THERMOPHILIC MICROORGANISMS AS SOURCE OF MOLECULES FOR BIOTECHNOLOGICAL APPLICATIONS

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Tutor: Prof. Patrizia Contursi
Co-tutor: Dr. Andrea Strazzulli
Coordinatore: Prof. Salvatore Cozzolino

Dedicata alla mia famiglia

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ABSTRACT

Background: The spore-forming lactic acid bacterium *Bacillus coagulans* MA-13 has been isolated from canned beans manufacturing and successfully employed for the sustainable production of lactic acid from lignocellulosic biomass. Among lactic acid bacteria, *B. coagulans* strains are generally recognized as safe (GRAS) for human consumption.

Low-cost microbial production of industrially valuable products such as lactic acid and various enzymes devoted to the hydrolysis of oligosaccharides and lactose is of great importance to the food industry. Specifically, α- and β-galactosidases are attractive for their ability to hydrolyze not-digestible galactosides present in the food matrix as well as in the human gastrointestinal tract.

Results: In this thesis, the potential of *B. coagulans* MA-13 as a source of metabolites and enzymes has been explored to improve the digestibility and the nutritional value of food. A combination of mass spectrometry analysis with conventional biochemical approaches has been employed to unveil the intra- and extra-cellular glycosyl hydrolases (GH) repertoire of *B. coagulans* MA-13 under diverse growth conditions.

The highest enzymatic activity was detected on β-1,4 and α-1,6-glycosidic linkages and the enzymes responsible for these activities were unambiguously identified as a β-galactosidase (GH42) and an α-galactosidase (GH36), respectively.

Whilst the former has been found only in the cytosol, the latter is localized also extracellularly. The export of this enzyme may occur through a not yet identified secretion mechanism since a typical signal peptide is missing in the α-galactosidase sequence. A full biochemical characterization of the recombinant β-galactosidase has been carried out and the ability of this enzyme to perform homo- and hetero-condensation reactions to produce galacto-oligosaccharides, has been demonstrated.

Conclusions: Probiotics that are safe for human use and are capable of producing high levels of both α-galactosidase and β-galactosidase are of great importance to the food industry. In this thesis, it has been proven the ability of *B. coagulans* MA-13 to over-produce these two enzymes thus paving the way for its potential use in food industry.

ABBREVIATION LIST

*Bc*GalB	*Bacillus coagulans* MA-13 β-galactosidase
CAZy	Carbohydrate-Active enZymes
dbCAN	Database for automated Carbohydrate-Active enzyme Annotation
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ESI-MS	Electro-Spray Mass Spectrometry
GH	Glycoside hydrolase
GOS	Galacto-oligosaccharides
GRAS	Generally Regarded as Safe
GS	Glyco-synthase
IPTG	Isopropyl-β-D-1-thiogalactopyranoside
k_{cat}	Catalytic constant
K_M	Michaelis-Menten constant
LAB	Lactic acid bacteria
LC-MS/MS	Liquid Chromatography-Tandem Mass Spectrometry
PNP-α-ara	*para*-Nitrophenyl-α-L-arabinofuranoside
PNP-α-fuc	*para*-Nitrophenyl-α-L-fucopyranoside
PNP-α-gal	*para*-Nitrophenyl-α-D-galactopyranoside
PNP-α-glu	*para*-Nitrophenyl-α-D-glucopyranoside
PNP-α-man	*para*-Nitrophenyl-α-D-mannopyranoside
PNP-α-rha	*para*-Nitrophenyl-α-L-rhamnopyranoside
PNP-β-fuc	*para*-Nitrophenyl-β-L-fucopyranoside
PNP-β-gal	*para*-Nitrophenyl-β-D-galactopyranoside
PNP-β-glu	*para*-Nitrophenyl-β-D-glucopyranoside
PNP-β-man	*para*-Nitrophenyl-β-D-glucopyranoside
PNP-β-xyl	*para*-Nitrophenyl-β-D-xylopyranoside
ONP-β-gal	*ortho*-Nitrophenyl-β-D-galactopyranoside
ONP-β-glu	*ortho*-Nitrophenyl-β-D-glucopyranoside
RAST	Rapid Annotation using Subsystem Technology
RFO	Raffinose family oligosaccharides
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TLC	Thin-Layer Chromatography
U	Hydrolytic Units
v_{max}	Maximal velocity

SUMMARY IN ITALIAN

I batteri lattici ed il genere *Bacillus*

I batteri lattici (LAB) sono Gram positivi con DNA a basso contenuto in G+C, che vengono definiti così perché capaci di produrre acido lattico tramite il processo di fermentazione.
Riconosciuti come GRAS (Generally Regarded as Safe) non sono nocivi per la salute dell'uomo e possono appartenere ai generi *Lactobacillus, Pediococcus, Leuconostoc,* e *Weissella* [1].
Tali microrganismi sono caratterizzati da cellule a forma di cocco o di bastoncello e presentano un metabolismo obbligatoriamente fermentativo. Producono composti ad alta energia mediante fosforilazione al livello del substrato perché non possiedono importanti componenti della catena di trasporto respiratoria come i citocromi; tuttavia, pur avendo un metabolismo tipicamente anaerobio, possono essere anche aero-tolleranti.
Di solito i batteri lattici hanno delle capacità biosintetiche piuttosto limitate e richieste nutrizionali molto complesse, per cui i terreni a base minerale devono essere arricchiti con vitamine e amminoacidi specifici per la crescita del batterio.
Nonostante ciò, i LAB sono riusciti a colonizzare diverse nicchie ecologiche [2] mediante l'acquisto di nuove funzioni metaboliche tramite il trasferimento orizzontale dei geni [3].
Infatti, sono largamente distribuiti in natura e gli habitat più rappresentativi sono la cavità orale, il tratto intestinale, il suolo, l'acqua, i cereali ed i prodotti fermentati (latte, carne e vegetali).
I LAB possono essere classificati anche in funzione dei prodotti di fermentazione che vengono ottenuti in seguito alla loro crescita.

Infatti, esistono microorganismi *omo-fermentanti* i quali producono esclusivamente acido lattico (oltre il 90% dei prodotti di fermentazione), e microorganismi *etero-fermentanti* i quali sono coinvolti nel processo di fermentazione etero-lattica, con secrezioni composte da acido lattico, etanolo, acetato, acido formico e succinato in funzione della tipologia di batterio fermentativo [4].

Oggigiorno, i LAB rivestono un ruolo cruciale in molteplici processi industriali del settore alimentare, poiché coinvolti nella produzione di acido lattico e altri acidi organici deboli a partire da diverse fonti di carbonio disponibili.

Tali microrganismi sono spesso utilizzati anche come conservanti naturali alimentari grazie alla capacità di produrre metaboliti antimicrobici e antifungini [5] che prolungano la conservabilità del prodotto, preservandolo dall'attacco di eventuali microrganismi patogeni.

Inoltre, l'utilizzo dei LAB è strettamente correlato anche alla capacità di incrementare le caratteristiche nutrizionali e aromatiche del prodotto, rendendolo più gradevole e digeribile [6].

In aggiunta, i LAB possono esercitare effetti benefici sull'uomo, migliorando l'assunzione dei nutrienti, arricchendo la flora intestinale, alleviando l'intolleranza al lattosio e modulando il sistema immunitario dell'ospite al fine di prevenire eventuali infezioni enteriche.

Infatti, rivestono il ruolo di probiotici in grado di influenzare direttamente la fisiologia dell'organismo ospite, quando vengono ingeriti in adeguate quantità [7].

Alcuni ceppi batterici vengono definiti anche “fabbriche cellulari” perché in grado di produrre delle sostanze benefiche per l’uomo che prendono il nome di prebiotici [8].

Tra i probiotici maggiormente utilizzati in commercio, il genere *Bacillus* è quello più rappresentativo con le diverse specie benefiche per la salute dell’uomo.

In particolare, la specie *Bacillus coagulans* è caratterizzata da batteri sporigeni a forma di bastoncello Gram positivo, presente naturalmente nel microbiota umano.

È rilevante la produzione di endospore da parte del batterio, in condizioni di stringenza legate a livelli ridotti di nutrienti nell’ambiente. Difatti, quest’ultime sono metabolicamente dormienti e resistenti alle radiazioni, alle sostanze chimiche (pH acido dello stomaco, bile) e al calore (umido e secco). Una volta raggiunto l’intestino umano, le spore possono germinare, e colonizzare il tratto intestinale proteggendolo da eventuali infezioni da patogeni.

Tale probiotico viene spesso utilizzato per il trattamento di disturbi gastrointestinali come diarrea acuta, intolleranza al lattosio, patologia epatica e ipercolesterolemia [9].

Bacillus coagulans MA-13

In questo progetto di dottorato, un ceppo batterico di *B. coagulans* denominato MA-13 è stato utilizzato per studiare la produzione di macromolecole biologiche aventi proprietà prebiotiche.

Il batterio *B. coagulans* MA-13 è stato isolato dagli scarti di lavorazione industriale dei fagioli. È un batterio termofilo ed acidofilo con un optimum di crescita di 55°C e pH 5.5, in condizioni di anaerobiosi facoltativa [10].

Noto come organismo modello per la produzione di acido lattico a partire da zuccheri esosi derivanti da biomassa lignocellulosica, è stata osservata la sua capacità di crescere su melassa, una ricca fonte di carbonio utilizzata nell'alimentazione animale.

Inoltre, il batterio presenta una buona tolleranza a sostanze nocive per la vitalità cellulare come furfurali, idrossimetilfurfurali e acido acetico che solitamente vengono rilasciati nell'idrolizzato durante i processi di pretrattamento delle biomasse.

Quando il batterio cresce in presenza di elevate concentrazioni di sostanze tossiche (circa il 95% di idrolizzato), si prolunga nel tempo il processo fermentativo senza però alterare la resa di produzione di acido lattico [11].

Al fine di migliorare la resa dell'acido lattico prodotto, la crescita cellulare condotta in anaerobiosi in condizioni ottimali di pH e di temperatura è stata implementata una fase di adattamento che prevede la crescita cellulare in presenza di diverse concentrazioni di idrolizzato (0%-30%-40%-50%) fino alla fase stazionaria per poi

raccogliere le cellule e far avvenire la fermentazione lattica in un un bioreattore.

Tutte le condizioni testate presentavano la stessa resa di acido lattico prodotto, ma nel caso delle cellule cresciute in presenza del 30% di idrolizzato avveniva una riduzione dei tempi del processo fermentativo che durava 12 ore (invece di 30 ore) e aumentava la produttività massima dell'acido lattico (2.1 g/L/h).

Il sequenziamento del genoma [12] e la successiva annotazione genica effettuata con i server RAST (https://rast.nmpdr.org/) e dbCAN (http://bcb.unl.edu/dbCAN2/), ha evidenziato una pletora di geni coinvolti nella produzione di glicosil idrolasi.

Le glicosil idrolasi (GH) sono una famiglia di enzimi che catalizzano l'idrolisi di un legame glicosidico tra due o più glicosidi al fine di liberare unità monosaccaridiche da zuccheri complessi.

Analisi degli estratti intra- ed extracellulari del batterio *B. coagulans* MA-13

Al fine di ricercare nuove glicosil idrolasi prodotte da MA-13, sono stati analizzati l'estratto intra- ed extracellulare.

In particolare, analisi spettrofotometriche preliminari sono state effettuate su entrambi gli estratti di cellule raccolte in fase esponenziale dopo crescita su un terreno ricco, sfruttando substrati artificiali caratterizzati dalla presenza di *para*- oppure *orto*-nitrofenolo nella loro struttura.

Questo particolare composto arilico sviluppa un colore giallo quando viene rilasciato in soluzione a seguito dell'idrolisi del legame glicosidico.

Dall'analisi condotta sull'estratto intracellulare, si evince che il batterio produce glicosil idrolasi in grado di esplicare la loro funzione idrolitica su composti quali PNP-α-gal, ONP-β-gal, PNP-β-gal, PNP-α-glu e PNP-α-ara.

Il dato sperimentale ottenuto è stato poi correlato all'analisi bioinformatica condotta sul *panel* di glicosil idrolasi del batterio.

È stato identificato un possibile coinvolgimento della GH13 sulla digestione del substrato PNP-α-glu, della GH42 sui substrati PNP-β-gal e ONP-β-gal ed infine della GH36 sul substrato PNP-α-gal.

L'analisi dell'estratto extracellulare ha invece evidenziato la produzione di un solo enzima appartenente alla famiglia GH36, probabilmente coinvolto nel segnale derivato dall'idrolisi del substrato PNP-α-gal.

Per identificare gli enzimi responsabili dell'idrolisi dei legami β-galattosidici, gli estratti cellulari sono stati dapprima analizzati per zimografia e poi analizzati tramite spettrometria di massa LC-ESI-MS/MS.

Una putativa GH42 è stata identificata come unico potenziale candidato della digestione dei substrati PNP-β-gal e ONP-β-gal. Inoltre, un incremento della produzione di β-galattosidasi (circa 30 volte) è stato osservato quando il batterio era cresciuto in un terreno minimo (0,1% di estratto di lievito) arricchito con 0,1% di lattosio.

Le stesse analisi sono state effettuate per ricercare anche enzimi coinvolti nella idrolisi dei legami α-galattosidici.

In particolare, una putativa GH36 è stata individuata in entrambi gli estratti cellulari come responsabile dell'attività idrolitica identificata tramite il substrato PNP-α-gal.
Per confermare la presenza di tale enzima nel surnatante, il batterio è stato cresciuto in terreno minimo (0,1% di estratto di lievito) arricchito con 1% di locust bean gum, (farina di semi di carrube) un galattomannano vegetale.
La presenza di galattomannano nel terreno di crescita influenzava la secrezione dell'enzima α-galattosidasi all'esterno della cellula batterica.

La caratterizzazione biochimica e l'analisi dei prodotti di transgalattosilazione della β-galattosidasi *Bc*GalB

Il gene E2E33_010705 codificante per una putativa β-galattosidasi GH42 (denominata *Bc*GalB) è stato amplificato tramite PCR e successivamente inserito nel vettore pET28b ed espresso in cellule di *Escherichia coli* ceppo (DE3) Rosetta. L'over-espressione della proteina e la conseguente purificazione tramite cromatografia d'affinità, sfruttando il tag di istidine presente all'estremità
C-terminale, hanno permesso di ottenere circa 10 milligrammi totali di enzima da un 1 litro di coltura batterica, con una resa finale dell'82%.
L'enzima purificato all'omogeneità è stato visualizzato poi su gel di poliacrilammide tramite SDS-PAGE, mostrando una singola banda in corrispondenza del peso molecolare di 75 kDa.

Il peso molecolare è stato anche confermato mediante analisi per spettrometria di massa.

Inoltre, un'attenta analisi tramite cromatografia per esclusione molecolare associata al light scattering QELS ha rivelato una struttura ad esamero della proteina presente in soluzione.

Dall'analisi bioinformatica condotta sulla sequenza amminoacidica della proteina ricombinante, è stata determinata la presenza di sette cisteine.

La proteina dunque, è stata risospesa in soluzione in presenza dell'agente riducente β-mercaptoetanolo. Quest'ultima è stata analizzata poi per SDS-PAGE da cui è risultato che la proteina era presente solo in forma monomerica, sottolineando così l'importante ruolo rivestito dalle cisteine nell'organizzazione dello stato oligomerico della proteina stessa.

In seguito, sono stati condotti studi sull'influenza della temperatura e del pH sull'attività enzimatica, utilizzando l'ONP-β-gal come substrato.

*Bc*GalB esplica la sua massima attività idrolitica quando viene saggiata a 60°C in tampone sodio citrato 100 mM, pH 5.0.

In aggiunta a tali informazioni raccolte sull'enzima, è stata valutata anche la sua stabilità a diversi intervalli di temperatura (4°C – 60°C) e di pH (4.0 – 8.0). In particolare, *Bc*GalB mantiene circa il 70% della sua attività catalitica in un intervallo di pH compreso tra 5.0 e 7.0, mentre si osserva un decremento a pH 4.0.

In concomitanza a ciò, alla sua temperatura ottimale (60°C) l'enzima presenta un'emivita di 4 ore associata ad un'ottima stabilità a 50°C mantenendo più del 60% della sua attività catalitica dopo 24 ore.

Dal punto di vista di possibili applicazioni industriali, *Bc*GalB rappresenta un buon candidato per il trattamento del siero del latte, essendo coinvolto nella digestione del al fine di produrre cibi e bevande privi di lattosio.

In letteratura, è noto che l'attività enzimatica delle β-galattosidasi è fortemente influenzata dalla presenza di ioni metallici in soluzione.

A tal proposito, l'attività enzimatica di *Bc*GalB è stata saggiata in presenza di differenti ioni metallici (Mg^{2+}, Ca^{2+}, Zn^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, K^{+}, Li^{+}, Cu^{2+}, Fe^{3+}, Na^{+}) alla concentrazione finale di 2 mM, dopo aver dializzato l'enzima con 10 mM di EDTA. Dai saggi enzimatici, è stato possibile determinare un decremento dell'attività di *Bc*GalB in presenza degli ioni Cu^{2+} e Fe^{3+}, che comportano cambiamenti nella struttura proteica, destabilizzandola. Gli altri ioni saggiati invece non influenzano l'attività catalitica dell'enzima.

Successivamente, è stato analizzato l'effetto inibitorio derivato dai prodotti di idrolisi del lattosio. Elevate concentrazioni di glucosio e di galattosio (circa 100 mM) possono influenzare negativamente l'attività catalitica di *Bc*GalB. Questo effetto inibitorio risulta essere ancora più marcato quando entrambi gli zuccheri sono presenti ad alte concentrazioni in soluzione (effetto non additivo).

In aggiunta a questi zuccheri, è stata testata l'attività enzimatica anche in presenza di elevate concentrazioni di xilosio ed arabinosio, osservando che *Bc*GalB mantiene circa il 66% della sua attività idrolitica.

La caratterizzazione biochimica di *Bc*GalB ha previsto anche studi condotti sugli effetti di surfattanti (SDS e Tween 20), agenti riducenti (DTT e β-mercaptoetanolo) ed agenti caotropici (urea e cloruro di guanidinio) sulla attività e stabilità proteica.

L’enzima mostra un significativo decremento della sua attività idrolitica in presenza di SDS, mentre mantiene circa il 65% della sua attività relativa in presenza di tutti gli altri agenti chimici.

Al fine di determinare la specificità di substrato di *Bc*GalB, è stata testata l’attività enzimatica su differenti substrati artificiali quali l’ONP-β-gal, il PNP-β-gal, il PNP-α-ara, il PNP-β-glu, il PNP-β-xyl ed il substrato naturale D-lattosio.

*Bc*GalB mostrava un’elevata affinità per il substrato ONP-β-gal (circa 4300 U/mg) rispetto ai substrati PNP-β-gal e PNP-α-ara (circa 796 U/mg e 328 U/mg rispettivamente). Nessuna attività è stata registrata sui substrati PNP-β-glu e PNP-β-xyl.

Le proprietà catalitiche dell’enzima sono state analizzate anche in presenza di lattosio come substrato naturale, determinando un’attività specifica pari a 1283 U/mg. Quest’ultima risulta essere una delle attività più elevate sul lattosio, riportate in letteratura.

Infine, l’attività di transgalattosilazione dell’enzima è stata studiata con lo scopo di determinare la produzione nuovi galattoligosaccaridi (GOS).

Dapprima sono state condotte reazioni di *omo*-condensazione utilizzando come coppia donatore/accettore di zuccheri soltanto il substrato preferenziale dell’enzima, l’ONP-β-gal. I prodotti di transgalattosilazione sono stati osservati per TLC (Thin Layer Cromatography) e in seguito analizzati per spettrometria di massa ESI-MS. Dagli spettri di tali reazioni è stato possibile determinare la formazione di di-, tri- e tetrasaccaridi composti da unità di galattosio.

Successivamente, è stata testata la capacità di *Bc*GalB di effettuare anche reazione di *etero*-condensazione utilizzando come donatore

di zuccheri il substrato ONP-β-gal e come accettore di zuccheri i substrati PNP-β-glu e PNP-β-xyl, rispettivamente.
Dalla coppia di substrati ONP-β-gal/PNP-β-glu è stata ottenuta la formazione di di- e trisaccaridi composti da un'unità di glucosio e una o due unità di galattosio, in funzione dello zucchero complesso considerato.
Anche nel caso della reazione di *etero*-condensazione in presenza del substrato PNP-β-xyl, si ottenevano di- e trisaccaridi composti da un'unità di xilosio e una o due unità di galattosio, in aggiunta ai prodotti di *omo*-condensazione citati in precedenza (tranne la formazione del tetrasaccaride).
Tali risultati hanno dimostrato che l'enzima è in grado di sfruttare diversi substrati come accettori di zuccheri, durante le reazioni di transgalattosilazione al fine di formare differenti GOS.
Infine, sono state condotte anche reazioni di transgalattosilazione utilizzando come donatore ed accettore di zuccheri il lattosio.
Anche in questo caso è stato possibile osservare la formazione di un nuovo GOS composto da un'unità di lattosio a cui è stato legato un'unità di galattosio, formando così un trisaccaride ex novo.

1. INTRODUCTION

1.1 Lactic acid bacteria and industrial applications

Lactic acid bacteria (LAB) are a wide group of microorganisms used as biocatalysts for the manufacturing of dairy products during the fermentation process. The majority of LAB comprise non-motile, microaerophilic Gram-positive bacteria (*Lactobacillus, Pediococcus, Leuconostoc,* and *Weissella*) belonging to the phylum *Firmicutes* and recognized as GRAS (Generally Regarded as Safe) for human consumption. Anaerobic *Bifidobacterium* genus is also considered as LAB but is not strictly related to other bacteria, since it belongs to the *Actinobacteria* phylum.

Nevertheless, *Bifidobacterium* and *Lactobacillus* are reported as the most representative probiotics, leading the production of functional foods in the dairy industry [1]. These microbes commonly thrive in the human and animal intestinal tracts, preventing pathogen infections and improving the digestibility of foods.

However, LAB can also colonize different natural habitats due to their good tolerance to environmental stresses and their capability to metabolize various carbon sources [2].

Genomic analysis of LAB, indeed, have shown their evolutionary advantage of adaption to different environmental niches through gain or loss of metabolic functions by gene transfer [3].

All these bacteria can produce lactic acid as final product from the fermentation of sugars and are recognized as homo-fermenters [4]. Instead, LAB employed for the production of ethanol, acetate, formic acid, and succinate are classified as hetero- or mixed acid-fermenters.

Moreover, LAB play a relevant role in the production of exopolysaccharides, aromatic compounds, sweeteners, [6] and bacteriocins [5].

From an industrial point of view, LAB are considered attractive tools for the development of molecules increasing flavor, aroma, health effects, and shelf life of fermented foods.

Indeed, these bacteria are often used as starter cultures in food industries, in order to reduce the risk of microbial contaminations and thus improve the end-product value [13]. The selective addition of LAB to raw materials can lead to the output of fermented products, increasing their organoleptic qualities and shortening the fermentation time.

Another biotechnological application of LAB is related to their use as delivery vehicles for therapeutic drugs and oral vaccines, exploiting their probiotic properties [7].

In the last decades, these microbes have attracted even greater attention as microbial cell factories, employed in the bio-based economy. In this regard, large consumption of fossil fuels has resulted in high amount of pollution, thus moving the white biotechnology research towards waste bioconversion [14].

It is noteworthy that, LAB can be considered as reservoirs of degradative enzymes that are involved in the decomposition of renewable feedstocks. Indeed, several families of glycoside hydrolases have been found in their genomes and have been reported in the Carbohydrate Active enZyme (CAzy) database (http://www.cazy.org/) [15].

Subsequently, the pentoses and hexoses obtained by the enzymatic digestion can be fermented into value-added products by LAB.

The wide availability of these “green” products can overcome the bad environmental impact of fossil-based products, ensuring the transition towards the bio-based economy.
Development of LAB as bio-factories and probiotics has determined an increasing knowledge about their genomics, metabolism, and physiology [8].
In this context, new LAB belonging to the non-pathogenic *Bacillus* species have been identified as probiotics. An attractive feature of these bacteria is the resistance to extremely harsh environments thanks to their ability to form spores and to grow under a relatively wide range of temperatures, usually up to ~ 60°C [16,17].
This novel group of LAB turns to be a very attractive tool for various industrial applications ranging from dairy to pharmaceutical fields, thanks to their biochemical properties.
Indeed, *Bacillus* species can improve the digestive health of humans through their probiotics effects as well as might increase the production of new functional foods in high-temperature processing.

1.2 *Bacillus coagulans* strain MA-13

The spore-forming *Bacillus coagulans* is a thermotolerant and acidophilic bacterium, considered as safe by the US Food and Drug Administration (FDA) and the European Union Food Safety Authority (EFSA) [18].
Hence, several strains of *B. coagulans* are employed in the microbiota modulation and immune responses of humans, thanks to their beneficial effects as pharmaceutical agents [19].

Their probiotic effects are also suited for the production of safe and functional foods [20].

From an industrial point of view, these microorganisms turned to be a source of thermostable enzymes, bacteriocins, and fermented products such as bioethanol and lactic acid.

