option as it cuts BC1960 in a way that together with XmnI, bands of around 3, 2 and 1 kbp would be present in the gel. On the other hand, AxE would produce bands of around 4 and 2 kbp using the same restriction enzymes making easier to distinguish between these two constructs.

DISCUSSION

Chitin Deproteination and Demineralization

According to the data obtained in this study, the remaining material after deproteination and demineralisation comprises around the 60% of shrimp powder. Several studies have established the chitin content of shrimp powder in around 15-40% of total dry weight, while protein in 20-40% and calcium carbonate in 20-50% (18). This wide range of chitin content in shrimps and prawns is determined by the

species as shown by Rødde et al. 2008 and Percot et al 2003 (8,19) in which *Pandalus borealis* and *Parapenaeopsis stylifera*, presented between 33%-40% of protein content and chitin around 20% while shrimp *Penaeus monodon*'s chitin content comprises around 10% of total weight (19). Besides, there was no significant evidence that shows changes in chitin composition for one species along the year.

Comparing these previously reported results with those obtained by this study, it can be claimed that the deproteination and demineralisation were not driven to completion as the resulting material is at least 20% higher in chitin content than those from the literature. This can be explained by the fact that the shrimp powder was not long enough exposed to the deproteination and the demineralisation solutions in order to eliminate all the extra components but only for the time visual evidence of carbon dioxide stopped bubbling in the demineralisation step and for the deproteination solution was pour out once the material was precipitated.

In order to test the purity of chitin; content of ash, lipids and proteins must be measured. A simple method which involves the heating of raw shells followed by coupled plasma-optical emission spectroscopy analysis has been described to elucidate the ash content. In addition, the method also comprises total nitrogen measurement by Kjeldahl using multiplying factor for protein determination and finally gravimetric analysis after Soxhler extraction with hexane for lipid measurement (20)

CD gene sequences and alignments

As previously mentioned, table 1 (11) shows a limited number of members from the carbohydrate esterase family 4 which have been biochemically characterized to be active on chitin-like molecules performing deacetylation. This information reveals that members of the CE4 are not specific for their natural substrate and can be applied for deacetylation of other molecules such as chitooligosaccharides, peptidoglycan and even acetylxylan which makes them versatile enzymes. Despite this is case for CE4 members, it has not been proven that they can be active on crystalline chitin and therefore in several studies researchers must have synthesized soluble chitin by adding a hydroxyethyl group in the carbon six of the N-acetylglucosamine monomers.

Having into account that not all the chitin CD are listed in table 1, protein BLAST was made for each sequence in order to verify which ones have similarities either with *Ar*CE4, BC1960, MRCD and AXE. As the results showed that only putative and hypothetical proteins were found, that stands in direct submission with no further biochemical test for chitin deacetylase activity. For instance, enzymes

BC0361 from *Bacillus cereus* and, BA0330 and BA0150 from *Bacillus anthracis.* are ORFs which do not have any natural substrate yet reported even thought their 3D structure have been elucidated and for the *Bacillus anthracis* ones, there is evidence about the role they play in cell wall shape and high salt concentration resistance by regulating osmotic stability (22)

In addition, other chitin deacetylases have been reported to belong to rhizobial bacteria, the most common and first described correspond to *Sinorhizobium meliloti* in which the enzyme NoDB was classified as the first member of the CE4 family. The role played by NoDB is mainly chitooligosaccharide modification by deacetylation (2-5-unit length) which serve as nodulation factor and induce the formation of nodules in leguminous (23).

Protein expression and Purification

Having into account that *Ar*CE4 and BC1960 were previously heterologous expressed in *E. coli*, it is not surprising that they were the only proteins which could be produced and purified in this work as shown in figure 7. Their molecular weight correspond to those cited in the literature, approximately 25 kDA and 30 kDA for *Ar*CE4 and BC1960 respectively (23, 24) despite there was some of these proteins in other fractions like FT and Wash fractions. This might be the consequence of high expression of the proteins and the binding capacity of using only 1 mL of nickel-sepharose column in the purification as the concentrations were constantly 15 µM and 71 µM for BC1960 and *Ar*CE4 respectively in different batches, even when increasing the volume of expression cultures 4 times. Evidence of this is that after re-doing purification of the remaining FT, the elution fraction still contained a remarkable amount of protein; therefore, increasing the volume of column would be sufficient to collect a higher amount of enzyme.