In this context, a novel strain of *B. coagulans,* named MA-13*,* has been recently isolated from the canned beans manufacturing residues.

In particular, MA-13 is a Gram-positive, facultative anaerobic, spore-forming, non-pathogenic, lactic acid-producing bacterium [10]. It can be classified as a thermophilic and acidophilic microorganism due to its good resistance at different pH and temperature values, showing its optimum growth at pH 5.5 and 55°C.

Furthermore, the genome sequencing of MA-13 has highlighted its ability to ferment lignocellulose-derived 6-carbon sugars to lactic acid molecules [12].

The capability of the bacterium to produce lactic acid has been also tested in presence of toxic compounds derived from the chemo-physical treatments of the lignocellulosic biomass [11].

In this regard, the microorganism has shown good tolerance to phenols, furfurals, acetic acid, and other hydrolysates at high concentrations in the cultivation medium. Under these conditions, the yield of lactic acid was preserved but the fermentation time was negatively affected by the high concentration of hydrolysates. The genomic analysis of MA-13 also revealed the presence of a wide repertoire of glycoside hydrolases.

These degradative enzymes are often involved in the decomposition of raw materials in simple sugars, thus leading to the uptake of nutrients.

Nevertheless, these classes of enzymes have gained more attention for the decomposition of natural feedstocks as an alternative to the use of harsh chemical-physical pretreatments [21].

In this context, the enzymatic synergism can represent a suitable tool for the decomposition of biomasses [22], alleviating the negative impact of the chemical processes on the environment.

These enzymes are also involved in food uptake, ameliorating the release of nutrients through the enzymatic digestion of complex foodstuffs. Their implementation in food and drink as supplements has been also recently investigated. Abundant availability and cost-effectiveness of these molecules have allowed obtaining new functional foods, aiding the maintenance of gut homeostasis and improving human health [23].

1.3 Glycoside Hydrolases

Glycoside Hydrolases (GHs named also glycosidases or carbohydrases with EC number 3.2.1.-) comprise a wide range of enzymes able to catalyze the hydrolysis of O-glycosidic bond between glycosides or between monosaccharides and aglyconic moieties.

From an evolutionary point of view, GH families are spread all over living organisms, except for some Archaea species and some unicellular eukaryotes [24]. It is noteworthy that, the set of genes

encoding for GH enzymes in each organism depends mainly on two factors: its taxonomic position and its ecological niche.

Moreover, horizontal transfer and duplication phenomena have also led to further diversity in the structure and catalytic functions of these proteins, especially for microorganisms.

Indeed, the modification of one amino might alter their substrate specificity among different strains of the same species.

Based upon the sequence similarity, GHs are gathered in over 168 families compiled into the CAzy database [25].

The IUB-MB (International Union of Biochemistry and Molecular Biology) nomenclature of GHs is mainly related to substrate stereospecificity and molecular mechanism.

The former allows to classify GHs in α- and β-hydrolases, depending on the stereospecificity of anomeric carbon inside the substrate. The latter has been originally proposed by Koshland et al. [26], through either double displacement (*retaining*) or single displacement (*inverting*) mechanisms (Fig. 1 and Fig. 2).

The *retaining* mechanism requires the presence of a nucleophile and an acid/base residue for the retention of the anomeric configuration of substrate with a double-displacement method during the hydrolysis reaction.

On the other hand, the *inverting* mechanism is characterized by two catalytic acid/base residues that provide the nucleophilic substitution at the anomeric center of the substrate, after enzymatic digestion.

However, these glycan processing enzymes can be also recognized as *exo*- and *endo*-hydrolases, according to their ability to cleave the linkages at the end or within the chain [27].

Moreover, the GH families are involved in the synthesis of glycosides, thus representing an attractive tool for glycan production at industrial scale. The products of transglycosylation reactions (or reverse hydrolysis processes) are oligosaccharides that are often employed as additives, prebiotics, and dietary carbohydrates in the food industry.

Low yields of these products, depending on the hydrolytic activity of GH enzymes, have led obtaining mutants of GHs by protein engineering. In this context, a new class of enzymes has been developed, named glycosynthases (GSs) able to synthesize glycosidic bonds without hydrolyzing them, thus improving the final yields of these low-cost products [28].

1.3.1 Catalytic mechanisms of glycoside hydrolases

The *retaining* mechanism requires the action of two catalytic residues named nucleophile and acid/base, respectively.

The first step (glycosylation step) involves the linkage between the nucleophile and the anomeric carbon of the substrate, forming a glycosyl-enzyme intermediate. The other residue plays the role of acid catalyst and protonates the glycosidic oxygen, thus promoting bond cleavage and departure of the leaving group [29].

During the second step (deglycosylation step), the glycosyl enzyme is hydrolyzed by a molecule of water.

In this regard, the residue acts as base deprotonating the water that subsequently attacks the glycosyl-enzyme intermediate (Fig. 1).

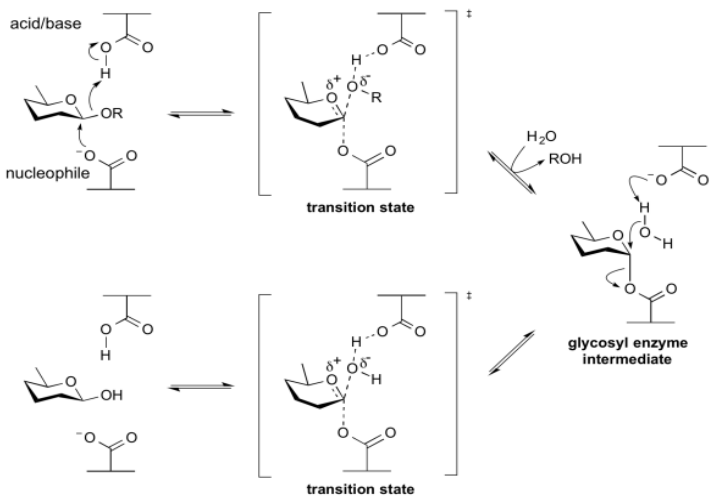


Fig. 1. *Retaining* mechanism of glycoside hydrolase from https://www.cazypedia.org/index.php/Glycoside_hydrolases.

This final step provides for the release of a product with the same anomeric configuration of the substrate.

Among the *retaining* GHs, a typical feature of these residues, which generally are carboxylic amino acids, is the distance of 5.5 Å apart [30].

On the other hand, the *inverting* mechanism is based on a single displacement step. Indeed, the hydrolysis of a glycoside and the inversion of the anomeric configuration of the final product are achieved by an oxocarbenium ion-like transition states. Two residues are required for this reaction, playing the role of general acid and general base, respectively.

These residues, which contain also carboxylic groups, are generally located 6-11 Å apart in the active site of the enzyme (Fig. 2).

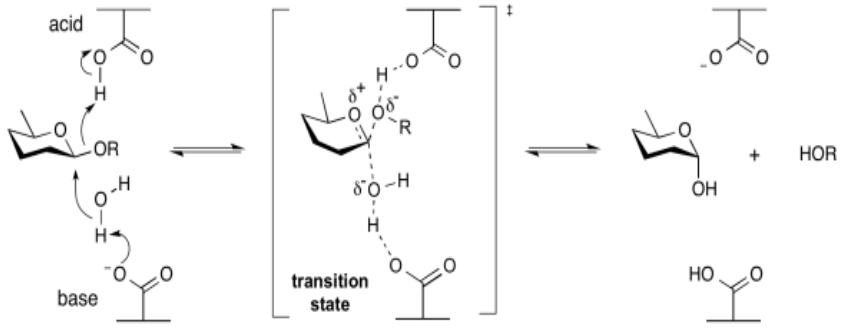


Fig. 2. *Inverting* mechanism of glycoside hydrolases from https://www.cazypedia.org/index.php/Glycoside_hydrolases.

1.3.2 Active site topology of glycoside hydrolases

The sequence-based classification of GHs is provided by the CAZy database in which the glycosidases are pooled into families based on the similarity of their aminoacidic sequences to biochemically characterized proteins.

It Is noteworthy that, the folding and the catalytic mechanism of enzymes are conserved among the members of the same families. In this regard, uncharacterized glycosidases can be associated with a specific family through a simple sequence-alignment, thus their structural features and catalytic mechanism can be inferred.

Although X-ray analysis has revealed that several tridimensional structures of GHs are highly conserved more than their sequences, these findings have also highlighted three different topologies of the active site of the enzymes [31].

These three topologies are related to the substrate specificity, regardless to whether of the enzyme can follow the *retaining* or *inverting* mechanism.

The first topology is called "*pocket or crater*" and it is employed for the release of sugar moieties at the non-reducing ends of saccharides. The e*xo*-glycosidases are all characterized by this specific active site that allows them to degrade complex substrates as the starch granules.

The second topology is named "*cleft or groove*" and it is a typical of *endo*-glycosidases that are involved in the digestion of large polymers. Indeed, this structure allows the enzymes to randomly bind the sugar units and cleave the linkages inside the chain.

Finally, the "*tunnel*" topology is very similar to the previous one but in this case, the cleft is covered by loops. This structure has been found in the cellobiohydrolases and leads to the cleavage of substrate with release of the final product of reaction, while the enzyme is still linked to the sugar chain, thus creating the conditions for processivity (Fig. 3).

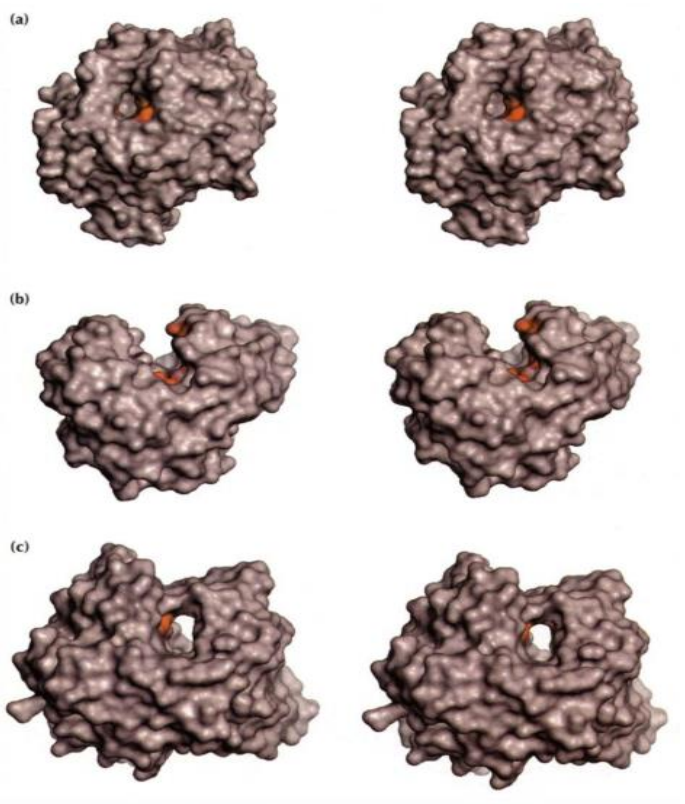


Fig. 3. Three kind of active site inside the glycoside hydrolases.
a. Topology *pocket* or *crater;* b. The structure *cleft* or gr*oove* of glycoside hydrolases; c. *Tunnel* disposition of the active site.
This figure is extracted from the paper [27] Davies, Gideon, and Bernard Henrissat. "Structures and mechanisms of glycosyl hydrolases." Structure 3.9 (1995): 853-859.

1.4 α-galactosidases

The α-galactosidases (EC: 3.2.1.22) are *exo*-glycosidases able to hydrolyze the non-reducing terminal ends of galacto-saccharides and galacto-lipids, releasing units of α-galactose. This class of enzymes was originally known as melibiase because of its ability to hydrolyze the α-1,6 linkage between galactose and glucose into

melibiose. Further analysis on its substrate specificity have displayed new hydrolytic properties on different kind of galactosides. Indeed, the α-galactosidases can be divided into two big groups: Group I and Group II [32].

Group I consists of enzymes that degrade specific substrates such as melibiose, raffinose, stachyose, and verbascose.

On the other hand, Group II is involved in the decomposition of galactomannans.

According to the CAZy classification, these activities are clustered into seven different GH families: GH4, GH27, GH31, GH36, GH57, GH97, and GH110. In this context, the enzymes of prokaryotic origin are distributed in all seven families. Structural analyses on members of these GH families have allowed to identify a typical $(\beta/\alpha)_8$ barrel for GH27, GH31, GH36 and GH97 families, a $(\beta/\alpha)_7$ barrel for GH57 family, a parallel β-helix structure for GH110 family and finally the GH4 family is characterized by a Rossmann fold structure. The GH4, GH27, GH31, GH36, GH57 and GH97 families follow a *retaining* mechanism, while the GH110 family exerts an *inverting* mechanism [33,34]. The N-terminus of the protein is often characterized by the presence of the active site, while a group of eight antiparallel β sheets is located at the C-terminus [35].

Furthermore, these enzymes have been purified by fungi, yeasts, and bacteria. The fungal and yeast α-galactosidases perform the hydrolysis of the substrate under acidic conditions [36,37]. These enzymes are often employed for the removal of galactose units from raffinose and stachyose from soybeans and other legumes in industrial processes [38].

The bacterial α-galactosidases exert their function under a wide range of pH, especially within neutral and alkaline values [39], having a good thermo-resistance at different temperatures [40–42]. These proteins are largely used for various biotechnological applications than the fungal and yeast counterparts due to their stability at different pH and temperature values and the large substrate specificities. Among these, thermostable enzymes are usually exploited for the decomposition of complex substrates in food and feed, sugar-beet manufacturing and paper-pulp industries, where high-temperature processes are required. Nevertheless, microbial α-galactosidases from psychrophilic microorganisms are also used for the manufacturing of aquatic foodstuffs thanks to their biochemical properties at low temperatures [43].

In the last decades, a deep knowledge of these enzymes through molecular techniques and structural modeling has open the way to enhance their performance with protein engineering.

Recombinant enzymes produced by efficient microbial cell factories systems have gained a huge industrial relevance, leading to large-scale production of them for different applications in food, feed and medical fields.

1.4.1 Food applications and bioprocesses of the α-galactosidases

In recent years, the microbial α-galactosidases have been considered a relevant biotechnological tool for various industrial areas, thus increasing their commercial use. The employment of these enzymes concerns the food-processing industry and the

saccharification of biomasses. Regarding to the food-processing, there are several foodstuffs as vegetables and cereals which are rich in complex sugars consisting mainly of raffinose and stachyose. These oligosaccharides are composed of units of galactose linked by α-1,6 glycosidic bonds to sucrose. Human uptake of these carbohydrates can induce gastro-intestinal diseases, due to the lack of a pancreatic α-galactosidase able to digest them. Indeed, these indigestible sugars are fermented into the colon and cause abdominal discomfort, flatulence, and diarrhea. Hence, they are recognized as anti-nutritive aliments [44].

To overcome this problem, it has been tested the use of microbial α-galactosidases in the active removal of stachyose and raffinose, thereby facilitating food digestibility. The enzymatic method is more effective in the maintenance of nutritional components like vitamins and minerals during the food-processing than the traditional one based on cooking or soaking foodstuffs. Generally, these enzymes are available as digestive supplements which can be taken orally [45].

It is noteworthy that, thermostable α-galactosidases are also employed for the treatment of beet sugar and soybeans at industrial scale [46]. These enzymes are required for exerting their hydrolytic activity at high temperatures since the manufacturing processes are performed up to 80°C. The ability of bacterial α-galactosidases to be active at physiological pH values of mammals and poultry intestines might allow their use as feed additives [47].

Another relevant application of α-galactosidases in the food field is related to the hydrolysis of galactomannans, such as locust bean and Guar.

This latter one is a polysaccharide composed of mannose and galactose residues joined together by α-1,6 glycosidic bonds. It is often used as a stabilizer for many foods (ice-creams, puddings, and cheese) without affecting their organoleptic properties. In this context, the α-galactosidases can depolymerize the substrate, working in synergy together with other degradative enzymes [48].
This combined action with other enzymes is also exploited for the pre-treatment of biomass [49].
For example, these enzymes can be employed in the deconstruction of complex biomasses such as the sugarcane bagasses, especially removing the galactosyl side groups present in their galactomannans thus improving the accessibility of mannan backbone to other degradative enzymes, such as α-mannanases and α-mannosidases. [50].
Finally, it has been also described the relevant role of these enzymes in the clarification of juice, enhancing the degradative activity of pectinolytic enzymes by cleaving galacturonan-based side chains [51].

1.5 Beta-galactosidases

The β-galactosidases (EC: 3.2.1.23) are involved in the digestion of β-galactosides, by the hydrolysis of the glycosidic bond between the terminal non-reducing β-galactose and the rest of the substrate [52]. Commonly known as lactase, the biological applications of this enzyme are mainly related to the hydrolysis of lactose in milk, thus releasing glucose and galactose units.

However, several β-galactosidases are unable to digest lactose and their biochemical characterization has been reported in literature [53].

The β-galactosidases are ubiquitous enzymes, naturally synthetized by different microorganisms including bacteria, archaea, yeasts, fungi, animals and plants [54].

According to the CAZy database, these proteins are collected into eight GH families: GH1, GH2, GH35, GH39, GH42, GH59, GH147, and GH165.

Their classification is based on sequence similarity, common structure domains, same catalytic mechanism and evolutionary connections.

It is worth noting that, all members of GH families mentioned above, are characterized by a $(\beta/\alpha)_8$ barrel-structure and hydrolyze the substrate following a *retaining* mechanism and the double-displacement steps are carried out by a couple of glutamic or aspartic residues, depending on the enzyme source.

Analyzing the number of sequences deposited in CAZy database, these β-galactosidases are mainly distributed among GH2, GH35, and GH42 families. The GH35 β-galactosidases have been isolated from fungi, plants and animals. On the other hand, the microbial β-galactosidases belong to GH2 and GH42 families. However, the microbial enzymes are more technologically relevant than the other ones thanks to their production at low costs with high yields and productivity.

Some GH42 β-galactosidases derived from thermophiles, have attracted industrial interests for their good thermostability in high-temperature processes.

Recent analyses about the structural conformation of these enzymes have been reported [55]. The crystal structure of these proteins has revealed the presence of generally three different domains: A, B, and C. Domain A is the catalytic one with a typical TIM barrel structure already observed also in the other GH families. This domain is involved in the substrate binding. Domain B showed a complex α/β structure and leads the protein stabilization. Domain C is characterized by an anti-parallel β-sandwich structure probably plays a role in the oligomerization state [56].

Concerning to their hydrolytic function, the β-galactosidases are the most studied commercialized proteins at industrial scale. The hydrolysis of lactose was massively exploited by dairy industries for generating lactose-free products [57]. The large consumption of these free-lactose food and drinks allows alleviating the gastro-intestinal disorders (nausea, abdominal discomfort, and diarrhea) due to lactose intolerance, which is a quite common condition.

Another relevant use of β-galactosidases is strictly related to the treatment of whey derived from milk and cheese-manufacturing processes. Whey degradation provides the simple sugars for the production of syrups used by bakery and confectionary industry.

Furthermore, the catalytic properties of these enzymes are also used in research fields. Indeed, β-galactosidases are used as reporter genes for many decades.

1.6 Galacto-oligosaccharides (GOS) synthesis

In the last decades, the ability to enzymatically synthetize new carbohydrates has been investigated. These enzymes can catalyze transglycosylation reactions in order to form new glycosidic bonds by transferring galactose units to sugar acceptors. The products of transglycosylation activity are new oligosaccharides, which can be used as prebiotics for the human gut. Carbohydrate synthesis has also been promoted by protein engineering, removing the hydrolytic activity through mutagenesis of nucleophiles and subsequently increasing the use of various non-natural substrates by the new class of enzymes named glycosynthases [58].

Among the oligosaccharides, the galacto-oligosaccharides (GOS) are non-digestible components of food, which are commercially produced from lactose by transglycosilation reactions, using β-galactosidases as biocatalysts [59]. In the last years, GOS have attracted the attention of dairy industries and cheese manufacturers, thanks to their prebiotic properties [60]. GOS can induce the same bifidogenic effects of human milk oligosaccharides, entailing growth benefits for the intestinal bacteria.

They can also stimulate the immune system and reduce the amount of cholesterol in the human blood, thus reducing the risk of cancer development.

Additionally, GOS can also increase the host defense, preventing the attack of pathogens. Moreover, they protect the human gastrointestinal tract, acting as decoy receptors for pathogens [61].

GOS production by β-galactosidases occurs in two different steps, following the *retaining* mechanism [62].

The first step implies the hydrolysis of a lactose molecule in order to form a galactosyl-enzyme complex with the subsequent release of glucose. In the second step, another molecule of lactose, acting as acceptor, interacts with the intermediate, thus obtaining a new oligosaccharide (Fig. 4).

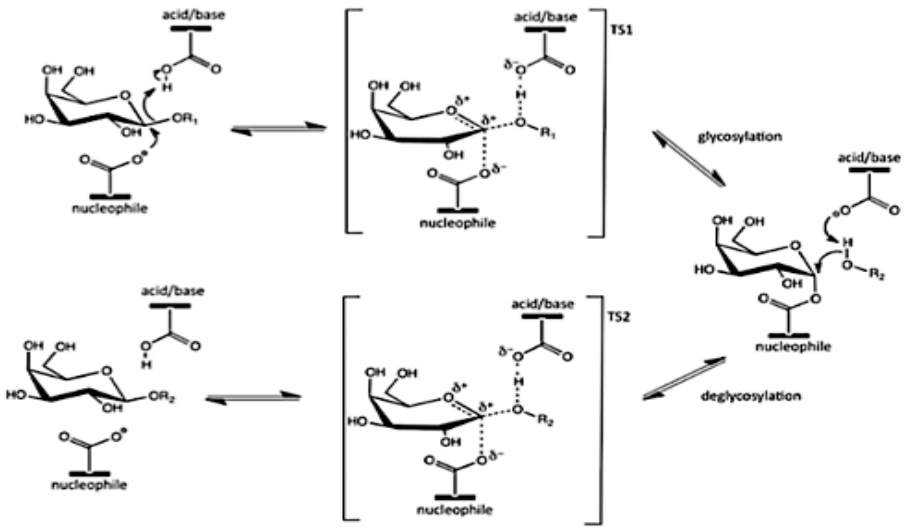


Fig. 4. *Retaining* mechanism of β-galactosidases in transgalactosylation reactions. This figure is extracted from the paper [62] Strazzulli, Andrea, et al. "Introducing transgalactosylation activity into a family 42 β-galactosidase." Glycobiology 27.5 (2017): 425-437.

It is worth noting that, the β-galactosidases can perform either hydrolysis or synthesis of carbohydrates depending on the nature of the acceptor (water or another mono-oligosaccharides respectively) [63] (Fig. 5).

Transglycosilation is a controlled kinetically reaction, where the GOS production is basically dependent upon the acceptor

concentration, temperature setting, and enzymatic stability [64]. If the acceptor is lactose, a high concentration of this substrate is required to achieve the maximum yield of GOS.

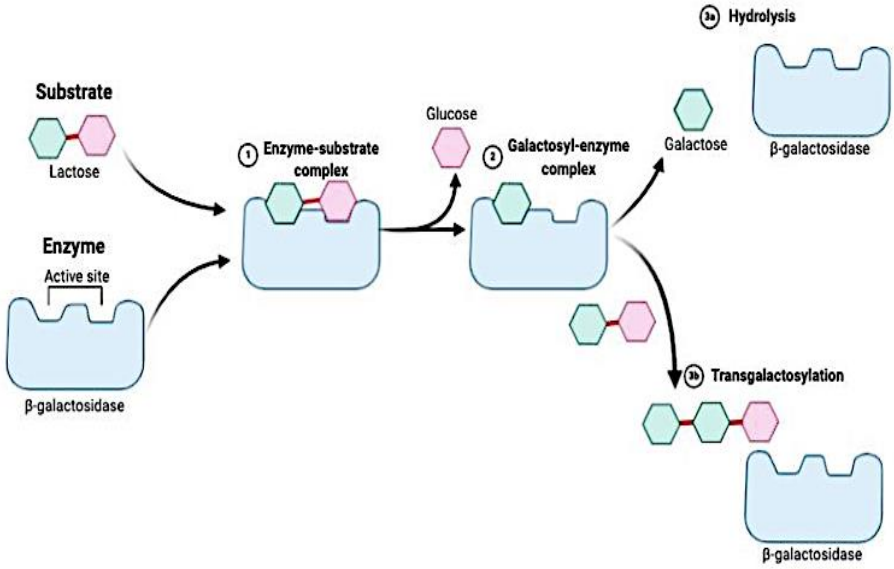


Fig. 5. Schematic representation of the GOS synthesis by transgalactosylation reaction.

According to the amount of lactose and the nature of the enzyme, the degree of polymerization and the saccharide composition of the GOS may differ.

In a lactose-saturated solution, the β-galactosidase catalyzes the synthesis of a trisaccharide as the first transglycosylated product. Then, the further addition of galactose units to the trisaccharide allows obtaining new GOS, depending on the number of transferred galactoses [65,66].

GOS production is often performed at high temperatures, in order to increase lactose solubility. The thermophilic β-galactosidases turned to be an attractive tool for the production of GOS and dairy goods at

industrial scale. The most commercially thermophilic β-galactosidases are obtained from the fungi *Aspergillus* spp., with an optimum of temperature at 50°C [67].

The advantages of using these thermozymes are also related to their tolerance to the optimal pH for lactose degradation (usually in a range of pH 2.5-5.4) [68]. Furthermore, the ability to synthetise GOS is maintained even when they are immobilized on specific supports during the transgalactosylation reactions [69].

Protein engineering has been effective in enhancing the efficiency to produce GOS and to modulate the glycosyl donor and acceptor specificity and reduce the product inhibition [70].

However, the main drawback of these microbial enzymes still remains their high costs of production at large-scale, even if they can be recovered and reused by immobilization [71]. Therefore, the characterization of new enzymes from probiotic sources with similar or enhanced biochemical properties, may provide an interesting low-cost alternative for the production of GOS.

Nowadays, the global GOS market is constantly growing because of the huge consumption of functional foods by consumers. The increasing production of GOS also depends on the employment of these oligosaccharides as food ingredients for infant nutrition. The main producers of GOS are the Japanese and European companies, which have launched many commercial products characterized by prebiotic properties [72]. Among these, Yakult Honsha (Japan), Nissin Sugar Manufacturing Company (Japan), Friesland Foods Domo (Netherlands) and Clasado Ltd. (United Kingdom) are the most representative industries involved in the GOS manufacturing processes [73]. All products supplemented with

GOS are often provided in form of powder and/or syrups. Development of new strategies for the incorporation of GOS in food and beverages are already in progress, in order to improve the organoleptic and prebiotic properties of these foodstuffs.

1.7 Aim of the work

One of the most challenging food consumption issues is how to ameliorate the digestibility of nutrients made up of complex sugars. Indeed, the intake of foods containing some not digestible galactosides is associated with their fermentation in the large intestine, thereby building up intestinal gas and discomfort.

In this context, α- and β-galactosidases are the enzymes able to catalyze the hydrolysis of α-1,6 and β-1,4 linkages in oligo- and polysaccharides containing D-galactopyranosides, respectively.

The aim of this thesis has been focused on the identification of new carbohydrate-active enzymes, exploiting the thermophilic bacterium *B. coagulans* MA-13 as microbial source.

The combination of mass spectrometry-based omics technologies and conventional biochemical approaches have allowed obtaining a GH enzymes spectrum, by analysing the intra- and extracellular extracts of the bacterium.

Among them, the hydrolytic properties of the α- and β-galactosidases of MA-13 have been investigated in order to improve the decomposition of oligosaccharides that are indigestible by the human gut and to produce prebiotics, such as galacto-oligosaccharides.

In particular, the expression of these proteins has been evaluated by growing MA-13 on a single and inexpensive carbon source.

The use of growth media containing renewable sources suitable to increase the enzyme expression could reduce the high costs related to large-scale of enzyme production by industries.

A fully biochemical characterization of the β-galactosidase from *B. coagulans* MA-13 was carried out.

Enzymatic activity has been assayed on different artificial and natural substrates.

Furthermore, the ability of the enzyme to synthetize prebiotics, such as galacto-oligosaccharides has been investigated, using lactose as a natural substrate.

Comprehensive studies on the transgalactosylation reactions have allowed analyzing the huge potential of the enzyme in the production of prebiotics, exploiting different sugar donors and acceptors.

2. MATERIALS AND METHODS

2.1 Cultivation conditions for detection of glycosyl hydrolases activity

Aliquots from *B. coagulans* MA-13 strain stored at −80°C with glycerol 30%, were grown under standard conditions i.e. in Luria-Bertani (LB) liquid medium at 55°C [10].

Cells were collected through centrifugation at 3,000 x g for 15 min and homogenized by sonication (Sonicator heat system Ultrasonic Inc.) for 10 min, alternating 30 s of pulse-on and 30 s of pulse-off. Clarification of cell extracts was obtained through centrifugation at 40,000 x g for 30 min at 4°C.

For analysis of the extracellular proteins, the supernatant was filtered under vacuum through 0.45 µm nylon membrane (Millipore). The filtrate (secretome) was concentrated 300-fold using an Amicon Ultrafiltration System (Millipore) with a 10 kDa cut-off nitrocellulose membrane (Millipore) at room temperature and a maximum pressure of 75 MPa.

Samples were stored at 4°C for further analysis. At least three independent biological replicates were carried out.

2.2 Functional annotation of *B. coagulans* MA-13 glycosyl hydrolases enzymes

Genome draft [12] was annotated by the Rapid Annotation Subsystem Technology (RAST) (https://rast.nmpdr.org/) e dbCAN metaserver (http://bcb.unl.edu/dbCAN2/) was used to generate a family classification from CAZy database [74].

The gene list was extracted by selecting the GH(s) reported in all the 3 databases used (HMMER, DIAMOND and Hotprep).

2.3 Activity screening of glycoside hydrolases

Cell extract and secretome of *Bacillus coagulans* MA-13 were screened for enzymatic activities over a panel of synthetic substrates: PNP-β-glu, ONP-β-gal, ONP-β-glu, PNP-α-glu, PNP-β-xyl, PNP-α-man, PNP-β-man, PNP-β-fuc, PNP-α-fuc, PNP-α-rha, PNP-β-gal, PNP-α-gal, PNP-α-ara.

Briefly, 0.8 µg of the intracellular and extracellular samples were added to the substrate (10 mM) in 100 mM citrate buffer pH 5.5 (final volume of 100 µl) and incubated in Synergy H4 Plate Reader at 55°C. Enzymatic activity was measured by detecting the release of nitrophenol at 405_{nm} every 10 minutes up to 15 hours.

All the activities were expressed in International Units (U), corresponding to the quantity of enzyme(s) able to release 1 µmole of PNP-OH (millimolar extinction coefficient, 18.5 mM^{-1} cm^{-1}) or ONP-OH per minute (millimolar extinction coefficient, 4.6 mM^{-1} cm^{-1}).

The experiments were carried out with at least three technical and biological replicates. The acceptable standard deviation was less than 20% of the mean.

Enzymatic activity on PNP-α-gal, ONP-β-gal, PNP-β-gal, PNP-α-ara, PNP-α-glu was investigated also through zymography in a 7% SDS PAGE as previously described [40].

After renaturation, the gel was incubated with 20 mM of each substrate at 55°C for a time ranging between 10 and 60 minutes, until a clear halo of hydrolysis was visible on the gel.
Activity bands were excised for identification of the enzyme(s) through mass spectrometry.

2.4 Selective growth conditions for expression of α- and β-galactosidases

B. coagulans MA-13 was grown under standard conditions up to exponential growth phase (0.5 OD_{600}/ml) and cells were collected through centrifugation at 3,000 x *g* for 15 minutes.
Pellets (25 OD_{600nm}) were washed with Milli-Q water before resuspension in 50 ml of selective media.
For extracellular detection of α-galactosidase, the medium contained 0.1% yeast extract (YE) and either 1% locust bean gum or diverse agri-food residues (such as rice hull).
Cells were collected along with the supernatant after 24 hours. Cell extracts were prepared by resuspending pellets in lysis buffer, i.e. B-PER solution (Thermo Fisher Scientific) plus lysozyme (1 mg/ml) and then incubated at 37°C for 1 hour.
The lysed cells were clarified through centrifugation at 40,000 x *g* for 20 minutes at 4°C. Intra- and extracellular α-galactosidase activities were analysed by testing 0.25 µg and 20 µl of supernatant on 150 µl PNP-α-gal substrate (10 mM), respectively.
The assays were incubated for 10 minutes under standard pH and T conditions (100 mM sodium citrate 5.5 and 55°C) and the reaction was stopped by adding 150 µl 0.5 M Na_2CO_3 before detection at

405_{nm}. As control, the supernatant was tested for β-galactosidase activity. The supernatants were analysed also through zymography on PNP-α-gal, as described above [40].

For over-expression of the native β-galactosidase, pellets (5.0 OD_{600nm}) of *B. coagulans* MA-13 were resuspended in two different media, either 0.1 % YE or 0.1% YE with 0.1% lactose.

Cells were harvested once they reached the early stationary phase (0.8 OD_{600nm}/ml) and pellets were resuspended in B-PER solution as described before.

For each sample, 5 µg of intracellular proteins were tested using 10 mM ONP-β-gal following the same procedure as described above.

2.5 Protein identification by LC-MS/MS analysis

Protein bands from SDS-PAGE corresponding to those positive in zymographic assays were excised and *in situ* digested with trypsin in 50 mM NH_4HCO_3, following treatment with 10 mM DTT (Sigma-Aldrich), for 45 min at 56°C and then with 55 mM iodoacetamide (Sigma-Aldrich) in the dark at room temperature for 30 min for cysteines reduction and alkylation, respectively.

Gel bands were then incubated with 10 ng/µl trypsin overnight at 37°C. Peptide mixtures were extracted from the gel, and then acidified by 20% trifluoroacetic acid (Sigma-Aldrich), and dried by a Speed-Vac system (Thermo Fisher Scientific, USA).

Peptide mixtures were resuspended in 0.2% Formic Acid and analysed by nano-LC-MS/MS on a 6530 Q-TOF LC/MS equipped with a CHIP-CUBE system and coupled with a capillary 1100 HPLC system (Agilent Technologies, Santa Clara, California, USA).

Each peptide sample was then fractionated with a gradient of eluent B (0,2% formic acid, 95% acetonitrile LC-MS Grade) from 5% to 75% for 100 min and eluent A (0,2% formic acid, 2% acetonitrile LC-MS Grade). Data Dependent Acquisition method was set as follows: MS scan range was from 300 to 2400 m/z; MS/MS scans from 100 to 2000 m/z were acquired for the five most abundant +2 or +3 charged precursor ions (top 5) in each MS scan, applying a dynamic exclusion window of 30 s.

LC-MS/MS raw data were processed and then employed for protein identification by using licensed Mascot software (Matrix Science, Boston, USA) to search in a protein-encoding genes (PEGs) database containing *B. coagulans* MA-13 predicted protein sequences. The main parameters employed for identifications were: mass tolerance value of 10 ppm for precursor ions and 0.6 Da for MS/MS fragments; trypsin as the proteolytic enzyme; missed cleavages maximum value of 1; Cys carbamidomethylation as fixed modifications; pyroglutamate (peptide N-terminal Gln) and Met oxidation as variable modifications. Candidates with at least 2 assigned peptides with an individual MASCOT score > 10 were considered significant for identification [75]. The identified proteins were compared to sequences present in a complete annotated database (UniProt) by using BLAST Search Form. Best alignments showing the minimum value for E-values were considered.

2.6 Cloning and sequencing of the β-galactosidase gene

A single colony of *B. coagulans* MA-13 was inoculated into LB liquid medium and genomic DNA was isolated using the LETS (lithium, EDTA, Tris, and SDS) buffer method [12,76].

The gene (locus tag: E2E33_010705), encoding for a putative β-galactosidase, was amplified by polymerase chain reaction (PCR) using the primers 5'GAGGAATGCGTGCCATGGTAAAAAAACAT3' (*Nco*I restriction site is underlined), 5'ATCCGGGCGCCTCGAGTTTTTCAATTAC3' (*Xho*I restriction site is underlined) and Taq DNA Polymerase (Thermo Fisher Scientific). The amplification was performed with an initial denaturation at 95°C for 3 min, followed by 25 cycles (95°C for 30 s, 58°C for 45 s and 72°C for 75 s) and a final extension step at 72°C for 10 min.

The PCR products were checked by agarose gel electrophoresis and subsequently purified with QIAquick PCR purification kit (Qiagen Spa, Milan, Italy).

Afterwards, the purified product was cloned in pCR4-TOPO-vector (TOPO TA CLONING Kit, Invitrogen) and its identity was confirmed by DNA sequencing (Eurofins Genomics).

The insert then was subcloned in pET28b (+) vector (Novagen) using *NcoI* and *XhoI* restriction enzymes and T4 DNA ligase (Promega).

2.7 Expression and purification of recombinant *Bc*GalB

The vector pET28b/*Bc*GalB containing the β-galactosidase gene was used for transforming *E. coli* Rosetta™(DE3) pLysS cells in order to express the recombinant protein bearing a C-terminus His-tag.

The transformants were selected on LB agar plates containing 50 µg/ml kanamycin and 33 µg/ml chloramphenicol.

A single colony was inoculated in 50 ml LB medium with antibiotics and incubated on an orbital shaker (180 rpm at 37°C).

Cells were diluted in 1 liter of LB at 0.06-0.08 OD_{600nm} and once the culture reached 0.5-0.6 OD_{600nm} protein expression was induced overnight by adding 0.5 mM of IPTG.

Cells were harvested by centrifugation at 4,000 x *g* and resuspended in 100 mM sodium-phosphate pH 8.0 supplemented with a protease inhibitor cocktail tablet (Roche).

Subsequently, the cells were disrupted by sonication (Sonicator: Heat System Ultrasonic, Inc.) for 10 min, alternating 30 s of pulse-on, and 30 s of pulse-off and the suspension was clarified by a centrifugation step at 40,000 x *g* for 30 min at 4°C.

*Bc*GalB was purified to the homogeneity by affinity chromatography on HisTrap column (1 mL, GE Healthcare) connected to an AKTA Explorer system.

The column was equilibrated with 100 mM of sodium-phosphate pH 8.0 and 500 mM of sodium chloride buffer and elution was performed with a linear gradient of imidazole (0-250 mM).

All the peak fractions were pooled and then dialyzed against 100 mM of sodium-phosphate pH 8.0 and 50 mM of sodium chloride (storage buffer).
Protein concentration was estimated by Bradford assay using bovine serum albumin as standard.
The monomeric molecular mass of *Bc*GalB was evaluated by SDS-PAGE analysis (12%) and purity degree was evaluated by staining the gel with Coomassie brilliant blue R-250.

2.8 Molecular weight determination of *Bc*GalB

The native molecular weight of *Bc*GalB was obtained by gel-filtration chromatography connected to Mini DAWN Treos light-scattering system (Wyatt Technology) equipped with a QELS (quasi-elastic light scattering) module mass value and hydrodynamic radius (Rh) measurements.
One milligram of protein (1 mg/ml) was loaded on a S200 column (16/60 GE Healthcare) with a flow-rate of 0.5 ml/min and equilibrated in 100 mM of sodium-phosphate pH 8.0, 1 mM DTT. Data were analyzed using Astra 5.3.4.14 software (Wyatt Technology).

2.9 pH and temperature profiles of *Bc*GalB

The optimal pH value was determined by assaying 10 ng ($\simeq$ 0.04 Hydrolytic Units, U) of *Bc*GalB at 60°C using ONP-β-gal as substrate in a pH range from 4.0 to 10.0.
The following buffers (each 100 mM): sodium citrate (4.0-6.0), sodium phosphate (6.0-8.0), and glycine-NaOH (8.6-10.0) were

used to prepare the different substrate mixtures containing 10 mM ONP-β-gal. The temperature dependence of *Bc*GalB activity was studied by assaying the enzyme from 30 to 90°C in 0.1 M sodium phosphate pH 6.0 on ONP-β-gal.

Once determined the pH and temperature dependence of the enzyme, all the subsequent assays were performed using a reaction mixture containing 10 mM ONP-β-gal, 100 mM sodium citrate buffer pH 5.0 and ≃ 0.04 U of *Bc*GalB.

Briefly, the substrate mix was incubated at 60°C for 3 minutes, before adding the enzyme. The reaction was stopped after 3 min of incubation, by the addition of cold sodium carbonate 1.0 M.

The concentration of the released ortho-nitrophenol (millimolar extinction coefficient, 4.6 mM^{-1} cm^{-1}) was evaluated by measuring the absorbance of the mixture at 405 nm.

The pH stability and thermal inactivation were analyzed by incubating the enzyme in sodium citrate (4.0-6.0), sodium phosphate (6.0-8.0) and at 45, 50, 55 and 60°C, respectively. Aliquots of *Bc*GalB were withdrawn at regular time intervals to measure the residual activity under standard conditions.

2.10 Effect of metal ions, chemicals, and monosaccharides on enzyme activity

To test the effect of metal ions on enzymatic activity, *Bc*GalB was dialysed in storage buffer supplemented with 10 mM EDTA for 2 hours to get rid of metal ions present in the protein preparation. Afterwards, EDTA was removed through extensive dialysis in storage buffer. *Bc*GalB was incubated with metal ions for 5 min at

room temperature (Mg^{2+}, Ca^{2+}, Zn^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, K^{+}, Li^{+}, Cu^{2+}, Fe^{3+}, Na^{+}) at 2 mM concentration and the enzymatic activity was measured under standard conditions with the addition of 2 mM of each metal ion in the mix assay.

In relation to the activity of *Bc*GalB on lactose, the effect of Ca^{2+} was also evaluated in a reaction mixture containing 2 mM of Ca^{2+}, 150 mM lactose and ≃0.04 U.

Furthermore, the inhibition effect of chemicals on *Bc*GalB activity was also tested. Non-ionic (Tween-20), ionic (SDS) detergents, reducing (DTT, β-mercaptoethanol), chelating (EDTA) and chaotropic (urea, guanidinium chloride) agents were added to the enzyme solution at 50 mM concentration for 5 min and residual activity was assayed under standard conditions.

Finally, the influence of monosaccharides on the enzymatic activity was studied too. IC_{50} (half maximal inhibitory concentration) was calculated by incubating the enzyme in the presence of D-xylose or D-arabinose or D-glucose or D-galactose or a mix of these two latter at different concentration values (0-100 mM), for 5 min at room temperature and assaying the enzymatic activity in the presence of the monosaccharides.

2.11 Substrate specificity and kinetic parameters of *Bc*GalB

The hydrolytic activity of *Bc*GalB was tested on several substrates: PNP-β-glu, ONP-β-glu, PNP-α-glu, PNP-β-xyl, PNP-α-man, PNP-β-man, PNP-β-fuc, PNP-α-fuc, PNP-α-rha, ONP-β-gal, PNP-β-gal, PNP-α-gal, PNP-α-ara and D-lactose.

The enzyme was incubated in presence of 10 mM of each substrate under standard assay conditions. When lactose was used, the amount of free-glucose released upon hydrolysis was determined using D-Glucose Assay Kit (GOPOD Format, Megazyme) according to the manufacturer's protocol.

One unit (U) is defined as the amount of enzyme required to release 1 µmol of glucose per min. In order to study the kinetic parameters of the enzyme, different concentration values of ONP-β-gal (0.1 to 20 mM) and lactose (0-500 mM) were tested.

The Michaelis-Menten constant (K_M) and V_{max} were calculated by non-linear regression analysis using GraphPad 9.0 Prism software.

2.12 Analysis of the transgalactosylation activity of *Bc*GalB by Thin-Layer Chromatography

The transgalactosylation experiments were performed to study homo- and hetero-condensation reactions under standard conditions (100 mM sodium citrate pH 5.0 and 60°C).

The final volume of all the reactions was 800 µl and contained 2.2 U (0.5 µg) of the enzyme mixed with 80 mM of ONP-β-gal or 35.0 U of *Bc*GalB (8 µg) with 160 mM lactose, respectively. For the hetero-condensation reactions, 40 mM ONP-β-gal was employed as donor and 40 mM PNP-β-glu or PNP-β-xyl as acceptors, in a final volume of 800 µl containing 2.2 U.

These reaction conditions were established after preliminary tests (data not shown) in which different donor: acceptor ratios as well as of the enzyme amounts, were tested. Aliquots of reaction mixtures were collected at different time intervals (up to 18 hours), and the reactions were stopped by incubation in dry ice for 5 min.

Control reactions without enzyme were included in the analysis. The products were analyzed by TLC on silica gel 60 (F254, 0.25 mm) plates (Merck, Darmstadt, Germany) and separated using ethyl acetate/methanol/ddH_2O (70:20:10 v/v) as eluent, or butanol/ethanol/ddH_2O (50:30:20 v/v), for the detection of GOS from lactose. Aliquots corresponding to 0.2-2.0% of the total reaction mixture were loaded onto the TLC plate.

For the detection of sugars, the TLC plates were soaked in a staining solution consisting of 4% of 1-naphthol in 10% sulphuric acid in ethanol followed by heating at 120°C.

2.13 ESI-MS analysis of the galacto-oligosaccharides (GOS)

Transgalactosylation products were analyzed by direct ESI-MS procedure from reactions carried out for 18 hours: all samples were diluted in 5% acetic acid and analyzed on a Q-ToF Premier (Waters, Milford, MA, USA), in positive mode, by direct injection into the ESI source at a flow of 10 µL/min.

The source parameters were set as follows: capillary voltage = 3 kV and cone voltage = 42 kV. The acquisition range was set between 100 and 1000 m/z.

All data were processed by using Mass Lynx 4.1 software (Waters, Milford, MA, USA).

3. RESULTS AND DISCUSSION

3.1 Annotation of glycosyl hydrolases

Whilst a full functional annotation of *B. coagulans* MA-13 genome is under way (manuscript in preparation), herein, the annotation of the GH(s) repertoire using dbCAN2 meta server, is shown [74] (Table 1). Seventeen enzymes have been identified, among which some families (GH3, GH15, GH32, GH36, GH42, GH70, GH73) are represented by a single member, whereas all the others include diverse glycosyl hydrolases.

A set of GH(s) potentially involved in starch degradation which includes GH13 and GH65 representatives, mirrors the isolation source of *B. coagulans* MA-13, i.e. canned beans manufacturing which is particularly rich in starch [10,77].

Three GHs members belonging to families 18 and 73 are related to the sporulation pathway of *B. coagulans.* Few Carbohydrate-Binding Modules (CBMs) were found in association with GH13 and GH18 members. The presence of a sucrose-6-phosphate hydrolase (GH32) is in line with the capability of *B. coagulans* MA-13 to use molasses as an inexpensive sucrose-rich carbon source [11].

Finally, GH36 and GH42 members have been identified and interestingly lactic bacteria producing both α- and β-galactosidases are relevant for the food industry [78].

NCBI Reference sequence	GH family	Signal peptide	RAST annotation
WP_195850490.1	GH3	N	β-glycosyl hydrolase
WP_019720988.1	CBM34 + GH13_20	N	Neopullulanase (EC 3.2.1.135)
WP_133536160.1	GH13_31	N	Oligo-1,6-glucosidase (EC 3.2.1.10)
WP_195850265.1	GH13_31	N	Oligo-1,6-glucosidase (EC 3.2.1.10)
WP_133536961.1	GH13_5	N	Glucan 1,4-α-maltohexaosidase (EC 3.2.1.98)
WP_195850265.1	CMB48 + CH13_9	N	1,4-α-glucan (glycogen) branching enzyme (EC 2.4.1.18)
WP_061575462.1	GH0	N	Phosphorylase b kinase regulatory subunit β
WP_133537568.1	CBM50 + GH18	N	Spore cortex-lytic enzyme, N-acetylglucosaminidase SleL
WP_133536804.1	CBM50 + GH18	N	spore peptidoglycan hydrolase (N-acetylglucosaminidase) (EC 3.2.1.-)
WP_133537667.1	GH32	N	Sucrose-6-phosphate hydrolase (EC 3.2.1.26)
WP_133537615.1	GH36	N	α-galactosidase (EC 3.2.1.22)
WP_133536219.1	GH42	N	β-galactosidase (EC 3.2.1.23)
WP_133536548.1	GH65	N	α,α-trehalose phosphorylase (2.4.1.64)
WP_133536158.1	GH65	N	Maltose phosphorylase (EC 2.4.1.8)
WP_133536578.1	GH65	N	Maltose phosphorylase (EC 2.4.1.8)
WP_133537168.1	GH70	Y (1-34)	hypothetical protein
WP_195850162.1	GH73	N	endo-β-N-acetylglucosaminidase (EC 3.2.1.96)

Table 1. Predicted GH in the genome of *B. coagulans* MA-13.

3.2 Detection of the intracellular and extracellular GH activities

Intracellular cell extracts and secretome of *B. coagulans* MA-13 were tested on a panel of artificial substrates to detect GH enzymatic activities.

Cells were cultivated in LB rich medium to detect a baseline of activities under standard growing conditions. Upon collection of cells at exponential growth phase (0.5–0.6 OD_{600nm}), 0.8 µg of total intracellular and extracellular protein preparations were assayed over the following substrates: PNP-β-glu, ONP-β-glu, PNP-α-glu, PNP-β-xyl, PNP-α-man, PNP-β-man, PNP-β-fuc, PNP-α-fuc, PNP-α-rha, ONP-β-gal, PNP-β-gal, PNP-α-gal, PNP-α-ara.

Intracellular enzymatic activities were revealed only on a subset of substrates, i.e., ONP-β-gal, PNP-β-gal, PNP-α-gal, PNP-α-glu, PNP-α-ara (Fig. 6). By comparing these findings with the annotated list of *B. coagulans* MA-13 GHs, the hydrolytic activity towards PNP-α-glu was traced back to representative(s) of the GH13 family (sub-family 31).