Additionally, there is no evidence in the SDS-PAGE of the presence of MRCD nor AXE as bands of around 46 kDa and 34 kDa respectively were absent. This can be related to difficulties on either the heterologous expression of the protein or the cloned sequence itself. Despite the recombinant sequence was properly selected from the database, this was the first attempt to express these two proteins in a heterologous host, requiring a longer period of incubation and other supplements for their natural host to grow than for *E. coli*. No information is given about other compounds attached to the AXE that are key for its activity except for the presence of $CoCl_2$; on the other hand, MRCD is a glycoprotein like all the fungi CD found to date and therefore their folding could not be properly made as the heterologous host does not have the system to perform the adequate the post-translational modifications required to form, for instance sulphide bonds, or adding the gycol group to the protein (25, 26, 27). Furthermore, only a few fungi proteins have been

successfully expressed in *E. coli* to date, named CD from *Colletotrichum lindemuthianum, Saccharomyces cerevisie, Aspergillus nidulans* and *Puccinia graminis* (28, 29, 30)

Enzyme activity

The results of the HPLC revealed that there was no detectable CD activity on either *Ar*CE4 or BC1960 as the high peaks presented in the chromatograms corresponded to imidazole, which was present in high concentration, 300 mM, after the elution from the Nickel-sepharose column. There is no evidence in the literature of high concentrations of imidazole affecting the enzyme activity of CD but it has been reported that synthetic catalyst with an imidazole motif can play an esterase-like role on *p*-nitrophenyl acetate and phenylalanine methyl ester (31). Furthermore, it has also been proven that synthesized acrylamides assembled with imidazole or benzimidazole motifs can affect negatively the activity of the enzyme histone deacetylase (32). This is especially interesting as the active site of the vast majority of CD possess two Histidine residues which stabilize the metal ion alongside one residue of Aspartate and that might has a significant impact in the deacetylase activity if the imidazole can be activated in a way that it can interacts with the amide bond of the chitin.

Furthermore, there have been previous reports on the CD activity of the enzymes used for this work. The one which was used as positive control, *Ar*CE4, was especially active on chitooligosaccharides of 5 monomers, apart from its natural substrate, acetylxylan, followed by chitosan from which it presented 0.32 and 0.28 nmol/min respectively. Besides, its activity was also tested on α-chitin and β-chitin from which value only was 0.001 nmol/min for β-chitin (24). On the other hand, BC1960 presented its best activity on chitooligosaccharides of 4 and 6 units length (23). These results showed that the CD used for this work have preference for short length compounds probably because the binding pockets of the enzymes can hardly access to the crystal structure of the chitin but for more soluble chitin derivate compounds the substrate is perform faster and easier due to the lack of crystalline structure. Methods for solubilizing chitin and for crystalline structure elimination have been tested, for instance, addition of hydroxyethyl groups in the carbon number six of the monomer in order to produce glycol chitin or ethylene glycol chitosan. The aforementioned method is preferred rather than solubilizing chitin or chitosan in organic solvents or high concentrated solutions of inorganic acids. This is due to the harmful, corrosive and scarcely degradable that these solutions can be, besides that enzymes are likely to be denature under such conditions (11,33)

In this sense, CD from fungi are more prompted to have deacetylase activity on crystalline chitin as in previous reports it has been shown that these enzymes can deacetylate 0.5 % of the molecule. The microorganisms from which these enzymes come from are *M. rouxii* ATCC24905 and *A. nidulans* FGSCA4 and they possess a carbohydrate subunit in their structure which comprises 30% of the total weight of the protein, which can be related to undone post-transcriptional changes and therefore non-proper folding of the enzymes when expressing and catalysing the deacetylation of chitin (34,35). Finally, a very interesting approach for enhancing the CD activity is co-expressing a chitinase from another host in order to make the amide bonds more accessible (34).

Conclusions

Apparently, performing deproteination of chitin before demineralization is more effective when trying to increase the purity of chitin although experiments to verify chitin purity must be performed to confirm it. Additionally, alignments made on proteins studied in this work, showed that no CD proven at laboratory scale were present in the results which means that only a few CD have been confirmed to be active on chitin, chitosan or chitooligosaccharides. In fact, activity on chitin itself is still a challenging task as no protein has been discovered or engineered to perform activity on the crystalline structure of chitin.

Chitin and chitosan were not able to be deacetylated in the range of the detection limit (above 0.05 g/L acetic acid) by none of the proteins *Ar*CE4 and BC1960 despite they were properly expressed. On the other hand, MRCD and AXE were not able to be expressed under the conditions presented in this work. Besides, using the supernatants of the cultures inoculated with sludge from waste water treatment plant (CDCT and WACT) did not show any deacetylase activity either. Given that the expression of MRCD and AXE was not possible, it is recommendable to try to perform the transformation again for these proteins and verify the proper insertion of the genes in the vector by electrophoresis. Doing so, pay special attention on the conditions of the reagents in order to get reliable results. In addition, dialysis of the purified proteins must be tested with the aim to decrease drastically the concentration of imidazole which might have been interfering with the enzyme activity of *Ar*CE4 and BC1960. Regarding CDCT and WACT, a more exhaustive research must be made on the protein profile and characterisation of these microorganisms in order to elucidate possible chitin deacetylases already reported in databases that could match with characterisation of these colonies. Finally, it would be interesting to perform a co-expression of CD

and chitinases, either in the same host or different ones in order to improve the CD performance.

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