However, the correlation with the activity on PNP-α-ara is not obvious (Table 1). The hydrolysis of β- (ONP-β-gal, PNP-β-gal) and α-galactosidic (PNP-α-gal) linkages might be linked to the GH42 and GH36 members, respectively (Fig. 6).

Indeed, *B. coagulans* MA-13 genome bears two genes, i.e., locus tag: E2E33_010705 (WP_133536219.1) and locus tag: E2E33_000265 (WP_133537615.1), encoding for a GH42 and for a GH36, respectively (Table 1). As shown in Fig. 6, the specific activity

recorded on ONP-β-gal and PNP-α-gal was significantly higher than on other substrates tested.

The presence of secreted GHs was verified by testing supernatants on the same substrates and the only relevant activity was detected on PNP-α-gal (Fig. 6). All together, these results indicate that enzymes hydrolyzing β- and α-galactosidic linkages represent the most relevant activities under standard growing conditions (LB medium). These enzymes catalyze the hydrolysis of terminally joined galactosidic residues in simple galactose-containing oligosaccharides as well as in complex polysaccharides and have the potential to improve the digestibility of some RFO-containing food and of milk-based products [69,79].

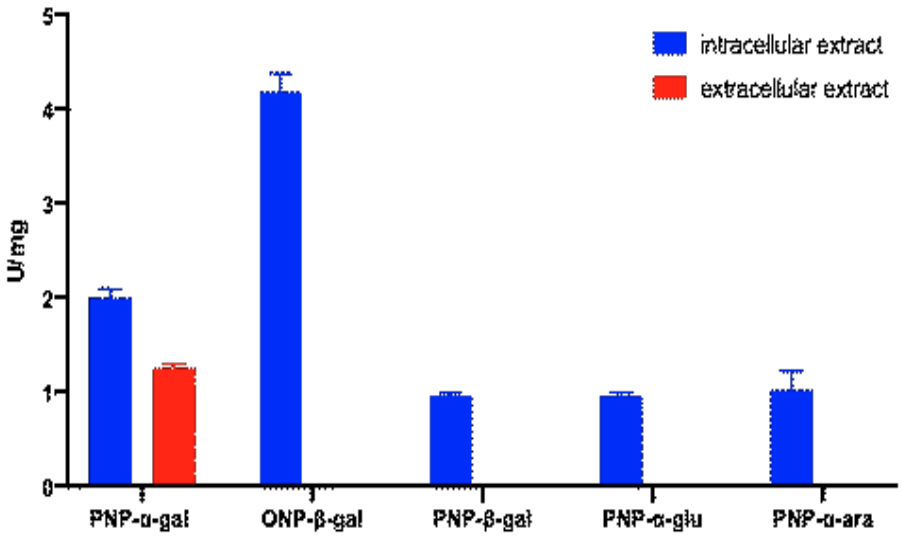


Fig. 6. Detection of enzymatic activities on different artificial substrates.

3.3 Identification of the hydrolytic activities through mass spectrometry

To identify the enzymes involved in the hydrolysis of β-galactosidic linkages, cell extracts were analysed through zymography.

Active-bands on ONP-β-gal and PNP-β-gal resided in the same upper gel region (not shown). These bands were excised, the proteins were in-gel trypsinized and the peptides were extracted and analysed by LC–ESI–MS/MS.

Proteins were identified by using MASCOT search engine to explore *B. coagulans* MA-13 protein database.

The sequences were compared to those present in a complete annotated database (UniProt) by using BLAST Search Form and the best alignments (minimum E-value) were obtained towards *B. coagulans* strain 36D1.

As expected, α-galactosidase (Uniprot code: G2TQE8) was identified both in intra- and extracellular protein extracts together with other unrelated co-migrating proteins.

The putative GH42 (Uniprot code: G2TH90) was recognized as the only enzyme potentially responsible for the hydrolytic activity on PNP-β-gal, since the other co-migrating proteins/enzymes clearly belonged to unrelated metabolic pathways (Table 2).

Reference Organism	UniProtKB Code	Reference Protein name	Gene	Score	Peptide number	Mass	E-value	Identity Percentage
Bacillus coagulans 36D1	G2TN44	Formate acetyltransferase	Bcoa_2164	928	29	85651	0.0	99,2
Bacillus coagulans 36D1	G2TNL9	Glutamine synthetase	Bcoa_3015	926	20	50860	0.0	98,8
Bacillus coagulans 36D1	G2TQJ0	Aldehyde-alcohol dehydrogenase	Bcoa_0619	861	29	96640	0.0	97,8
***Bacillus coagulans* 36D1**	**G2TQE8**	**Alpha-galactosidase**	**Bcoa_0573**	**667**	**23**	**83465**	**0.0**	**96**
Bacillus coagulans 36D1	G2TQZ8	Peptidase M30, hyicolysin	Bcoa_0856	499	15	61938	0.0	100
***Bacillus coagulans* 36D1**	**G2TH90**	**Beta-galactosidase**	**Bcoa_0772**	**370**	**13**	**76362**	**0.0**	**96**
Bacillus coagulans 36D1	G2TIA6	Elongation factor Tu	tuf	355	9	32604	0.0	98,2
Bacillus coagulans 36D1	G2TP75	Glyceraldehyde-3-phosphate dehydrogenase	Bcoa_2217	271	6	31823	0.0	99,3
Bacillus coagulans 36D1	G2TRD5	Iron-containing alcohol dehydrogenase	Bcoa_0993	223	5	39621	0.0	97,3
Bacillus coagulans 36D1	G2TPG6	Pyruvate kinase	Bcoa_2517	163	7	63000	0.0	99,6
Bacillus coagulans 36D1	G2TIB1	DNA-directed RNA polymerase subunit beta'	rpoC	159	8	134018	0.0	99,5
Bacillus coagulans 36D1	G2TIB2	DNA-directed RNA polymerase subunit beta	rpoB	154	8	127660	0.0	99,2
Bacillus coagulans 36D1	G2TIA7	Elongation factor G	fusA	135	6	75356		98,8
Bacillus coagulans 36D1	G2THL9	ATP-dependent zinc metalloprotease FtsH	ftsH	58	2	73952	0.0	98,3
Bacillus coagulans 36D1	G2THL0	Inosine-5'-monophosphate dehydrogenase	guaB	58	3	52703	0.0	100
Bacillus coagulans 36D1	G2TLE3	Dihydrolipoyl dehydrogenase	Bcoa_0220	58	3	50069	0.0	99,6
Bacillus ginsengihumi	A0A0A6XYQ6	Nitrate reductase (quinone)	narZ	33	2	125334	0.0	99,5

Table 2. Intracellular proteins active on PNP-β-gal as revealed by gel zymography, were identified through LC-MS/MS method.

The only exception was a GH36 member (Uniprot code: G2TQE8), which, based on CAZy classification, is however not predicted to be active on PNP-β-gal substrate. Hence, the presence of this enzyme is explainable with similar migration properties to GH42 in the zymography gel.

From a first inspection of proteins identified within bands active on ONP-β-gal with at least two peptides, no enzymes linked to the hydrolysis of β-1–4 linkages were found. Decreasing the detection threshold up to one peptide, a β-galactosidase (Uniprot code: G2TQE8) was detected (Table 3).

Overall, the results obtained from enzymatic screening and mass spectrometry analysis indicated the presence of a single enzyme (GH42, accession number: MBF8418755) involved in the hydrolysis of β-linkages.

The enzyme specific activity associated with ONP-β-gal and PNP-β-gal was particularly high (Fig. 6), thus suggesting that either the enzyme was over-expressed under basal growth conditions or its specific activity was significantly high.

Reference Organism	UniProtKB Code	Reference Protein name	Gene	Score	Peptide number	Mass	E-value	Identity Percentage
Bacillus coagulans 36D1	G2TIB2	DNA-directed RNA polymerase subunit beta	rpoB	689	25	133181	0.0	99,4
Bacillus coagulans 36D1	G2TIB1	DNA-directed RNA polymerase subunit beta'	rpoC	678	25	134506	0.0	99,6
Bacillus coagulans 36D1	G2TN44	Formate acetyltransferase	Bcoa_2164	577	16	85425	0.0	99,2
Bacillus coagulans 36D1	G2TNL9	Glutamine synthetase	Bcoa_3015	262	9	50673	0.0	98,8
Bacillus coagulans 36D1	G2TRD5	Iron-containing alcohol dehydrogenase	Bcoa_0993	72	2	39449	0.0	99,1
Bacillus coagulans 36D1	G2THL9	ATP-dependent zinc metalloprotease FtsH	ftsH	66	3	73952	0.0	98,2
Bacillus coagulans 36D1	G2TQE8	Alpha-galactosidase	Bcoa_0573	58	4	83653	0.0	95,9
Bacillus coagulans 36D1	G2THR0	Acyl-CoA dehydrogenase domain-containing protein	Bcoa_1422	24	2	41430	0.0	98,2
***Bacillus coagulans* 36D1**	**G2TH90**	**Beta-galactosidase**	**Bcoa_0772**	**17**	**1**	**76362**	**0.0**	**96**

Table 3. Intracellular proteins active on ONP-β-gal as revealed by gel zymography, were identified through LC-MS/MS method.

To assess the culture conditions suitable to further increase the expression levels of β-galactosidase, the induction profile of this enzyme using a selective medium was analysed. By adding 0.1% lactose into a minimal medium (0.1% yeast), a significant increase (~30-fold) of the β-galactosidase activity was observed (Fig. 7).

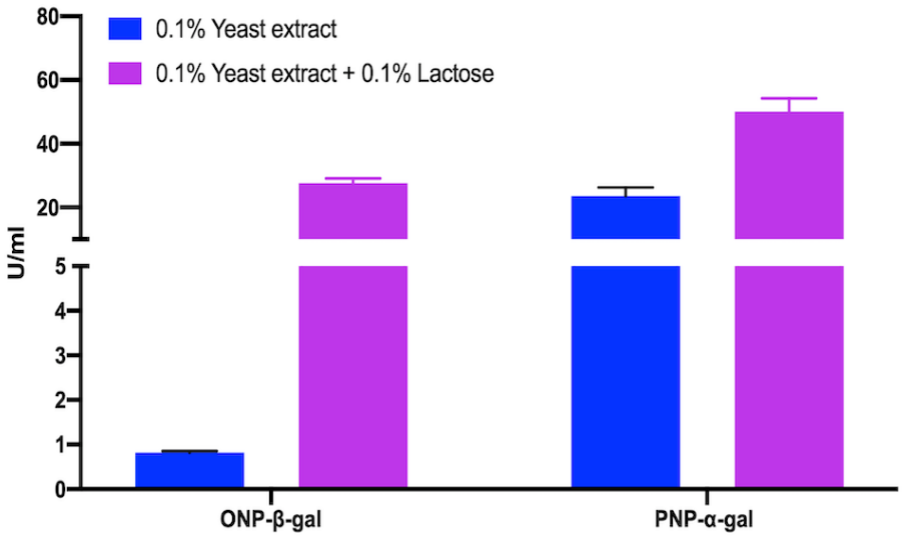


Fig. 7. Detection of intracellular enzymatic activities on ONP-b-gal and PNP-α-gal from *B. coagulans* MA-13 cells grown in the presence of lactose.

This result is not surprising considering that most β-galactosidases play a major role in lactose metabolism and this substrate is the best carbon source to induce their maximum production in Gram+ and Gram- bacteria [80,81].

Furthermore, both cell extracts and secretome of *B. coagulans* MA-13 cells grown in LB medium were tested on PNP-α-gal, since hydrolytic activity on α-linkages was detected inside and outside the cells (Fig. 6).

The activity bands of intra- and extra-cellular proteins displayed the same electrophoretic mobility, lying within the 130–180 KDa gel region.

As shown by mass spectrometry analysis, the GH 36 (Uniprot code: G2TQE8) was found in both samples (Table 4,5) suggesting that it might exert its hydrolytic activity on intracellular and extracellular α-1–6 galactans.

The list of intracellular proteins identified through mass spectrometry analysis included also another GH enzyme (namely, an arabinogalactan endo-β-1,4-galactanase), however, this latter was not found in the annotated *B. coagulans* MA-13 genome (Table 5).

The remaining co-migrating proteins identified in the extracellular and intracellular samples were related to other metabolic pathways.

Reference Organism	UniProtKB Code	Reference Protein name	Gene	Score	Peptide number	Mass	E-value	Identity Percentage
Bacillus coagulans 36D1	G2TIB1	DNA-directed RNA polymerase subunit beta'	rpoC	4590	98	134506	0.0	99,6
Bacillus coagulans 36D1	G2TIB2	DNA-directed RNA polymerase subunit beta	rpoB	4295	86	133181	0.0	99,5
Bacillus coagulans 36D1	G2TN44	Formate acetyltransferase	Bcoa_2164	2804	58	85425	0.0	99,1
Bacillus coagulans 36D1	G2TP79	Enolase	eno	2169	32	46832		
***Bacillus coagulans* 36D1**	**G2TQE8**	**Alpha-galactosidase**	**Bcoa_0573**	**1749**	**36**	**83653**	**0.0**	**95,3**
Bacillus coagulans 36D1	G2TP01	ATP-dependent helicase/nuclease subunit A	addA	1630	46	141931	0.0	95,9
Bacillus ginsengihumi	A0A0A6XYQ6	Nitrate reductase (quinone)	narZ	1116	34	139501	0.0	99,3
Bacillus coagulans 36D1	G2TJP0	Chromosome partition protein Smc	smc	846	25	135444	0.0	97,4
Bacillus coagulans 36D1	G2THL0	Inosine-5'-monophosphate dehydrogenase	guaB	752	19	52565	0.0	100
Bacillus coagulans 36D1	G2TRD5	Iron-containing alcohol dehydrogenase	Bcoa_0993	717	12	39449	0.0	97,6
Bacillus coagulans 36D1	G2TLE5	Transketolase central region	Bcoa_0222	501	11	35649	0.0	99,4
Bacillus coagulans 36D1	G2TLE4	Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex	Bcoa_0221	457	13	47549	0.0	95,6
Bacillus coagulans 36D1	G2TNL9	Glutamine synthetase	Bcoa_3015	433	10	50673	0.0	98,8
Bacillus coagulans 36D1	G2TMC2	Dynamin family protein	Bcoa_3013	400	12	136164	0.0	94
Bacillus coagulans 36D1	G2TIA6	Elongation factor Tu	tuf	388	8	43363	0.0	98,2
Bacillus coagulans 36D1	G2TLE6	Pyruvate dehydrogenase E1 component subunit alpha	pdhA	382	9	41266	0.0	98,6
Bacillus coagulans 36D1	G2TMZ6	Uncharacterized protein	Bcoa_2035	373	12	158325	0.0	91,7
Bacillus coagulans 36D1	G2TLE3	Dihydrolipoyl dehydrogenase	Bcoa_0220	360	13	49959	0.0	99,7
Bacillus coagulans 36D1	G2TP75	Glyceraldehyde-3-phosphate dehydrogenase	Bcoa_2217	316	6	36259	0.0	99,3
Bacillus coagulans 36D1	G2TP02	ATP-dependent helicase/deoxyribonuclease subunit B	addB	284	11	134349	0.0	97,6
Bacillus coagulans 36D1	G2TQJ0	Aldehyde-alcohol dehydrogenase	Bcoa_0619	224	7	95305	0.0	98,2
Bacillus coagulans 36D1	G2THN3	Transcription-repair-coupling factor	mfd	209	7	133878	0.0	96,7
Bacillus coagulans 36D1	G2TIT3	Thiamine pyrophosphate enzyme, central domain protein	Bcoa_1350	182	5	62263	0.0	99,1
Bacillus coagulans 36D1	G2TKL2	NADH:flavin oxidoreductase/NADH oxidase	Bcoa_2324	153	5	37470	0.0	96,4
Bacillus coagulans 36D1	G2TL37	Aconitate hydratase	Bcoa_3175	122	5	100322	0.0	99,1
Bacillus coagulans 36D1	G2TPG6	Pyruvate kinase	Bcoa_2517	58	2	62669	0.0	100
Bacillus coagulans 36D1	G2TQ29	CDP-glycerol:poly(Glycerophosphate) glycerophosphotransferase	Bcoa_0454	47	2	137932	0.0	98,1

Table 4. Intracellular proteins active on PNP-α gal as revealed by gel zymography, were identified through LC-MS/MS method.

Reference Organism	UniProtKB Code	Reference Protein name	Gene	Score	Peptide number	Mass	E-value	Identity Percentage
Bacillus coagulans 36D1	G2TIB1	DNA-directed RNA polymerase subunit beta'	rpoC	2055	63	134506	0.0	99,6
Bacillus coagulans 36D1	G2TIB2	DNA-directed RNA polymerase subunit beta	rpoB	1558	47	133181	0.0	99,4
***Bacillus coagulans* 36D1**	**G2TQE8**	**Alpha-galactosidase**	**Bcoa_0573**	**1349**	**38**	**83653**	**0.0**	**95,3**
Bacillus coagulans 36D1	G2TQZ8	Peptidase M30, hyicolysin	Bcoa_0856	892	22	134261	0.0	82,5
Bacillus coagulans 36D1	G2THC3	Arabinogalactan endo-beta-1,4-galactanase	Bcoa_0805	603	17	129,04	4.2e-44	75,9
Bacillus coagulans 36D1	G2TN44	Formate acetyltransferase	Bcoa_2164	373	12	85425	0.0	99,2
Bacillus coagulans 36D1	G2THL0	Inosine-5'-monophosphate dehydrogenase	guaB	132	5	52565	0.0	100
Bacillus coagulans 36D1	G2TNL9	Glutamine synthetase	Bcoa_3015	89	4	50673	0.0	99,1
Bacillus coagulans 36D1	G2TPG6	Pyruvate kinase	Bcoa_2517	49	2	62669	0.0	98,1
Bacillus coagulans 36D1	G2TP01	ATP-dependent helicase/nuclease subunit A	addA	45	2	141931	0.0	96,1

Table 5. Extracellular proteins active on PNP-α gal as revealed by gel zymography, were identified through LC-MS/MS method.

The analysis of the protein sequence did not highlight any typical signal peptide (Tat or Sec system) at the N-terminus of α-galactosidase through dbCAN database (Table 1), thus raising questions on how this protein is actually secreted and why this enzyme has a dual cellular localization. A reasonable explanation is that *B. coagulans* MA-13 exploits a leader-less secretion system, namely ESAT-6 Secretion System (ESS), which has been discovered in Firmicutes and Actinobacteria [82–84]. In this system, proteins lacking a canonical signal peptide can be secreted through the combined action of two molecular components, namely EcsA and EcsB. The relative genes are both present in the *B. coagulans* MA-13 genome (Fig.8) and are arranged in a cluster, likewise for other *B. coagulans* strains (not shown) and bacteria [82–84].

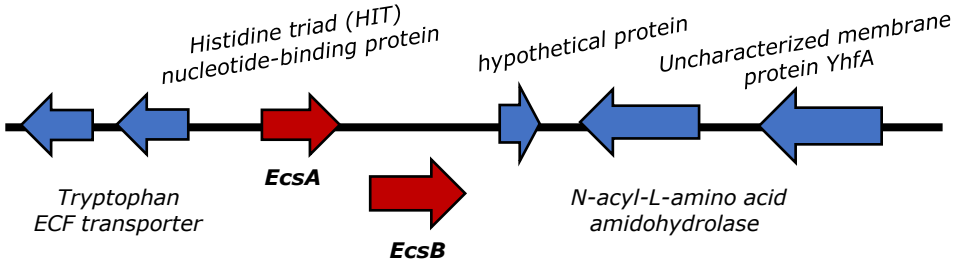


Fig. 8. Genetic organization of EcsA and EcsB cluster in *B. coagulans* MA-13 genome.

In some cases, a third molecular partner (EcsC) is associated with the same cluster, but its role seems to be dispensable for the secretion pathway [82]. Moreover, many of the proteins secreted through ESS share some distinguishing and conserved features that include a WXG amino acid motif in the central region of the protein.

Interestingly, this motif has been identified in the middle of the sequence (W_{368} and G_{370}) of the α-galactosidase (Accession number: MBF8416840, 730 aa) as well in the enolase (Uniprot code: G2TP79) which was found extracellularly along with the α-galactosidase (Table 5).

To further confirm the presence of this enzyme in the supernatant, *B. coagulans* MA-13 was grown in a minimal medium supplemented with galactomannans (locust bean gum). These are insoluble polymers that cannot be translocated inside cells and bear α-1,6-linkages, thus being natural potential substrates of α-galactosidases.

Enzymatic assays carried out on the supernatants using PNP-α-gal as a substrate, revealed that α-galactosidase was induced (about fourfold) in the presence of galactomannans compared to the control cells cultivated only in yeast (Fig. 9a).

Moreover, the analysis of cell extract indicated that the levels of intracellular and extracellular enzymatic activities were similar.

Conversely, the distribution of α-galactosidase was strongly biased toward its intracellular localization when yeast was used as the only carbon source (Fig. 9a), thus suggesting that the presence in the medium of a galactose-containing polymer, such as locust bean gum, plays a role in the secretion of α-galactosidase.

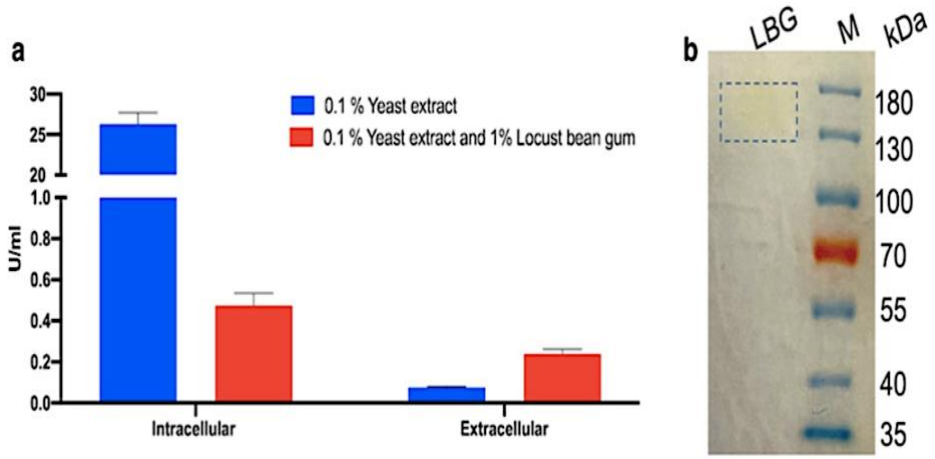


Fig. 9. Detection of α-galactosidase activity. **a**. Enzymatic assays of intracellular and extracellular extract of *B. coagulans* MA-13 on PNP-α-gal, after growth on selective medium containing locust bean gum. **b.** Zymogram of supernatants from *B. coagulans* MA-13 cells grown on locust bean gum medium, using PNP-α-gal as substrate.

All together, these findings, along with the lack of a mannanase gene in *B. coagulans* MA-13 genome (Table 1), strongly support the hypothesis that this microorganism can rely solely on the activity of an external α-galactosidase to metabolize these galactomannans.

By assaying the supernatants of locust bean gum grown cells through zymography, the α-galactosidase activity was promptly revealed (Fig. 9b) and a similar result was obtained by using other complex carbon sources derived from agri-food wastes, such as rice hull (not shown).

Enzymes identification by mass spectrometry was hindered by a strong contamination of polymers probably deriving from the substrates used for the detection of the in-gel activity. However, the electrophoretic mobility of this band (within the 130–180 KDa gel region) was identical to that identified as GH36 (Uniprot code:

G2TQE8, Table 4,5) thus indicating that the enzymatic activity revealed by zymography, can be ascribed to the same protein.

Besides our experimental evidences, the extracellular localization of the α-galactosidase has been previously described for another closely related *B. coagulans* strain [85,86] as well as for other soil microorganisms [87] and for *Bacillus megaterium* [88].

It is known that galactomannans are present in seeds of bean and, in general, RFOs (raffinose, stachyose, and verbascose) that contain α 1–6-linked galactose units, are particularly abundant in these legumes [89].

Since *B. coagulans* MA-13 was isolated from manufactured canned beans, the α-galactosidase, along with the β-galactosidase might be key enzymes for the host metabolism. Indeed, manufacturing bean wastes represent a lactose-free environment. However, other genes encoding GH42 enzymes from prokaryotes are unlikely to encounter lactose, suggesting that the substrate for these enzymes in their natural environment, might also be more complex oligo- and polysaccharides [90].

The experimental evidence of the induction of β-galactosidase expression upon exposure to lactose prompted us to analyze the effect of this inexpensive substrate also on α-galactosidase production.

Indeed, previous studies have reported the induction of β-galactosidase on galactose-containing oligosaccharides or galactose [91]. Hence, the enzymatic activity on PNP-α-gal was also measured in lactose medium and a twofold induction was observed (Fig. 7).

However, it is not clear whether the true inducer of *B. coagulans* MA-13 α-galactosidase is lactose or galactose; indeed, the latter might be produced at high intracellular concentration as a hydrolysis product of the over-expressed β-galactosidase in the presence of lactose.

Application wise, the setup of growth conditions suitable for the expression of both β-and α-galactosidases is highly relevant and only a few studies have described the production of both enzymes by the same strain [91–93].

Thoroughly biochemical characterization of a closely related recombinant α-galactosidase from *B. coagulans* ATCC 7050 (identity percentage 97.4%) has been recently published [85].

Therefore, we focused on the study of the β-galactosidase enzyme, since there is no evidence about the ability of β-galactosidases from other *B. coagulans* strains to produce GOS upon transgalactosylation reactions.

3.4 Sequence analysis, cloning and expression of *Bc*GalB

The gene (E2E33_010705) encoding for the putative β-galactosidase (herein named as *Bc*GalB), has been identified within a cluster of genes encoding for a *lacI* family regulator, a hypothetical Major Facilitator Superfamily Transporter related to multi-drug resistance mechanisms and other small hypothetical proteins.

This genetic arrangement is also present in *B. coagulans* ATCC 7050. The gene is, therefore, not included in an operon encoding

also for a lactose-permease and a transacetylase, likewise the well-known *E. coli lac*-operon.

Specifically, the hypothetical galactose-lactose permease encoding sequence is quite distant (≃7,000 nt) from the *Bc*GalB gene This suggests that its expression might not be subjected to the same regulative circuit of the *lac* operon, which consists of the concomitant over-expression of the permease after exposure of cells to lactose. Accordingly, repression of *Bc*GalB in lactose-free medium, as described for *E. coli,* was not observed; rather, the enzyme was constitutively expressed under standard growth conditions and the induction fold in the presence of lactose was significant but quite low if compared to other systems (Fig. 6) [94].

There is no report about any transcriptional cross-regulation which might account for the genetic proximity of β-galactosidase gene to choline-operon. The only functional connection has been found in a β-galactosidase from *Streptococcus mitis*, which bears a Choline Binding Domain (CBD) at its C-terminus. However, this β-galactosidase uses CBD domain as an attachment anchor to molecular components (such as lipo-teichoic acids) to bind to cell-wall. Instead, *Bc*GalB has an intracellular localization and therefore this genetic juxtaposition remains murky (Fig. 10) [95].

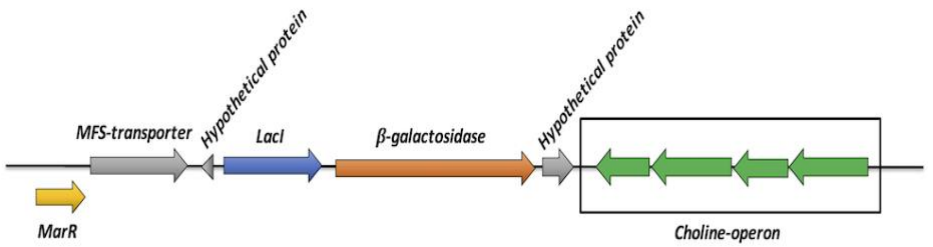


Fig. 10. β-galactosidase genomic context in *Bacillus coagulans* MA-13.

*Bc*GalB bears three typical domains of the GH42 family as suggested by CD-Search and other reports [96,97]. E2E33_010705 was amplified by PCR from the genomic DNA of *B. coagulans* MA-13 and expressed in *E. coli* Rosetta (DE3) pLysS cells as a soluble, intracellular histidine-tagged protein (C-terminus).

The overexpression system and purification method applied were quite efficient, since the enzyme was purified to homogeneity by His-trap affinity chromatography, (~ 10 mg for 1 L of culture) with a yield of 82% (Table 6).

BcGalB Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification fold	Yield (%)
Cellular extract not transformed	28.44	1422.0	50.0	/	/
Cellular extract not induced	67.70	3615.2	53.4	/	/
Cellular extract induced	209.40	52978.2	253.0	1.00	100
Affinity chromatography	10.30	43550.2	4229.0	16.72	82.2

Table 6. Purification table of *Bc*GalB.

As revealed by the SDS-PAGE analysis (Fig. 11), *Bc*GalB migrated as a single band with an apparent molecular mass of~75 kDa.

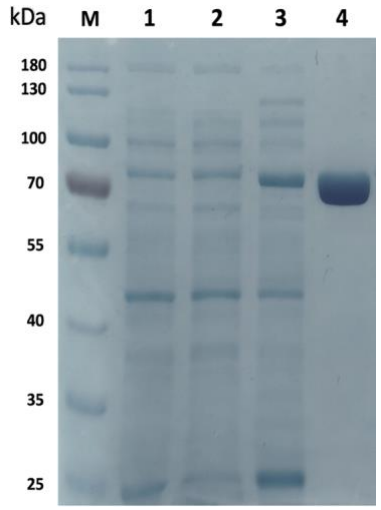


Fig. 11. SDS-PAGE analysis of *Bc*GalB. M. Molecular mass markers: 1 *E. coli* BL21 (DE3) Rosetta cellular extract not transformed; 2. *E. coli* BL21 (DE3) Rosetta pET28B/*Bc*GalB cellular extract not-induced; 3. *E. coli* BL21 (DE3) Rosetta pET28B/*Bc*GalB cellular extract induced over-night with 0,5 mM IPTG; 4. His-Trap affinity chromatography.

This concurred with the molecular mass of *Bc*GalB deduced from the nucleotide sequence of the E2E33_010705 gene and the identity of the protein was verified by mass spectrometry (data not shown). The recombinant protein was analyzed by size-exclusion chromatography coupled with a triple-angle light scattering QELS. This analysis revealed that *Bc*GalB is a hexamer in solution (not shown).

Since seven cysteines are present on the *Bc*GalB sequence, the enzyme was analyzed on SDS-PAGE in the presence of β-mercaptoethanol as a reducing agent (Fig. 11). *Bc*GalB was present only in monomeric form under this condition, thus pointing

to the role of at least some of the cysteines in the oligomerization state.

It is worth noting that β-galactosidases can be found in diverse oligomeric forms, such as dimeric (halophilic *Haloferax alicantei* [98]), trimeric (thermophilic *Geobacillus stearothermophilus* [55]), tetrameric (acidophilic archaeon *Sulfolobus solfataricus* [99]) and hexameric (hyperthermophilic *Thermotoga maritima* [100]) arrangements.

This latter structure is uncommon among thermophilic GH42 members, whereas some GH2 β-galactosidases exhibit this supramolecular organization.

To the best of our knowledge, the correlation between the hexameric structure and biochemical features of β-galactosidases is not obvious although a general correlation between oligomeric states and thermal stability has been proposed for thermophilic enzymes [22,40].

3.5 Characterization and stability properties of *Bc*GalB

The influence of pH and temperature on the enzymatic activity was evaluated using ONP-β-gal as a substrate. After testing the enzyme in intervals 4.0–10.0, the optimal pH was set at 5.0 (Fig. 12a).

Interestingly, *Bc*GalB retained 70% of its activity from 5.0 to 7.0 whilst a sharp decrease was observed at pH 4.0 (Fig. 12a).

Despite this drop at acidic pH values, the enzyme exhibited a significant stability at different pH values, ranging from acidic to alkaline ones.

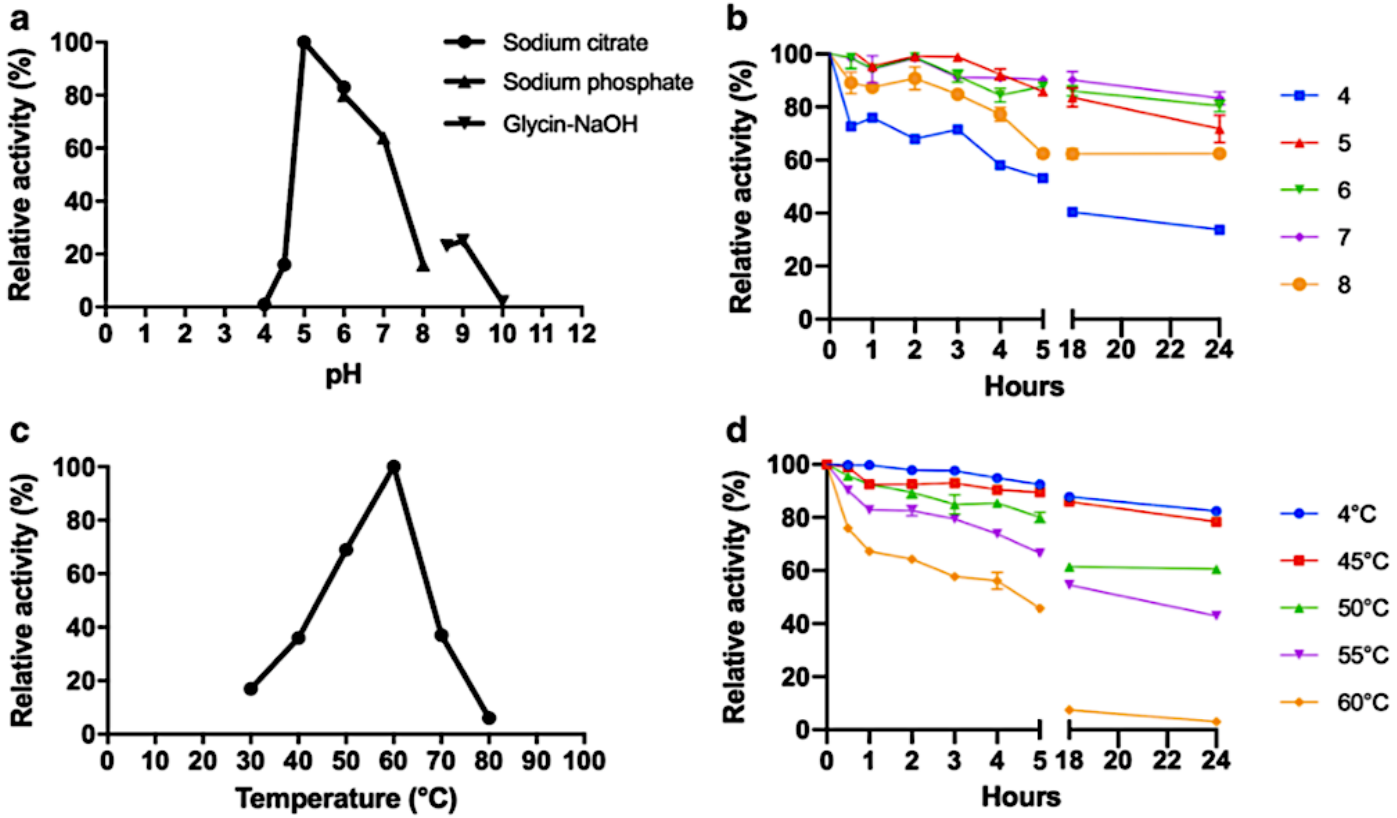


Fig. 12. Effect of pH and temperature on the enzymatic activity. **a**. The pH dependence was evaluated in different buffers ranging from pH 4.0 to pH 10.0. **b**. The pH stability was studied by incubating *Bc*GalB in different ranging from pH 4.0 to 8.0 up to 24 h. **c**. Temperature optimum was determined by testing the enzyme in the range 30-80 °C. **d**. For thermostability studies, the recombinant enzyme was incubated at different temperatures ranging from 4 to 60 °C up to 24 h.

As shown in Fig. 12b, the enzyme retained more than 70% of its activity up to 24 h in the pH range from 5.0 to 7.0 (Fig.13).

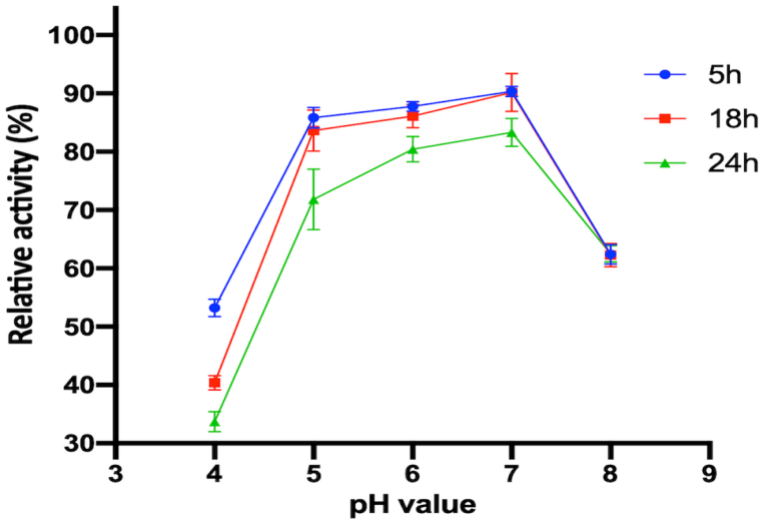


Fig. 13. Relative activity of *Bc*GalB after 5, 18 and 24 hours of incubation at different pH values.

The exploitation of β-galactosidases in the dairy industry is related to the optimal pH for hydrolysis [101]. Lactose is a hygroscopic sugar characterized by low solubility that causes crystallization as well as technological issues for certain products in the dairy industry.

The solubility and sweetness can be increased by lactose hydrolysis into the two glucose and galactose units [102].

Hence, the feature of stability in a wide range of pH values points to *Bc*GalB as a suitable tool for slightly acid and/or sweet hydrolysis of whey.

From an industrial point of view, the enzymes should be stable both at low (preventing the proliferation of microorganisms and nutrients

in milk) and at high temperatures (pasteurization) [102]. The dependence of *Bc*GalB on temperature was studied and the maximal activity was found at 60°C (Fig. 12c), which is quite similar to that of β-galactosidases from other *B. coagulans* strains [96,103,104].

Moreover, *Bc*GalB exhibited high stability at a temperature of 50°C given that approximately 60% of its initial activity was retained after incubation for up to 24 h (Fig. 12d). Moreover, the half-life at its optimal temperature was 4 h (Fig. 12d). The loss of activity at 60°C is counterbalanced by the high specificity activity of *Bc*GalB (i.e., about 4300 U/mg, Table 6) meaning that the catalytic performance of the enzyme is still consistent for an efficient hydrolysis at high temperature by employing small quantities of protein.

Interestingly, the thermophilic nature and thermal stability of *Bc*GalB is exploitable for the production of lactose-free dairy products by coupling the thermization to the hydrolysis of lactose preventing microbial contamination, decreasing viscosities of the substrate solution and reducing the cost of the whole process [102].

Finally, enzymes employed in the preparation of lactose-free products are positively selected for their relatively high activity at neutral pH and stability at low temperature [102]. In this regard, the high specific activity of *Bc*GalB at neutral pH and its stability at 4°C for up to several months match the chemical physical requirements of this biotechnological application.

3.6 Effects of metal ions and monosaccharides on *Bc*GalB activity

It is well known that ions affect the catalytic performance of β-galactosidases. For instance, the activity of yeast enzymes isolated from *Kluyveromyces lactis* and *K. fragilis* depends on the presence of Mn^{2+} or Na^{+}, and Mn^{2+}, Mg^{2+}, K^{+}, respectively [105]. Moreover, some metal ions such as Ca^{2+}, Mg^{2+} and Mn^{2+} can act as cofactors for β-galactosidases and their presence might significantly enhance their activities. Finally, it has been reported that Ca^{2+} and heavy metals inhibit the enzyme activity of several β-galactosidases. For the examination of the metal ion requirements, *Bc*GalB was assayed in the presence of 2 mM mono- and divalent ions after dialysis of the enzyme in 10 mM EDTA.

Results from this study were overall in agreement with former analyses conducted on other *B. coagulans* β-galactosidases (Fig. 14) [96,103,104].

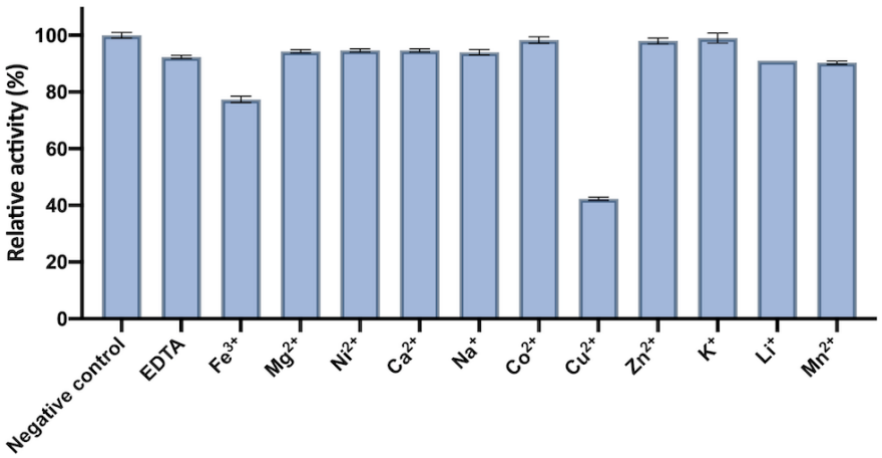


Fig. 14. Effect of metal ions on the enzymatic activity of *Bc*GalB.

Since Ca^{2+} is one of the prime elements in milk, dairy-industries processes would benefit from enzymatic activities not affected by Ca^{2+}. In this regard, the negligible effect of this ion up to 2 mM on the hydrolytic capability of *Bc*GalB, makes this enzyme an attractive candidate in these applications. Cu^{2+} is the only ion affecting the enzyme activity (60% reduction), as reported for other β-galactosidases. Indeed, some metal ions, such as Fe^{3+} and Cu^{2+}, could inactivate the enzyme by inducing structural changes upon interaction with the protein [106,107]. In order to foresee the employment of *Bc*GalB in the manufacturing of lactose-free products, the effect of galactose and glucose on enzyme activity was also studied.

The inhibitory effect exerted by the lactose hydrolysis products on *Bc*GalB activity seems different from previous studies since glucose affected the *Bc*GalB enzymatic activity more than galactose (Fig. 15).

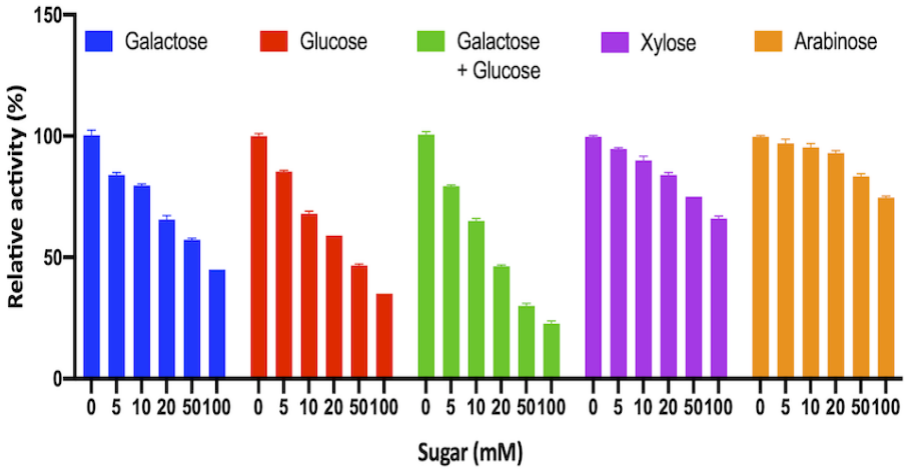


Fig. 15. Inhibitory effect of sugars on *Bc*GalB hydrolytic activity.

Moreover, since lactose hydrolysis produces equimolar amounts of the two sugar units, we resolved to investigate the combined influence of galactose and glucose. A stronger decrease of the enzymatic activity was observed especially at high concentrations of the sugars although the effect is not additive.

Furthermore, xylose and arabinose were included in these experiments since the former is an acceptor of transgalactosylation reactions whereas the latter is one of the substrates of *Bc*GalB (see below). These two monosaccharides had a minor effect on the enzymatic activity compared to galactose and glucose, since *Bc*GalB retained at least 66% of the activity at the highest concentrations tested (Fig. 15).

Finally, as part of the general biochemical characterisation of *Bc*GalB, the effect of surfactants (SDS and Tween 20), reducing (DTT and β-mercaptoethanol) and chaotropic (urea and guanidine chloride) agents, was studied.

The enzyme activity significantly decreased only in the presence of SDS whereas it retained at least 65% of the relative activity when tested with all the other agents (Table 7).

Compound (50 mM)	Relative activity (%)
SDS	17.9
Tween 20	65.7
β-mercaptoethanol	76.2
DTT	64.5
Urea	79.0
Guanidine chloride	73.1

Table 7. Relative activity of *Bc*GalB in presence of chemicals.

3.7 Catalytic properties of *Bc*GalB

The hydrolytic activity of *Bc*GalB was tested on different *ortho*- or *para*-nitrophenyl synthetic glycosides as well as on natural polysaccharide substrates and specificity of the enzyme was determined by carrying out individual reactions with each of the compounds as indicated in Material and Methods section.

As shown in Table 8, the highest specific activity was recorded on ONP-β-gal, whereas the enzyme performed less efficiently on *para*-substituted substrates. As shown in Fig. 6 analysis of the intracellular cell extract revealed the presence of enzyme(s) able to hydrolyze PNP-α-ara.

Substrate	Specific activity (U/mg)
ONP-β-gal	4373.4 ± 77.6
PNP-β-gal	795.9 ± 3.9
PNP-α-ara	328.5 ± 11.6
PNP-β-xyl	Not detected
PNP-β-glu	Not detected
D-lactose	1283.0 ± 24.7

Table 8. Substrate specificity of *Bc*GalB.

Interestingly, a lower but still significant activity of *Bc*GalB was found on this substrate suggesting that the enzyme is endowed with an ancillary activity on PNP-β-ara. Then, the observed enzymatic activity in the cell extract can be traced back, at least in part, to *Bc*GalB (Fig. 6; Table 8).

This accessory activity is surprising, since it has never been described for other thermophilic GH 42 β-galactosidases [58] and it will be a matter of further investigation. Some β-galactosidases can support the growth of environmental microorganisms from hot springs, soils and hypersaline sites where lactose is not present and rather plant biomasses are preferential carbon and energy sources. Since *B. coagulans* MA-13 was isolated from beans processing waste, it is conceivable that *Bc*GalB may be also involved in the hydrolysis of arabino-derived oligosaccharides *in vivo* (Table 8).

Lactose, which is the natural substrate for most β-galactosidases, is translocated inside cells through specific lactose-transporters [52]. Therefore, the hydrolytic performance of *Bc*GalB on this substrate was also studied and the specific activity was found to be 1283 U/mg, which is a quite high value compared to β-galactosidases from other *B. coagulans* strains [96,103,104].

The kinetic parameters of *Bc*GalB were evaluated using both the preferred artificial substrate and lactose under standard reaction conditions (Table 9).

Substrate	K_M (mM)	k_{cat} (s^{-1})	k_{cat} / K_M (s^{-1} /mM)
ONP-β-gal	0.723	5466.7	756.2
PNP-α-ara	5.77	564.0	97.7
D-lactose	136.2	1603.7	11.8

Table 9. Kinetic parameters of *Bc*GalB.

Results of this analysis highlighted that *Bc*GalB showed the highest affinity towards ONP-β-gal (K_M=0.72 mM) and interestingly this value is among the lowest determined so far among mesophilic and thermophilic β-galactosidases [69,97,108]. Moreover, even among closely related β-galactosidases from other *B. coagulans* strains, *Bc*GalB displays the highest affinity towards this substrate [96,103,104].

Interestingly, the enzymatic activity on lactose was not affected by Ca^{2+} and even a slight increase (114%) was recorded (data not shown). The K_M was found to be higher than for the artificial substrate; however, previous studies have revealed that most GH42 β-galactosidases prefer to hydrolyze chromogenic substrates while showing weaker lactose hydrolysis activity.

Although GH2 β-galactosidases perform better than GH42 representatives on lactose hydrolysis, *Bc*GalB exhibits a significant specific activity toward this substrate [62,96,103,104].

Accordingly, *B. coagulans* MA-13 is able to grow on lactose by over-producing *Bc*GalB (Fig. 7), whereas several prokaryotes possessing a GH42 gene are unable to utilize this substrate [90]. This indicates that *Bc*GalB can sustain the host metabolism through hydrolysis of either lactose or more complex oligosaccharides.

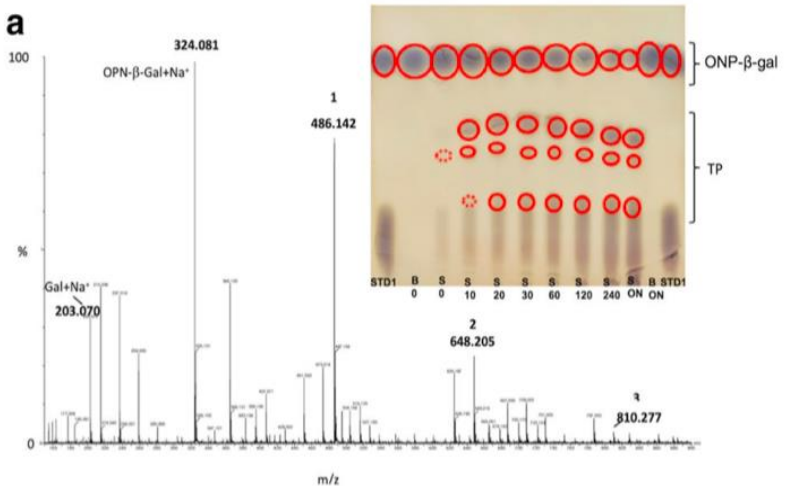
3.8 Transgalactosylation activity of *Bc*GalB

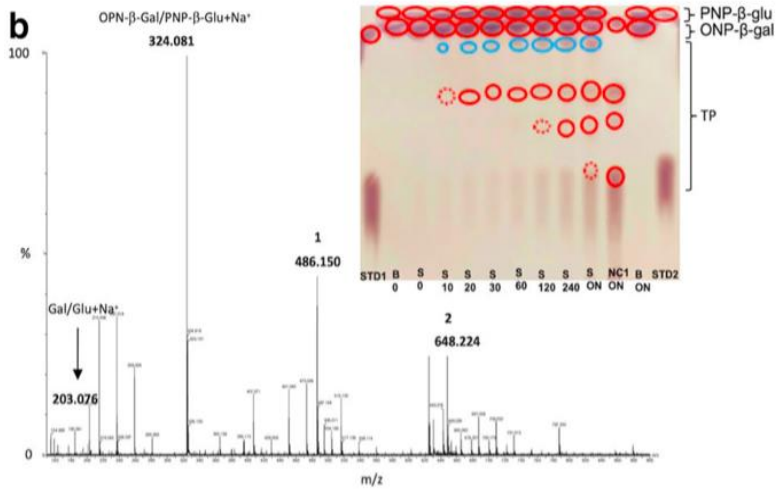
The transgalactosylation activity of *Bc*GalB was evaluated using ONP-β-gal substrate in either auto- or hetero condensation reactions, in this latter case, with different acceptors.

When ONP-β-gal was used as a donor and acceptor, TLC analysis revealed the synthesis of products of homo- galactosylation products already after 10 min of reaction (Fig. 16a, lane S 10). Moreover, additional signals were clearly visible after 20 min (Fig. 16a, lane S 20), demonstrating that in the early stages of the reaction the donor was promptly consumed in favor of the synthesis of transgalactosylation products (lower red circles).

More importantly, these compounds were not hydrolyzed by *Bc*GalB up to 18 h (Fig. 16a, lane S ON) although their complete hydrolysis was observed after the addition of fresh *Bc*GalB to the transgalactosylation mixture (data not shown).

Hence, the persistence of the transgalactosylation products up to 18 h may be due to the combined effect of the partial inactivation of *Bc*GalB occurring after 4 h at 60°C (Fig. 12d), with the inhibitory effect on the enzymatic activity due to D-galactose accumulation (Fig. 15).

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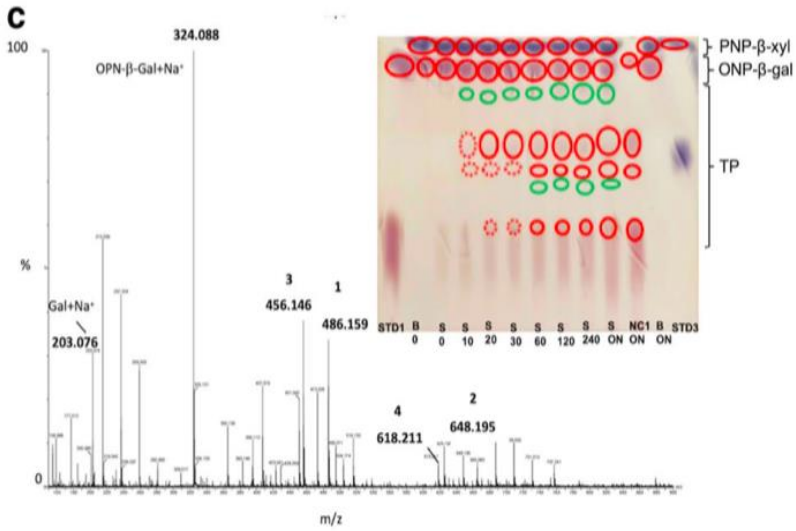
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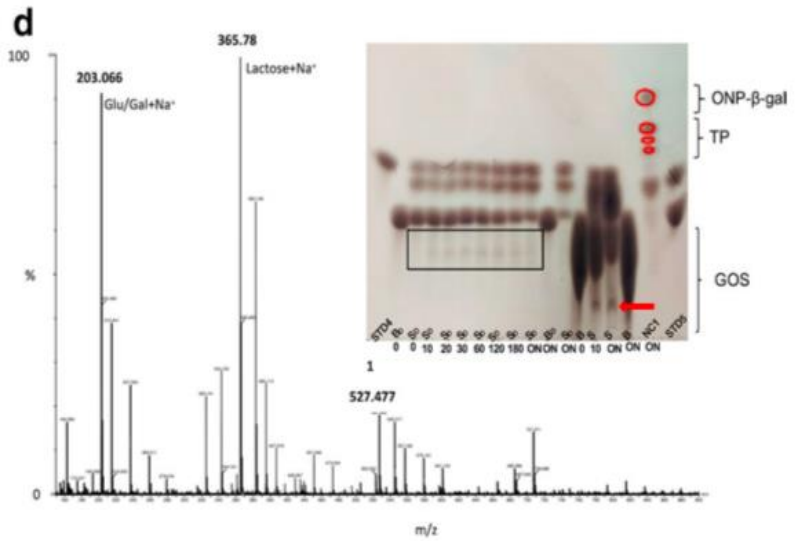

Fig. 16. Time course of transgalactosylation reaction detected by TLC analysis. Homo-condensation reactions using ONP-β-gal as donor and acceptor (**a**). Hetero-condensation reactions performed with PNP-β-glu (**b**) and PNP-β-xyl (**c**) as acceptors and ONP-β-gal as donor. Transgalactosylation reactions using D-lactose as substrate (**d**). Red, blue and green circles show the UV signals obtained from the arylic group of ONP-β-gal, PNP-β-glu, and PNP-β-xyl respectively. *STD1*: Standard with ONP-β-gal and D-galactose, *STD2*: Standard with PNP-β-glu and D-glucose, *STD3*: Standard with PNP-β-xyl and D-xylose, *STD4*: Standard with D-glucose, *STD5*: Standard with D-galactose and D-lactose, *S*: Samples collected at different times (min), *B*: Blank at different times, S_D: Sample diluted, B_D: Blank diluted, *TP*: Transgalactosylation products

*Bc*GalB is also able to synthetize hetero-oligosaccharides with PNP-β-glu and PNP-β-xyl as acceptors and ONP-β-gal as a donor. Indeed, signals that can be traced back to the formation of hetero-oligosaccharides (highlighted in blue, Fig. 16b and in green Fig. 16c), were identified together with transgalactosylation products with migration properties similar to those found in homo-condensation reactions (Fig. 16b, c). The transgalactosylation products were analysed by ESI–MS (Table 10) after carrying out all the reactions for 18 h.

In all spectra, the galactose as the product of the hydrolytic activity of *Bc*GalB was detected together with the substrate(s) (ONP-β-gal, ONP-β-glu, PNP- β-xyl). For homo-condensation reactions, m/z values of 486.142, 648.205 and 810.277 corresponding to the sodium adducts of the disaccharide, trisaccharide and tetrasaccharide, were observed (Fig. 16a; Table 10). These transgalactosylation products contained galactose unit(s) (m/z=162) and the galactose residue of ONP-β-gal (m/z = 324).

In the presence of glucose as acceptor, ESI–MS analysis revealed the formation of two transgalactosylation products (Fig. 16b, blue circles) counting for an increase in mass of one or two hexoses (Gal/Glu) (Fig. 16b; Table 10).

These reaction products could result from both homo- and hetero-condensation of glucose and galactose molecules, although they were not distinguishable by ESI–MS analysis because of their identical molecular weight (Table 10).

It is noteworthy that hetero-condensation products were synthetized when either xylose or glucose were used, thus demonstrating that *Bc*GalB displays a broad acceptor specificity in transgalactosylation reactions.

Since from a biotechnological perspective, the ONP-β-gal is useless as a donor in industrial processes, the natural, plentiful and inexpensive substrate lactose was employed as the glycosyl donor and acceptor in the synthesis of glycoconjugates.

Acceptor:donor	Transgalactosylation products	MNa^+ Theoretical (Da)	MNa^+ observed (Da)
(ONP-β-Gal: ONP-β-Gal)	1. (ONP-β-Gal + Gal) Na^+	486.408	486.142
	2. (ONP-β-Gal + 2 Gal) Na^+	648.565	648.205
	3. (ONP-β-Gal + 3Gal) Na^+	810.722	810.277
(ONP-β-Gal/PNP-β-Glu: ONP-β-Gal)	1. (ONP-β-Gal/PNP-β-Glu + Gal/Glu) Na^+	486.408	486.150
	2. (ONP-β-Gal/PNP-β-Glu + 2Gal/2Glu) Na^+	648.565	648.224
(PNP-β-Xyl:ONP-β-Gal)	1. (ONP-β-Gal + Gal) Na^+	486.408	486.159
	2. (ONP-β-Gal + 2Gal) Na^+	648.565	648.195
	3. (PNP-β-Xyl + Gal) Na^+	456.382	456.146
	4. (PNP-β-Xyl + Gal + Gal) Na^+	618.539	618.211
(D-Lactose:D-Lactose)	1. (Lactose + Gal) Na^+	527.477	527.202

Table 10. Transgalactosylation products identified by ESI-MS.

A high initial lactose concentration of 160 mM was chosen to enhance GOS synthesis over hydrolysis. TLC analysis revealed the presence of hydrolysis products as well as of several GOS signals already after 10 min of incubation (Fig. 16d, lanes S_D0 to S_D ON, S10 and S ON).

This result indicates that *Bc*GalB is able to produce GOS at the expenses of lactose hydrolysis in a short time range. As the reaction proceeded, lactose was consumed, and glucose and galactose were formed following lactose hydrolysis.

However, a concurrent increase of GOS amount was not observed, as revealed by the intensity of the spots (Fig. 16d, lanes S10-S ON). This indicates that the two reactions were in a dynamic equilibrium in which GOS production reached a plateau before lactose was completely hydrolyzed.

ESI–MS analysis revealed a transgalactosylation product (m/z value of 527.2, Table 10) consisting of a lactose molecule increased by one galactose unit (S ON, Fig. 16d) along with the corresponding signal to the D-lactose substrate.

Collectively, these data indicate that the thermophilic *Bc*GalB is effective in the production of GOS from lactose. Moreover, lactose solubility in water is rather low in comparison to other carbohydrates; therefore, achieving a lactose concentration high enough to promote transgalactosylation reactions is a difficult task.

Since lactose solubility increases exponentially with temperature, GOS synthesis can benefit from carrying out reactions with thermostable enzymes and thermophilic microorganisms.

4. CONCLUSION

Probiotic food production relies on the use of *Bifidobacterium*, lactic acid bacteria (LAB) as well as *Saccharomyces* species. However, some *Bacillus* species have been tested as probiotics, but their use is not as widespread as for traditional LAB and yeasts.

An attractive feature of *Bacillus spp.* is the resistance to extremely harsh environments thanks to their ability to form spores and to grow under a relatively wide range of temperatures, usually up to ~60°C. Within *Bacillus* genus*, B. coagulans* has been firstly discovered in spoiled canned milk and afterwards in other food sources.

Recently, a novel thermophilic *B. coagulans* strain, designed as MA-13, has been isolated from canned beans manufacturing and shown to be able to produce lactic acid from lignocellulose biomass.

B. coagulans MA-13 turned out to be exceptionally resistant to extreme conditions, such as toxic compounds derived from the thermo-acidic treatment of lignocellulose, thus pointing to this microorganism as a good candidate as probiotic especially when harsh conditions are required for food manufacturing.

Indeed, *B. coagulans* MA-13 is a versatile strain with the potential to be employed in industrial processes aimed not only at the production of value-added chemicals from lignocellulose but also of products/enzymes suitable for various industrial food applications.

In particular, this work shows the capability of this microorganism to overproduce under standard growth conditions α- and β-galactosidases that are key enzymes for improving the nutritional value of RFO- and lactose containing food.

Moreover, the expression of these two enzymes can be simultaneously increased in the presence of a natural and inexpensive substrate such as lactose which is abundant in dairy wastes (i.e., whey).

Interestingly enough, *Bc*GalB is able to produce GOS from artificial and natural (lactose) substrates as well as to perform homo- and hetero- condensation reactions.

GOS are produced by transgalactosylation reactions, in which the glycosyl group of one or more D-galactosyl units is transferred onto another mono-or oligosaccharide acceptor yielding different GOS mixtures formed by di-, tri-, tetrasaccharides.

Consequently, β-galactosidase producing microbes capable of performing transgalactosylation, can be used as microbial cell factories to produce GOS molecules for the selective stimulation of the gut microbiota.

All together these features point to *B. coagulans* MA-13 as a good candidate for the valorization of dairy waste products and for eco-friendly and sustainable production of GOS by using whole cells.

APPENDIX I:

Exploitation of *Bacillus coagulans* strain MA-13 as model system for plastic biodegradation

1. INTRODUCTION

1.1 Thermoplastics

The global use of plastics is constantly growing every year, but the awareness about their negative impact on ecosystems increased too [109]. Due to low production costs, these polymers are often not reused and the mismanagement of plastic waste results in their accumulation in the environment as pollutants with toxic effects [110]. Traditional disposal methods of these materials are based on solid waste incineration with the release of CO_2 and dioxins by burning processes [111].

Therefore, the study of mechanisms involved in plastic degradation polymers is strongly required to find “green” solutions to environmental pollution.

Thermoplastics are synthetic or semi-synthetic polymers that can be melted and recast almost indefinitely. They are cheap, lightweight, strong, durable and resistant to corrosion and are widely used in industry and agriculture fields. The main plastic debris is represented by disposable utensils, plastic bags and packaging [112]. Among thermoplastics high-density polyethylene (HDPE), low-density polyethylene (LPDE), polyethylene terephthalate (PET) and polyvinyl alcohol (PVA) are the most commonly used ones [113].

Polyethylene is petrochemical-based thermoplastics that has shaped the landscape of the packaging and manufacturing industry. Although LDPE and HDPE are both ethylene polymers, they differ in several properties since the former has more branching than the latter. LDPE is widely used in plastic bags as its low density makes it light and flexible, while HDPE, is harder and offers higher strength

and better heat resistance. For these features, it has been successfully employed for 3D printing filaments as well as for toys and plastic chairs production.

Although PET is one of the most recycled thermoplastics, the accumulation of this polymer in nature represents a long-standing problem that requires urgent solutions for its impact on the environment [114]. PET can be synthetized by esterification reactions between terephthalic acid (TPA) and ethylene glycol (EG) units [115] and is widely used for the production of bottles and containers, thanks to its durability and chemical resistance. Unlike the other plastic polymers, the collection rates of PET are very high all around the world. However, the overall of PET recycled and reused is still lower than the PET produced every day.

Differently from the solid pollutants described above, PVA is a water-soluble vinyl polymer belonging to microplastics that represent a serious threat to the marine environment. Indeed, the accumulation of PVA as large amounts of foam on the water surface, can prevent the recycling of oxygen, thus turning to be a toxic compound to living organisms [116]. PVA is obtained by the removal of acetate groups from vinyl acetate polymers after hydrolysis [117]. PVA is one of the most flexible synthetic polymers that offers good resistance to humidity and is often used for the production of paper coating, adhesives, and textiles polymers by industries [118]. In recent years, the improper waste management and the over utilization of plastics for various anthropic purposes have fostered the development of new strategies for plastic degradation. Indeed, the degradation of all these polymers can be achieved through abiotic or biotic approaches.

The former is based on physics and chemical methods such as high temperature and UV irradiation [119–121]. On the other hand, the latter relies on the action of microorganisms able to utilize these polymers as carbon and/or energy source.

Interestingly several bacterial and fungal strains are able to grow in the presence of plastic substrates [116,122,123]. This biotic approach can be carried out using pure strains or microbial communities, thus exploiting a high degradative efficiency especially for polymers that require a concerted action of different enzymatic activities.

Previous studies have demonstrated that laccases play a relevant role in PE degradation by oxidation. These enzymes are copper oxidases able to degrade PE in small molecules [124]. PE decomposition can be also achieved by the hydrolytic activities of alkane hydroxylases and esterases [125,126]. In particular, the esterases are involved in the hydrolysis of PET molecules too [127]. In addition, the serine hydrolases such as cutinases and lipases have shown a good capability of hydrolyzing PET [128]. Indeed, many cutinase-like hydrolase enzymes have been reported in literature as “PETases”. These enzymes are able to degrade PET in TPA and EG monomers [129,130]. As concerning the PVA-hydrolyzing enzymes, they carry out two catalytic mechanisms such as hydrolysis and oxidation of PVA molecules. Indeed, the acetyl groups of PVA chains are cleaved first by the esterases and then the microbial assimilation of PVA is increased by oxidases [131].

1.2 Aim of the work

Microbial degradation could represent a promising and cheap alternative to chemical processes, thus reducing the high amounts of plastics released in the environment in an eco-friendly way. The use of thermophilic microorganisms could represent an advantage in this process, since it is expected that the association of high temperature of growth to microbial decomposition could ameliorate the efficiency of the plastic decomposition. Therefore, the ability of the thermophilic bacterium *B. coagulans* MA-13 to grow in the presence of LDPE, PET and PVA has been investigated.

This strain has been proven to be resistant to lignocellulose biomass-derived inhibitors, thus pointing to MA-13 as a promising candidate in decontamination and detoxification applications. Moreover, other *Bacillus* species have been proven to be involved in the decomposition of plastic polymers [116,122,132].

2. MATERIALS AND METHODS

2.1 Strain and culture medium

The experiments were performed using the thermophilic and acidophilic bacterium *B. coagulans* strain MA-13 [10] which grows optimally at 55°C and pH 5.5.

An aliquot of frozen cultures was inoculated in Luria-Bertani medium which contained 10.0 g tryptone (AppliChem), 10.0 g NaCl (AppliChem) and 5.0 g yeast extract (AppliChem) per liter.

To set up growth conditions for plastic degradation, MA-13 was first inoculated in LB medium (100 ml) and then 5 OD_{600} were transferred to 50 ml of minimal medium (screening medium, SC) in 250-ml flask after washing the cell pellet. SC medium is composed of 0.1% of yeast extract and 0.5% of each single plastic substrate PVA (Sigma Aldrich) or LDPE (Sigma Aldrich) or PET (Sigma Aldrich).

LPDE and PET were sterilized using ethanol at 70% before addition to added to the medium. Conversely, PVA was dissolved in the medium by autoclaving sterilization cycle (120°C for 20 minutes).

In order to get PVA pre-adapted cells, pre-cultures were started from a frozen glycerol stock and grown in 100 ml of LB medium using 500-ml flasks in a shaking incubator at 55°C and 180 rpm.

5 OD_{600} of these pre-cultures were centrifuged at 3000 x g for 10 min. Pellets were resuspended in 50 ml of SC-PVA medium and cells were cultivated at 55°C and 180 rpm for 5 days. The optical density of each culture was regularly measured spectrophotometrically at 600 nm and the volume was adjusted by adding sterile water. Subsequently, a frozen culture of these pre-adapted cells was stored at −80°C with glycerol 30%.

To test the capability of the bacterium to grow under selective conditions, i.e. using PVA as sole carbon source. Cells were enriched in LB medium and then transferred (5 OD_{600}) to 50 ml of M9 medium supplemented with 0.5% of PVA. The M9 medium (per liter) was prepared as follows: 12.8 g $Na_2HPO_4 \times 7H_2O$ (Sigma Aldrich), 3.0 g KH_2PO_4 (Sigma Aldrich), 0.5 g NaCl, 1.0 g NH_4Cl (Sigma Aldrich), 240.7 mg $MgSO_4$ (Sigma Aldrich) and 11.1 mg $CaCl_2$ (Sigma Aldrich).

The cultivation time was extended up to 24 hours and 120 hours by monitoring the optical density of the culture at 600 nm.

2.2 Polyvinyl alcohol degradation assay

Cells were collected at 18 hours and analysed through centrifugation at 3000 × g for 15 min, and subsequently removed from the culture broth.

PVA concentration was estimated on the supernatants using a spectrophotometry method [133] which relies on the detection of PVA molecules after the formation of chemical bonds with this specific dye.

In particular, standard PVA solutions (ranging from 0.5 g/L to 5.0 g/L in 0.5 ml of M9 or SC) were supplemented with 0.75 ml of 4% boric acid (Sigma Aldrich) and 0.15 ml of I_2-KI (12.7 g/l I_2 and 25.0 g/l KI, Lugol solution, Carl Roth). The final volume of the solution (1.4 ml) was diluted to 2.5 ml with distilled water and shacked at 50 rpm for 30 min at room temperature. By spectrophotometer analysis, the PVA concentration was finally determined at 690 nm.

The decrease of PVA concentration in the supernatants during *B. coagulans* growth was measured following the same procedure

described above by using aliquots (0.5 ml) of the supernatant instead of the standard PVA solutions.

The amount of degraded PVA has been calculated through the calibration curve.

3. RESULTS AND DISCUSSION

The ability of the thermophilic bacterium *B. coagulans* MA-13 has been investigated as a model system for plastic decomposition. The genome sequencing of MA-13 [12], has revealed the presence of esterases potentially involved in plastic degradation [134]. With this purpose we have first tested the cultivability of MA-13 on three different thermoplastics, by using the SC medium supplemented with either LDPE or PET or PVA. Under these conditions we only observed growth on PVA-SC medium.

This is not surprising since LDPE and PET polymers are recalcitrant thermoplastics and therefore their biotic decomposition is usually not efficient [129,135]. Furthermore, their degradation is often carried out by microbial consortia in which the synergistic actions of several bacteria can cooperate to make the surface of these polymers accessible to plastic-degradative enzymes [122,134,135].

However, some physio-chemical methods can be applied for modulating the plastic surface as reported recently [119,136].

Therefore, UV pretreatment could render the plastic debris attainable to digestion by microorganisms.

Subsequent analyses were conducted only on PVA.

A pre-adaptation strategy was used to test the adaptability of MA-13 to activate the plastic degrading metabolic pathways. For this purpose, cells were grown in SC-PVA medium and diluted to 0.1 OD for 5 days. Then a frozen culture of these pre-adapted cells was prepared and used to compare the rate growth of pre-adapted and not pre-adapted cultures. However, as shown in Fig. 17, growth profiles were overlapping thus indicating that the pre-adaptation strategy did not work.

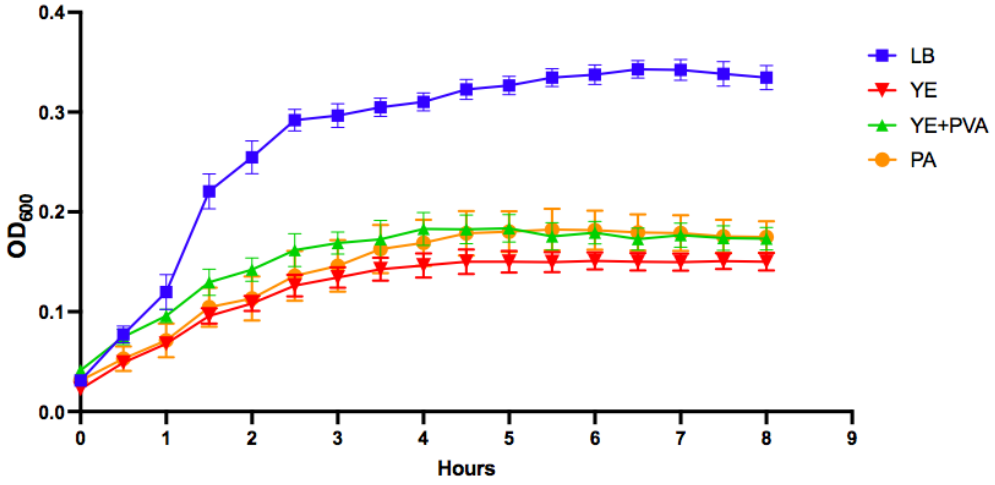

Fig. 17. Cellular growth of pre-adapted and not pre-adapted cells of *B. coagulans* MA-13 after incubation of 8 hours.
LB: Luria-Bertani medium (positive control); YE: Yeast Extract medium (negative control); YE+PVA: Minimal medium enriched with PVA; PA: pre-adapted cells cultured in minimal medium enriched with PVA.

In order to boost the physiology of MA-13 towards plastic degradation, MA-13 was grown in an even more selective medium containing only PVA as carbon source. Preliminary experiments suggest that MA-13 is able to use PVA to support its growth. (data not shown).

After assessing the ability of MA-13 to grow in the presence of PVA, we resolved to quantify the actual PVA degradation of MA-13.

Extracellular extracts were tested with Lugol solution.

As shown in Fig. 18, there is no significant difference between the amount of PVA consumed upon MA-13 growth in two different minimal media, since about 4-6% of degradation was revealed in both cases. Although the PVA reduction is quite limited under our growth conditions, the results were reproducible in biological replicates. Comprehensive studies will be required to understand

the enzymatic mechanisms that lead to the microbial decomposition of PVA.

Moreover, MA-13 will be exploited in association with other thermophilic microorganisms from our lab collection to set up artificial consortia to improve the digestibility of PVA.

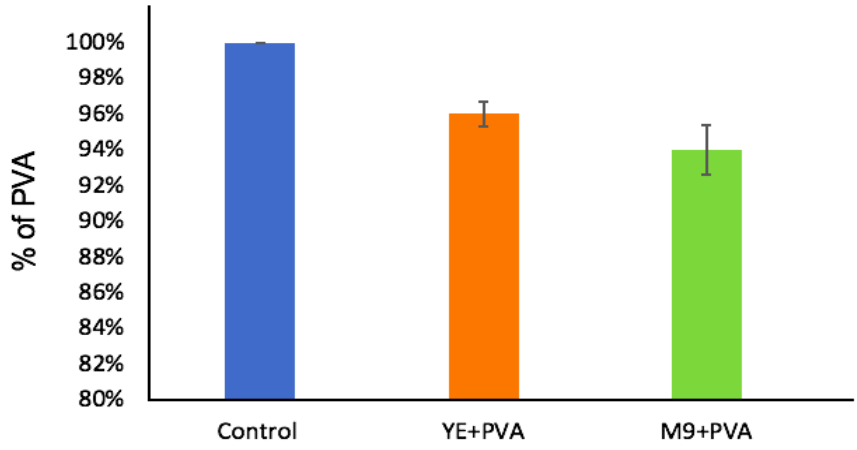

Fig. 18. PVA degradation assay.
Control: PVA dissolved in water; YE+PVA: minimal medium with yeast extract and PVA; M9+PVA: minimal medium with M9 and PVA.

4. CONCLUSION

During the third year, I have spent one month in the laboratory of the Prof. Antranikian at the Institute of Technical Microbiology of Technische Universitat Hamburg (TUHH) in Harburg (Hamburg, Germany).

During the foreign period of my PhD project, I was interested to analyze plastic biodegradation using *B. coagulans* MA-13 as a model system.

In this context, I tried to grow *B. coagulans* MA-13 on LDPE, PET and PVA substrates.

Unfortunately, I had to come back to Italy because of Covid outbreak. Nevertheless, I kept doing these experiments in Italy and I have observed the capability of *B. coagulans* MA-13 to grow in presence of PVA as substrate.

Although the bacterium seems to be not able to grow on LDPE and PET polymers, physio-chemical pre-treatments of these materials could improve their biotic decomposition.

Instead, the effects of microbial degradation have been evaluated on PVA. Preliminary results suggest that in particular, a certain degree of PVA decomposition is carried out by MA-13.

The major goal of future biochemical analysis will be to identify the enzymatic mechanisms involved in PVA degradation.

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TECHNISCHE UNIVERSITÄT HAMBURG · KASERNENSTR. 12 · D-21073 HAMBURG

PROF. DR. G. ANTRANIKIAN
TECHNISCHE UNIVERSITÄT HAMBURG

TECHNISCHE MIKROBIOLOGIE

KASERNENSTRASSE 12
D-21073 HAMBURG
TEL: 040 - 42878-3117
FAX: 040 - 42878-2582
E-MAIL: ANTRANIKIAN@TUHH.DE

Università degli Studi di Napoli Federico II
Complesso universitario Monte S. Angelo
Dipartimento di Biologia
Via Cinthia
80126 Napoli
Italy

Hamburg, March 31, 2020

Attendance of Mr. Ferdinando Sansone

Dear Madam / Sir,

This is to confirm that Mr. Ferdinando Sansone, born May 30, 1992, was a guest researcher in the Institute of Technical Microbiology at the TUHH, Hamburg, Germany, for the period from March 1 to 31, 2020. This research work is part of his PhD thesis.

Unfortunately, due to the corona crisis he could not stay longer.

Sincerely,

G. Antranikian

Prof. Dr. Dr. h. c. Garabed Antranikian

APPENDIX II:
List of communications

1. F. Sansone, M. Aulitto, A. Strazzulli, S. Fusco, M. Moracci, S. Bartolucci, P. Contursi **"Expression, purification and biochemical characterization of a novel beta-galactosidase from thermophilic *Bacillus coagulans* strain MA-13"** 2nd Workshop BIO/10 17th May 2019 in Naples (Italy) (poster session)
2. F. Sansone, M. Aulitto, A. Strazzulli, S. Fusco, M. Moracci, S. Bartolucci, P.Contursi **"Characterization of a new beta-galactosidase from thermophilic *Bacillus coagulans* strain MA13 for biotechnological applications"** Riunione Nazionale "A.Castellani" dei Dottorandi di Ricerca in Discipline Biochimiche 2019, 3-7th June 2019 in Brallo di Pregola, Pavia, Italy (talk session)
3. F. Sansone, M.Aulitto, A. Strazzulli, S. Fusco, M. Moracci, S. Bartolucci, P. Contursi **"Identification of a promising beta-galactosidase from thermophilic *Bacillus coagulans* strain MA-13 for enzymatic degradation of hemicelluloses"** 60th SIB congress Lecce, 18-20th September, 2019 (poster session)
4. F. Sansone, M. Aulitto, A. Strazzulli, S. Fusco, M. Moracci, S. Bartolucci, P. Contursi **"β-galactosidase from the thermophilic *Bacillus coagulans* strain MA-13: Biochemical characterization for enzymatic degradation of hemicelluloses"** School of Enzyme discovery and Engineering for Biotechnological applications, 3-5th December 2019, Naples, Italy (talk session

APPENDIX III:
Publications

Aulitto, M., Andrea, S., Sansone, F., Cozzolino, F., Monti, M., Moracci, M., ... & Contursi, P. **Prebiotic Properties of Bacillus Coagulans MA 13: Production of Galactoside Hydrolyzing Enzymes and Characterization of the Transglycosylation Properties of a GH42 β-Galactosidase.** Microb Cell Fact. (2021) 20;71

Aulitto et al. Microb Cell Fact (2021) 20:71
https://doi.org/10.1186/s12934-021-01553-y

Microbial Cell Factories

RESEARCH Open Access

Prebiotic properties of *Bacillus coagulans* MA-13: production of galactoside hydrolyzing enzymes and characterization of the transglycosylation properties of a GH42 β-galactosidase

Martina Aulitto[1,2], Andrea Strazzulli[1,3], Ferdinando Sansone[1], Flora Cozzolino[4,5], Maria Monti[4,5], Marco Moracci[1,3,6], Gabriella Fiorentino[1,7], Danila Limauro[1,7], Simonetta Bartolucci[1] and Patrizia Contursi[1,3,7*]

Abstract

Background: The spore-forming lactic acid bacterium *Bacillus coagulans* MA-13 has been isolated from canned beans manufacturing and successfully employed for the sustainable production of lactic acid from lignocellulosic biomass. Among lactic acid bacteria, *B. coagulans* strains are generally recognized as safe (GRAS) for human consumption. Low-cost microbial production of industrially valuable products such as lactic acid and various enzymes devoted to the hydrolysis of oligosaccharides and lactose, is of great importance to the food industry. Specifically, α- and β-galactosidases are attractive for their ability to hydrolyze not-digestible galactosides present in the food matrix as well as in the human gastrointestinal tract.

Results: In this work we have explored the potential of *B. coagulans* MA-13 as a source of metabolites and enzymes to improve the digestibility and the nutritional value of food. A combination of mass spectrometry analysis with conventional biochemical approaches has been employed to unveil the intra- and extra- cellular glycosyl hydrolase (GH) repertoire of *B. coagulans* MA-13 under diverse growth conditions. The highest enzymatic activity was detected on β-1,4 and α-1,6-glycosidic linkages and the enzymes responsible for these activities were unambiguously identified as β-galactosidase (GH42) and α-galactosidase (GH36), respectively. Whilst the former has been found only in the cytosol, the latter is localized also extracellularly. The export of this enzyme may occur through a not yet identified secretion mechanism, since a typical signal peptide is missing in the α-galactosidase sequence. A full biochemical characterization of the recombinant β-galactosidase has been carried out and the ability of this enzyme to perform homo- and hetero-condensation reactions to produce galacto-oligosaccharides, has been demonstrated.

Conclusions: Probiotics which are safe for human use and are capable of producing high levels of both α-galactosidase and β-galactosidase are of great importance to the food industry. In this work we have proven the ability of *B. coagulans* MA-13 to over-produce these two enzymes thus paving the way for its potential use in treatment of gastrointestinal diseases.

*Correspondence: contursi@unina.it
[1] Department of Biology, University of Naples Federico II, 80126 Naples, Italy
Full list of author information is available at the end of the article

Keywords: *Bacillus coagulans*, α-galactosidase, β-galactosidase, Transgalactosylation, Galacto-oligosaccharides, Prebiotics, Thermophilic

Background

Probiotic food production relies on the use of *Bifidobacterium*, lactic acid bacteria (LAB) as well as *Saccharomyces* species [1]. However, some *Bacillus* species have been tested as probiotics but their use is not as widespread as for traditional LAB and yeasts [2]. An attractive feature of *Bacillus spp* is the resistance to extremely harsh environments thanks to their ability to form spores and to grow under a relatively wide range of temperatures, usually up to ~ 60 °C [3, 4]. Within *Bacillus* genus, *B. coagulans* has been firstly discovered in spoiled canned milk and afterwards in other food sources. Recently, a novel thermophilic *B. coagulans* strain, designed as MA-13, has been isolated from canned beans manufacturing and shown to be able to produce lactic acid from lignocellulose biomass [5]. *B. coagulans* MA-13 turned out to be exceptionally resistant to extreme conditions, such as toxic compounds derived from the thermo-acidic treatment of lignocellulose, thus pointing to this microorganism as a good candidate as probiotic especially when harsh conditions are required for food manufacturing [5, 6].

One of the most challenging food consumption issues is how to ameliorate the digestibility of nutrients made up of complex sugars [7]. Indeed, the intake of foods containing some not-digestible galactosides is associated with their fermentation in the large intestine, thereby building up intestinal gas and discomfort. Among these oligosaccharides, raffinose family oligosaccharides (RFOs) (i.e. stachyose, raffinose, verbascose) are abundant in legumes and consist of α-(1,6)-D-galactose unit(s) [8], linked to sucrose. Moreover, β-(1,4)-D-galactose carbohydrates, such as lactose, are mainly present in dairy products [9].

In this context, α-galactosidases (EC 3.2.1.22) and β-galactosidases (EC 3.2.1.23) catalyze the hydrolysis of α-1,6 and β-1,4 linkages in oligo- and polysaccharides containing D-galactopyranosides, respectively. Since these enzymes are often lacking in the human intestine, it would be highly beneficial to find alternative means to deliver them into the digestive system [9]. Probiotic LAB and *Bifidobacteria*, which reside normally in the small intestine, might be used as source of digestive enzymes such as α- and β-galactosidases [10]. Previous studies have demonstrated that *Bifidobacteria* and *Lactobacilli* spp. can produce these enzymes; nevertheless, only few studies have focused on the production of both α- and β-galactosidases by the same strain [10–12]. These works have demonstrated that some probiotic microorganisms are able to express both the enzymes simultaneously when metal ions [12] or different nutrients [10, 11] were added to cultures media; however, the production of α- and β-galactosidases upon exposure to a single and inexpensive carbon source, has not yet been described. Considering the economic aspects related to large-scale enzyme production, the use of growth media containing renewable sources suitable to increase enzyme expression and reduce the cost of industrial processes, would be of great interest [13].

Among the enzymes active on not-digestible oligosaccharides, β-galactosidases are attractive not only for the hydrolysis of β-galactosyl linkages, but also for their ability to synthesize prebiotics, such as galactooligosaccharides (GOS) [14]. These are produced by transgalactosylation reactions, in which the glycosyl group of one or more D-galactosyl units is transferred onto another mono-or oligosaccharide acceptor yielding different GOS mixtures formed by di-, tri-, tetra-, and pentasaccharides [15]. Consequently, β-galactosidase producing microbes capable of performing transgalactosylation, can be used as microbial cell factories to produce GOS molecules for the selective stimulation of the gut microbiota [12].

Thermophilic microorganisms are of general interest for both basic [16–24] and applicative research [25–28]. Among them, several thermophilic strains of *B. coagulans* have been isolated and some glycosyl hydrolytic enzymes have been characterized [29–33]. Nevertheless, comprehensive studies about the intracellular and extracellular GH enzymes spectrum of this microorganism as well as the characterisation of the transglycosylation potential of β-galactosidase enzymes are lacking. In the present work, *B. coagulans* MA-13 was explored as a cell factory for the production of enzymes with the potential to produce GOS as well as to improve the digestibility and nutritional value of foods.

Methods

Cultivation conditions for detection of glycosyl hydrolases activity

Aliquots from *B. coagulans* MA-13 strain stored at − 80 °C were grown under standard conditions i.e. in Luria–Bertani liquid medium at 55 °C [5]. Cells were collected through centrifugation at 3000 × *g* for 15 min and homogenized by sonication (Sonicator heat system Ultrasonic Inc.) for 10 min, alternating 30 s of pulse-on and 30 s of pulse-off. Clarification of cell extracts was obtained through centrifugation at 40,000 × *g* for 30 min

at 4 °C. For analysis of the extracellular proteins, the supernatant was filtered under vacuum through 0.45 µm nylon membrane (Millipore). The filtrate (secretome) was concentrated 300-fold using an Amicon Ultrafiltration System (Millipore) with a 10 kDa cut-off nitrocellulose membrane (Millipore) at room temperature and a maximum pressure of 75 MPa. Samples were stored at 4 °C for further analysis. At least three independent biological replicates were carried out.

Functional annotation of *B. coagulans* MA-13 Glycosyl Hydrolase enzymes

Genome draft [34] was annotated by the Rapid Annotation Subsystem Technology (RAST) and dbCAN metaserver was used to generate a family classification from CAZy database [35]. The gene list was extracted by selecting the GH(s) reported in all the 3 databases used (HMMER, DIAMOND and Hotprep).

Screening of GH activities

Cell extract and secretome of *Bacillus coagulans* MA-13 were screened for enzymatic activities over a panel of synthetic substrates: *para*-Nitrophenyl-β-D-glucopyranoside (PNP-β-glu), *ortho*-Nitrophenyl-β-D-galactopyranoside (ONP-β-gal), *ortho*-Nitrophenyl-β-D-glucopyranoside (ONP-β-glu), *para*-Nitrophenyl-α-D-glucopyranoside (PNP-α-glu), *para*-Nitrophenyl-β-D-xylopyranoside (PNP-β-xyl), *para*-Nitrophenyl-α-D-mannopyranoside (PNP-α-man), *para*-Nitrophenyl-β-D-mannopyranoside (PNP-β-man), *para*-Nitrophenyl-β-L-fucopyranoside (PNP-β-fuc), *para*-Nitrophenyl-α-L-fucopyranoside (PNP-α-fuc), *para*-Nitrophenyl-α-L-rhamnopyranoside (PNP-α-rha), *para*-Nitrophenyl-β-D-galactopyranoside (PNP-β-gal), *para*-Nitrophenyl-α-D-galactopyranoside (PNP-α-gal), *para*-Nitrophenyl-α-L-arabinofuranoside (PNP-α-ara). Briefly, 0.8 µg of the intracellular and extracellular samples were added to the substrate (10 mM) in 100 mM citrate buffer pH 5.5 (final volume of 100 µl) and incubated in Synergy H4 Plate Reader at 55 °C. Enzymatic activity was measured by detecting the release of nitrophenol at 405 nm every 10 min up to 15 h. All the activities were expressed in International Units (U), corresponding to the quantity of enzyme(s) able to release 1 µmole of PNP-OH (millimolar extinction coefficient, 18.5 mM^{-1} cm^{-1}) or ONP-OH per minute (millimolar extinction coefficient, 4.6 mM^{-1} cm^{-1}). The experiments were carried out with at least three technical and biological replicates. The acceptable standard deviation was less than 20% of the mean.

Enzymatic activity on PNP-α-gal, ONP-β-gal, PNP-β-gal, PNP-α-ara, PNP-α-glu was investigated also through zymography in a 7% SDS PAGE as previously described [36]. After renaturation, the gel was incubated with 20 mM of each substrate at 55 °C for a time ranging between 10 and 60 min, until a clear halo of hydrolysis was visible on the gel. Activity bands were excised for identification of the enzyme(s) through mass spectrometry.

Selective Growth Conditions for expression of α- and β-galactosidases

B. coagulans MA-13 was grown under standard conditions up to exponential growth phase (0.5 OD_{600}/ml) and cells were collected through centrifugation at 3000 × *g* for 15 min. Pellets (25 OD_{600nm}) were washed with milliQ water before resuspension in 50 ml of selective media. For extracellular detection of α-galactosidase, the medium contained 0.1% yeast extract (YE) and either 1% locust bean gum or diverse agri-food residues (such as rice hull). Cells were collected along with the supernatant after 24 h. Cell extracts were prepared by resuspending pellets in lysis buffer, i.e. B-PER solution (Thermo Fisher Scientific) plus lysozyme (1 mg/ml) and then incubated at 37 °C for 1 h. The lysed cells were clarified through centrifugation at 40,000 × *g* for 20 min at 4 °C. Intra- and extracellular α-galactosidase activities were analysed by testing 0.25 µg and 20 µl of supernatant on 150 µl PNP-α-gal substrate (10 mM), respectively. The assays were incubated for 10 min under standard pH and T conditions (100 mM sodium citrate 5.5 and 55 °C) and the reaction was stopped by adding 150 µl 0.5 M Na_2CO_3 before detection at 405 nm. As control, the supernatant was tested for β-galactosidase activity. The supernatants were analysed also through zymography on PNP-α-gal, as described above [36].

For over-expression of the native β-galactosidase, pellets (5.0 OD_{600nm}) of *B. coagulans* MA-13 were resuspended in two different media, either 0.1% YE or 0.1% YE with 0.1% lactose. Cells were harvested once they reached the early stationary phase (0.8 OD_{600nm}/ml) and pellets were resuspended in B-PER solution as described before. For each sample, 5 µg of intracellular proteins were tested using 10 mM ONP-β-gal following the same procedure as described above.

Protein identification by LC–MS/MS analysis

Protein bands from SDS-PAGE corresponding to those positive in zymographic assays were excised and in situ digested with trypsin in 50 mM NH_4HCO_3, following treatment with 10 mM DTT (Sigma-Aldrich), for 45 min at 56 °C and then with 55 mM iodoacetamide (Sigma-Aldrich) in the dark at room temperature for 30 min for cysteines reduction and alkylation, respectively. Gel bands were then incubated with 10 ng/µl trypsin overnight at 37 °C. Peptide mixtures were extracted from the gel, and then acidified by 20% trifluoroacetic acid

(Sigma-Aldrich), and dried by a Speed-Vac system (Thermo Fisher Scientific, USA). Peptide mixtures were re-suspended in 0.2% Formic Acid and analysed by nano-LC–MS/MS on a 6530 Q-TOF LC/MS equipped with a CHIP-CUBE system and coupled with a capillary 1100 HPLC system (Agilent Technologies, Santa Clara, California, USA). Each peptide sample was then fractionated with a gradient of eluent B (0,2% formic acid, 95% acetonitrile LC–MS Grade) from 5 to 75% for 100 min and eluent A (0,2% formic acid, 2% acetonitrile LC–MS Grade). Data Dependent Acquisition method was set as follows: MS scan range was from 300 to 2400 m/z; MS/MS scans from 100 to 2000 m/z were acquired for the five most abundant +2 or +3 charged precursor ions (top 5) in each MS scan, applying a dynamic exclusion window of 30 s.

LC–MS/MS raw data were processed and then employed for protein identification by using licensed Mascot software (Matrix Science, Boston, USA) to search in a protein-encoding genes (PEGs) database containing *B. coagulans* MA-13 predicted protein sequences. The main parameters employed for identifications were: mass tolerance value of 10 ppm for precursor ions and 0.6 Da for MS/MS fragments; trypsin as the proteolytic enzyme; missed cleavages maximum value of 1; Cys carbamidomethylation as fixed modifications; pyroglutamate (peptide N-terminal Gln) and Met oxidation as variable modifications. Candidates with at least 2 assigned peptides with an individual MASCOT score > 10 were considered significant for identification [37].

The identified proteins were compared to sequences present in a complete annotated database (UniProt) by using BLAST Search Form. Best alignments showing the minimum value for E- values were considered.

Cloning and sequencing of the β-galactosidase gene

A single colony of *B. coagulans* MA-13 was inoculated into LB liquid medium and genomic DNA was isolated using the LETS (lithium, EDTA, Tris, and SDS) buffer method [34, 38]. The gene (locus tag: E2E33_010705), encoding for a putative β-galactosidase, was amplified by polymerase chain reaction (PCR) using the primers 5′GAGGAATGCGTGCCATGGTAAAAAACAT3′ (*Nco*I restriction site is underlined), 5′ATCCGGGCG CCTCGAGTTTTTCAATTAC3′ (*Xho*I restriction site is underlined) and Taq DNA Polymerase (Thermo Fisher Scientific). The amplification was performed with an initial denaturation at 95 °C for 3 min, followed by 25 cycles (95 °C for 30 s, 58 °C for 45 s and 72 °C for 75 s) and a final extension step at 72 °C for 10 min. The PCR products were checked by agarose gel electrophoresis and subsequently purified with QIAquick PCR purification kit (Qiagen Spa, Milan, Italy). Afterwards, the purified product was cloned in pCR4-TOPO-vector (TOPO TA CLONING Kit, Invitrogen) and its identity was confirmed by DNA sequencing (Eurofins Genomics). The insert then was subcloned in pET28b(+) vector (Novagen) using *NcoI*/*XhoI* restriction enzymes and T4 DNA ligase (Promega).

Expression and purification of recombinant *Bc*GalB

The vector pET28b/*Bc*GalB containing the β-galactosidase gene was used for transforming *E. coli* Rosetta™(DE3) pLysS cells in order to express the recombinant protein bearing a C-terminus His-tag. The transformants were selected on LB agar plates containing 50 μg/ml kanamycin and 33 μg/ml chloramphenicol. A single colony was inoculated in 50 ml LB medium with antibiotics and incubated on an orbital shaker (180 rpm at 37 °C). Cells were diluted in 1 L of LB at 0.06–0.08 OD_{600nm} and once the culture reached 0.5–0.6 OD_{600nm} protein expression was induced overnight by adding 0.5 mM of IPTG. Cells were harvested by centrifugation at 4000 × *g* and resuspended in 100 mM sodium-phosphate pH 8.0 supplemented with a protease inhibitor cocktail tablet (Roche). Subsequently, the cells were disrupted by sonication (Sonicator: Heat System Ultrasonic, Inc.) for 10 min, alternating 30 s of pulse-on, and 30 s of pulse-off and the suspension was clarified by a centrifugation step at 40,000 × *g* for 30 min at 4 °C. *Bc*GalB was purified to the homogeneity by affinity chromatography on HisTrap column (1 mL, GE Healthcare) connected to an AKTA Explorer system. The column was equilibrated with 100 mM of sodium-phosphate pH 8.0 and 500 mM of sodium chloride buffer and elution was performed with a linear gradient of imidazole (0–250 mM). All the peak fractions were pooled and then dialyzed against 100 mM of sodium-phosphate pH 8.0 and 50 mM of sodium chloride (storage buffer). Protein concentration was estimated by Bradford assay using bovine serum albumin as standard. The monomeric molecular mass of *Bc*GalB was evaluated by SDS-PAGE analysis (12%) and purity degree was evaluated by staining the gel with Coomassie brilliant blue R-250.

Molecular weight determination of *Bc*GalB

The native molecular weight of *Bc*GalB was obtained by gel-filtration chromatography connected to Mini DAWN Treos light-scattering system (Wyatt Technology) equipped with a QELS (quasi-elastic light scattering) module mass value and hydrodynamic radius (Rh) measurements. One milligram of protein (1 mg/ml) was loaded on a S200 column (16/60 GE Healthcare) with a flow-rate of 0.5 ml/min and equilibrated in 100 mM of sodium-phosphate pH 8.0, 1 mM DTT. Data were analyzed using Astra 5.3.4.14 software (Wyatt Technology).

pH and temperature profiles of *Bc*GalB

The optimal pH value was determined by assaying 10 ng (≃ 0.04 Hydrolytic Units, U) of *Bc*GalB at 60 °C using ONP-β-gal as substrate in a pH range from 4.0 to 10.0. The following buffers (each 100 mM): sodium citrate (4.0–6.0), sodium phosphate (6.0–8.0), and glycine–NaOH (8.6–10.0) were used to prepare the different substrate mixtures containing 10 mM ONP-β-gal. The temperature dependence of *Bc*GalB activity was studied by assaying the enzyme from 30 to 90 °C in 0.1 M sodium phosphate pH 6.0 on ONP-β-gal.

Once determined the pH and temperature dependence of the enzyme, all the subsequent assays were performed using a reaction mixture containing 10 mM ONP-β-gal, 100 mM sodium citrate buffer pH 5.0 and ≃ 0.04 U of *Bc*GalB. Briefly, the substrate mix was incubated at 60 °C for 3 min, before adding the enzyme. The reaction was stopped after 3 min of incubation, by the addition of cold sodium carbonate 1.0 M. The concentration of the released ortho-nitrophenol (millimolar extinction coefficient, 4.6 mM^{-1} cm^{-1}) was evaluated by measuring the absorbance of the mixture at 405 nm. The pH stability and thermal inactivation were analyzed by incubating the enzyme in sodium citrate (4.0–6.0), sodium phosphate (6.0–8.0) and at 45°, 50°, 55° and 60 °C, respectively. Aliquots of *Bc*GalB were withdrawn at regular time intervals to measure the residual activity under standard conditions.

Effect of metal ions, chemicals, and monosaccharides on enzyme activity

To test the effect of metal ions on enzymatic activity, *Bc*GalB was dialysed in storage buffer supplemented with 10 mM EDTA for 2 h to get rid of metal ions present in the protein preparation. Afterwards, EDTA was removed through extensive dialysis in storage buffer. *Bc*GalB was incubated with metal ions for 5 min at room temperature (Mg^{2+}, Ca^{2+}, Zn^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, K^{+}, Li^{+}, Cu^{2+}, Fe^{3+}, Na^{+}) at 2 mM concentration and the enzymatic activity was measured under standard conditions with the addition of 2 mM of each metal ion in the mix assay. In relation to the activity of *Bc*GalB on lactose, the effect of Ca^{2+} was also evaluated in a reaction mixture containing 2 mM of Ca^{2+}, 150 mM lactose and ≃0.04 U.

Furthermore, the inhibition effect of chemicals on *Bc*GalB activity was also tested. Non-ionic (Tween-20), ionic (SDS) detergents, reducing (DTT, β-mercaptoethanol), chelating (EDTA) and chaotropic (urea, guanidinium chloride) agents were added to the enzyme solution at 50 mM concentration for 5 min and residual activity was assayed under standard conditions.

Finally, the influence of monosaccharides on the enzymatic activity was studied too. IC_{50} (half maximal inhibitory concentration) was calculated by incubating the enzyme in the presence of D-xylose or D-arabinose or D-glucose or D-galactose or a mix of these two latter at different concentration values (0–100 mM), for 5 min at room temperature and assaying the enzymatic activity in the presence of the monosaccharides.

Substrate specificity and kinetic parameters of *Bc*GalB

The hydrolytic activity of *Bc*GalB was tested on several substrates: PNP-β-glu, ONP-β-glu, PNP-α-glu, PNP-β-xyl, PNP-α-man, PNP-β-man, PNP-β-fuc, PNP-α-fuc, PNP-α-rha, ONP-β-gal, PNP-β-gal, PNP-α-gal, PNP-α-ara and D-lactose. The enzyme was incubated in presence of 10 mM of each substrate under standard assay conditions. When lactose was used, the amount of free-glucose released upon hydrolysis was determined using D-Glucose Assay Kit (GOPOD Format, Megazyme) according to the manufacturer's protocol. One unit (U) is defined as the amount of enzyme required to release 1 µmol of glucose per min. In order to study the kinetic parameters of the enzyme, different concentration values of ONP-β-gal (0.1 to 20 mM) and lactose (0–500 mM) were tested. The Michaelis–Menten constant (K_M) and V_{max} were calculated by non-linear regression analysis using GraphPad 9.0 Prism software.

Analysis of the transgalactosylation activity of *Bc*GalB by Thin-Layer Chromatography (TLC)

The transgalactosylation experiments were performed to study homo- and hetero-condensation reactions under standard conditions (100 mM sodium citrate pH 5.0 and 60 °C). The final volume of all the reactions was 800 µl and contained 2.2 U (0.5 µg) of the enzyme mixed with 80 mM of ONP-β-gal or 35.0 U of *Bc*GalB (8 µg) with 160 mM lactose, respectively. For the hetero-condensation reactions, 40 mM ONP-β-gal was employed as donor and 40 mM PNP-β-glc or PNP-β-xyl as acceptors, in a final volume of 800 µl containing 2.2 U. These reaction conditions were established after preliminary tests (data not shown) in which different donor:acceptor ratios as well as of the enzyme amounts, were tested. Aliquots of reaction mixtures were collected at different time intervals (up to 18 h), and the reactions were stopped by incubation in dry ice for 5 min. Control reactions without enzyme were included in the analysis. The products were analyzed by TLC on silica gel 60 (F254, 0.25 mm) plates (Merck, Darmstadt, Germany) and separated using ethyl acetate/methanol/ddH_2O (70:20:10 v/v) as eluent, or butanol/ethanol/ddH_2O (50:30:20 v/v), for the detection of GOS from lactose. Aliquots corresponding to 0.2–2.0% of the total reaction mixture were loaded onto the TLC plate. For the detection of sugars, the

TLC plates were soaked in a staining solution consisting of 4% of 1-naphthol in 10% sulphuric acid in ethanol followed by heating at 120 °C.

ESI–MS Analysis of the galactooligosaccharides (GOS)

Transgalactosylation products were analyzed by direct ESI–MS procedure from reactions carried out for 18 h: all samples were diluted in 5% acetic acid and analyzed on a Q-ToF Premier (Waters, Milford, MA, USA), in positive mode, by direct injection into the ESI source at a flow of 10 µL/min. The source parameters were set as follows: capillary voltage = 3 kV and cone voltage = 42 kV. The acquisition range was set between 100 and 1000 m/z. All data were processed by using Mass Lynx 4.1 software (Waters, Milford, MA, USA).

Results and discussion

The aim of this work was to exploit the potential of *B. coagulans* MA-13 as a source of enzymes to improve the hydrolysis of oligosaccharides that are indigestible by the human gut, and to produce prebiotics, such as galactooligosaccharides. A combination of mass spectrometry-based omics technologies with conventional biochemical approaches has been employed to investigate on the applicative potential of *B. coagulans* MA-13 in these biotechnological contexts.

Screening and identification of the glycosyl hydrolases activities of *Bacillus coagulans* MA-13

Annotation of glycosyl hydrolases

Whilst a full functional annotation of *B. coagulans* MA-13 genome is under way (manuscript in preparation), herein, the annotation of the GH(s) repertoire using dbCAN2 meta server, is shown [35] (Table 1). Seventeen enzymes have been identified, among which some families (GH3, GH15, GH32, GH36, GH42, GH70, GH73) are represented by a single member, whereas all the others include diverse glycosyl hydrolases. A set of GH(s) connected to starch degradation which includes GH13 and GH65 representatives, mirrors the isolation source of *B. coagulans* MA-13, i.e. canned beans manufacturing which is particularly rich in starch [5, 39].

Three GHs members belonging to families 18 and 73 are related to the sporulation pathway of *B. coagulans*. Few Carbohydrate-Binding Modules (CBMs) were found in association with GH13 and GH18 members. The presence of a sucrose-6-phosphate hydrolase (GH32) is in line with the capability of *B. coagulans* MA-13 to use molasses as an inexpensive sucrose-rich carbon source [6]. Finally, GH36 and GH42 members have been identified and interestingly lactic bacteria producing both α- and β-galactosidases are relevant for the food industry [10].

Table 1 Predicted GH in the genome of *B. coagulans* MA-13

NCBI Reference Sequence	GH family	Signal Peptide	RAST annotation
WP_195850490.1	GH3	N	β-glycosyl hydrolase
WP_019720988.1	CBM34 + GH13_20	N	Neopullulanase (EC 3.2.1.135)
WP_133536160.1	GH13_31	N	Oligo-1,6-glucosidase (EC 3.2.1.10)
WP_195850265.1	GH13_31	N	Oligo-1,6-glucosidase (EC 3.2.1.10)
WP_133536961.1	GH13_5	N	Glucan 1,4-α-maltohexaosidase (EC 3.2.1.98)
WP_195850265.1	CBM48 + GH13_9	N	1,4-α-glucan (glycogen) branching enzyme (EC 2.4.1.18)
WP_061575462.1	GH0	N	Phosphorylase b kinase regulatory subunit β
WP_133537568.1	CBM50 + GH18	N	Spore cortex-lytic enzyme, N-acetylglucosaminidase SleL
WP_133536804.1	CBM50 + GH18	N	spore peptidoglycan hydrolase (N-acetylglucosaminidase) (EC 3.2.1.-)
WP_133537667.1	GH32	N	Sucrose-6-phosphate hydrolase (EC 3.2.1.26)
WP_133537615.1	GH36	N	α-galactosidase (EC 3.2.1.22)
WP_133536219.1	GH42	N	β-galactosidase (EC 3.2.1.23)
WP_133536548.1	GH65	N	α,α-trehalose phosphorylase (2.4.1.64)
WP_133536158.1	GH65	N	Maltose phosphorylase (EC 2.4.1.8)
WP_133536578.1	GH65	N	Maltose phosphorylase (EC 2.4.1.8)
WP_133537168.1	GH70	Y (1–34)	hypothetical protein
WP_195850162.1	GH73	N	endo-β-N-acetylglucosaminidase (EC 3.2.1.96)

The automated CAZyme annotation has been carried out using dbCAN2 metaserver, integrated with HMMER, DIAMOND and Hotprep databases [35]. The presence of signal peptides in the proteins are reported as: Y = 100%, N = 0%. *GH* Glycosyl hydrolases, *CBM* carbohydrate-binding modules

Screening of the intracellular and extracellular GH activities

To discover GH enzymatic activities, intracellular cell extracts and secretome of *B. coagulans* MA-13 were tested on a panel of artificial substrates. Cells were cultivated in LB rich medium with the purpose of detecting a baseline of activities under standard growing conditions. Cultures were collected at exponential growth phase (0.5–0.6 OD_{600nm}) and 0.8 µg of total intracellular and extracellular protein preparations were assayed over the following substrates: PNP-β-glu, ONP-β-glu, PNP-α-glu, PNP-β-xyl, PNP-α-man, PNP-β-man, PNP-β-fuc, PNP-α-fuc, PNP-α-rha, ONP-β-gal, PNP-β-gal, PNP-α-gal, PNP-α-ara.

Intracellular enzymatic activities were revealed only on a subset of substrates, i.e. ONP-β-gal, PNP-β-gal, PNP-α-gal, PNP-α-glu, PNP-α-ara (Fig. 1). By comparing these findings with the annotated list of *B. coagulans* MA-13 GHs, the hydrolytic activity towards PNP-α-glu was traced back to representative(s) of the GH13 family (subfamily 31). However, the correlation with the activity on PNP-α-ara is not obvious (Table 1). The hydrolysis of β- (ONP-β-gal, PNP-β-gal) and α-galactosidic (PNP-α-gal) linkages might be linked to the GH42 and GH36 members, respectively (Fig. 1). Indeed, *B. coagulans* MA-13 genome bears two genes, i.e. locus tag: E2E33_010705 (WP_133536219.1) and locus tag: E2E33_000265 (WP_133537615.1), encoding for a GH42 and for a GH36, respectively (Table 1). As shown in Fig. 1, the specific activity recorded on ONP-β-gal and PNP-α-gal was significantly higher than on other substrates tested.

The presence of secreted GHs was verified by testing supernatants on the same substrates and the only relevant activity was detected on PNP-α-gal (Fig. 1). All together, these results indicate that enzymes hydrolyzing β- and α-galactosidic linkages represent the most relevant activities under standard growing conditions (LB medium). These enzymes catalyze the hydrolysis of terminally joined galactosidic residues in simple galactose-containing oligosaccharides as well as in complex polysaccharides and have the potential to improve the digestibility of some RFO-containing food and of milk-based products [14, 15].

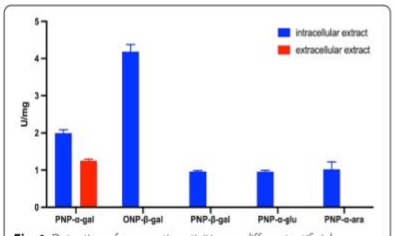

Fig. 1 Detection of enzymatic activities on different artificial substrates

Identification of the hydrolytic activities through mass spectrometry

To identify the enzymes involved in the hydrolysis of β-galactosidic linkages, cell extracts were analysed through zymography. Active-bands on ONP-β-gal and PNP-β-gal resided in the same upper gel region (not shown). These bands were excised, the proteins were in-gel trypsinized and the peptides were extracted and analysed by LC–ESI–MS/MS. Proteins were identified by using MASCOT search engine to explore *B. coagulans* MA-13 protein database. The sequences were compared to those present in a complete annotated database (UniProt) by using BLAST Search Form and the best alignments (minimum E-value) were obtained towards *B. coagulans* strain 36D1. As expected, α-galactosidase (Uniprot code: G2TQE8) was identified both in intra- and extracellular protein extracts together with other unrelated co-migrating proteins. The putative GH42 (Uniprot code: G2TH90) was recognized as the only enzyme potentially responsible for the hydrolytic activity on PNP-β-gal, since the other co-migrating proteins/enzymes clearly belonged to unrelated metabolic pathways (Additional file 1: Table S1). The only exception was a GH36 member (Uniprot code: G2TQE8), that, based on CAZy classification, is however not predicted to be active on PNP-β-gal substrate. Hence, the presence of this enzyme is explainable with similar migration properties to GH42 in the zymography gel. From a first inspection of proteins identified within bands active on ONP-β-gal with at least two peptides, no enzymes linked to the hydrolysis of β-1–4 linkages were found. Decreasing the detection threshold up to one peptide, a β-galactosidase (Uniprot code: G2TQE8) was detected (Additional file 1: Table S1). Overall, the results obtained from enzymatic screening and mass spectrometry analysis indicated the presence of a single enzyme (GH42, accession number: MBF8418755) involved in the hydrolysis of β-linkages. The enzyme specific activity associated to ONP-β-gal and PNP-β-gal was particularly high (Fig. 1), thus suggesting that either the enzyme was over-expressed under basal growth conditions or its specific activity was significantly high. To assess the culture conditions suitable to further increase the expression levels of β-galactosidase, the induction profile of this enzyme using a selective medium was analysed. By adding 0.1% lactose into a minimal medium (0.1% yeast), a significant increase (~30-fold) of the β-galactosidase activity was observed (Additional file 2: Figure S1). This result is not surprising considering that most β-galactosidases play a major role in lactose

metabolism and this substrate is the best carbon source to induce their maximum production in Gram + and Gram- bacteria [40, 41].

Furthermore, both cell extracts and secretome of *B. coagulans* MA-13 cells grown in LB medium were tested on PNP-α-gal, since hydrolytic activity on α-linkages was detected inside and outside the cells (Fig. 1). The activity bands of intra- and extra-cellular proteins displayed the same electrophoretic mobility, lying within the 130–180 KDa gel region. As shown by mass spectrometry analysis, the GH 36 (Uniprot code: G2TQE8) was found in both samples (Additional file 1: Table S1) suggesting that it might exert its hydrolytic activity on intracellular and extracellular α-1–6 galactans. The list of intracellular proteins identified through mass spectrometry analysis included also another GH enzyme (namely, an arabinogalactan endo-β-1,4-galactanase), however, this latter was not found in the annotated *B. coagulans* MA-13 genome (Additional file 1: Tale S1 and Table 1). The remaining co-migrating proteins identified in the extracellular and intracellular samples were related to other metabolic pathways (Additional file 1: Table S1).

The analysis of the protein sequence did not highlight any typical signal peptide (Tat or Sec system) at the N-terminus of α-galactosidase through dbCAN database (Table 1), thus raising questions on how this protein is actually secreted and why this enzyme has a dual cellular localization. A reasonable explanation is that *B. coagulans* MA-13 exploits a leader-less secretion system, namely ESAT-6 Secretion System (ESS), which has been discovered in Firmicutes and Actinobacteria [42–44]. In this system, proteins lacking a canonical signal peptide can be secreted through the combined action of two molecular components, namely EcsA and EcsB. The relative genes are both present in the *B. coagulans* MA-13 genome (Additional file 2: Figure S2) and are arranged in a cluster, likewise for other *B. coagulans* strains (not shown) and bacteria [42–44]. In some cases, a third molecular partner (EcsC) is associated to the same cluster but its role seems to be dispensable for the secretion pathway (42). Moreover, many of the proteins secreted through ESS share some distinguishing and conserved features that include a WXG amino acid motif in the central region of the protein. Interestingly, this motif has been identified in the middle of the sequence (W_{368} and G_{370}) of the α-galactosidase (Accession number: MBF8416840, 730 aa) as well in the enolase (Uniprot code: G2TP79) which was found extracellularly along with the α-galactosidase (Additional file 1: Table S1).

To further confirm the presence of this enzyme in the supernatant, *B. coagulans* MA-13 was grown in a minimal medium supplemented with galactomannans (locust bean gum). These are insoluble polymers that cannot be translocated inside cells and bear α-1,6-linkages, thus being natural potential substrates of α-galactosidases. Enzymatic assays carried out on the supernatants using PNP-α-gal as a substrate, revealed that α-galactosidase was induced (about fourfold) in the presence of galactomannans compared to the control cells cultivated only in yeast (Fig. 2a). Moreover, the analysis of cell extract indicated that the levels of intracellular and extracellular enzymatic activities were similar. Conversely, the distribution of α-galactosidase was strongly biased toward its intracellular localisation when yeast was used as the only carbon source (Fig. 2a), thus suggesting that the presence in the medium of a galactose-containing polymer, such as locust bean gum, plays a role in the secretion of α-galactosidase. All together, these findings, along with the lack of a mannanase gene in *B. coagulans* MA-13 genome (Table 1), strongly supports the hypothesis that this microorganism can rely solely on the activity of an external α-galactosidase to metabolise

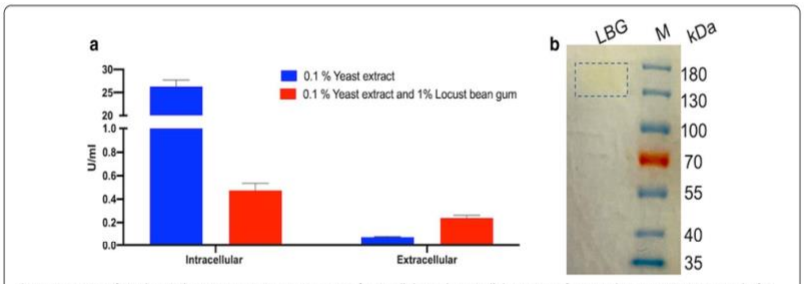

Fig. 2 Detection of α-galactosidase activity. **a** Enzymatic assays of intracellular and extracellular extract of *B. coagulans* MA-13 on PNP-α-gal, after growth on selective medium containing locust bean gum. **b** Zymogram of supernatants from *B. coagulans* MA-13 cells grown on locust bean gum medium, using PNP-α-gal as substrate

these galactomannans. By assaying the supernatants of locust bean gum grown cells through zymography, the α-galactosidase activity was promptly revealed (Fig. 2b) and a similar result was obtained by using other complex carbon sources derived from agri-food wastes, such as rice hull (not shown). Enzymes identification by mass spectrometry was hindered by a strong contamination of polymers probably deriving from the substrates used for the detection of the in-gel activity. However, the electrophoretic mobility of this band (within the 130–180 KDa gel region) was identical to that identified as GH36 (Uniprot code: G2TQE8, Additional file 1: Table S1) thus indicating that the enzymatic activity revealed by zymography, can be ascribed to the same protein. Besides our experimental evidences, the extracellular localization of the α-galactosidase has been previously described for another closely related *B. coagulans* strain [31, 45] as well as for other soil microorganisms [46] and for *Bacillus megaterium* [47]. It is known that galactomannans are present in seeds of bean and, in general, RFOs (raffinose, stachyose, and verbascose) that contain α 1–6-linked galactose units, are particularly abundant in these legumes [9]. Since *B. coagulans* MA-13 was isolated from manufactured canned beans, the α-galactosidase, along with the β-galactosidase might be a key enzyme for the host metabolism. Indeed, manufacturing bean wastes represent a lactose-free environment. However, other genes encoding GH42 enzymes from prokaryotes are unlikely to encounter lactose, suggesting that the substrate for these enzymes in their natural environment, might also be more complex oligo- and polysaccharides [48].

The experimental evidence of the induction of β-galactosidase expression upon exposure to lactose prompted us to analyze the effect of this inexpensive substrate also on α-galactosidase production. Indeed, previous studies have reported the induction of this enzyme on galactose-containing oligosaccharides or galactose [49]. Hence, the enzymatic activity on PNP-α-gal was also measured in lactose medium and a twofold induction was observed (Additional file 2: Figure S1). However, it is not clear whether the true inducer of *B. coagulans* MA-13 α-galactosidase is lactose or galactose; indeed, the latter might be produced at high intracellular concentration as a hydrolysis product of the over-expressed β-galactosidase in the presence of lactose. Application wise, the setup of suitable growth conditions suitable for the expression of both β-and α-galactosidases is highly relevant and only a few studies have described the production of both enzymes by the same strain [11, 12, 49].

A thoroughly biochemical characterization of a closely related recombinant α-galactosidase from *B. coagulans* ATCC 7050 (identity percentage 97.4%) has been recently published [31]. Therefore, we focused on the study of the β-galactosidase enzyme, since there is no evidence about the ability of β-galactosidases from other *B. coagulans* strains to produce GOS upon transglycosylation reactions.

Sequence analysis, cloning and expression of *Bc*GalB

The gene (E2E33_010705) encoding for the putative β-galactosidase (herein named as *Bc*GalB), has been identified within a cluster of genes encoding for a *lacI* family regulator, a hypothetical Major Facilitator Superfamily Transporter related to multi-drug resistance mechanisms and other small hypothetical proteins. This genetic arrangement is also present in *B. coagulans* ATCC 7050. The gene is, therefore, not included in an operon encoding also for a lactose-permease and a transacetylase, likewise the well-known *E. coli lac*-operon. Specifically, the hypothetical galactose-lactose permease encoding sequence is quite distant (≃7,000 nt) from the *Bc*GalB gene, thus suggesting that its expression might not be subjected to the same regulative circuit of the *lac* operon, which consists of the concomitant over-expression of the permease after exposure of cells to lactose. Accordingly, repression of *Bc*GalB in lactose-free medium, as described for *E. coli*, was not observed; rather, the enzyme was constitutively expressed under standard growth conditions and the induction fold in the presence of lactose was significant but quite low if compared to other systems (Fig. 1) [50].

There is no report about any transcriptional cross-regulation which might account for the genetic proximity of β-galactosidase gene to choline-operon. The only functional connection has been found in a β-galactosidase from *Streptococcus mitis*, which bears a Choline Binding Domain (CBD) at its C-terminus. However, this β-galactosidase uses CBD domain as an attachment anchor to molecular components (such as lipo-teichoic acids) to bind to cell-wall. Instead, *Bc*GalB has an intracellular localization and therefore this genetic juxtaposition remains murky (Fig. 3) [51]. *Bc*GalB bears three typical domains of the GH42 family as suggested by CD-Search and other reports [33, 52]. E2E33_010705 was amplified by PCR from the genomic DNA of *B. coagulans* MA-13 and expressed in *E. coli* Rosetta™(DE3) pLysS cells as a soluble, intracellular histidine-tagged protein (C-terminus). The overexpression system and purification method applied were quite efficient, since the enzyme was purified to homogeneity by His-trap affinity chromatography, (~10 mg for 1 L of culture) with an yield of 82% (Additional file 3: Table S2). As revealed by the SDS-PAGE analysis (Additional file 2: Figure S3), *Bc*GalB migrated as a single band with an apparent molecular mass of ~75 kDa. This concurred with the molecular

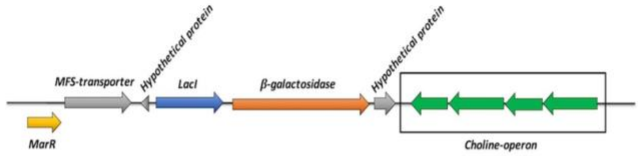

Fig. 3 β-galactosidase genomic context in *Bacillus coagulans* MA-13

mass of *Bc*GalB deduced from the nucleotide sequence of the E2E33_010705 gene and the identity of the protein was verified by mass spectrometry (data not shown). The recombinant protein was analyzed by size-exclusion chromatography coupled with a triple-angle light scattering QELS. This analysis revealed that *Bc*GalB is a hexamer in solution (not shown). Since seven cysteines are present on the *Bc*GalB sequence, the enzyme was analyzed on SDS-PAGE in the presence of β-mercaptoethanol as a reducing agent (Additional file 2: Figure S3). *Bc*GalB was present only in monomeric form under this condition, thus pointing to the role of at least some of the cysteines in the oligomerization state. It is worth noting that β-galactosidases can be found in diverse oligomeric forms, such as dimeric (halophilic *Haloferax alicantei* [53]), trimeric (thermophilic *Geobacillus stearothermophilus* [54]), tetrameric (acidophilic archaeon *Sulfolobus solfataricus* [55]) and hexameric (hyperthermophilic *Thermotoga maritima* [56]) arrangements. This latter structure is uncommon among thermophilic GH42 members, whereas some GH2 β-galactosidases exhibit this supramolecular organization. To the best of our knowledge, the correlation between the hexameric structure and biochemical features of β-galactosidases is not obvious although a general correlation between oligomeric states and thermal stability has been proposed for thermophilic enzymes [36, 57].

Characterization and stability properties of *Bc*GalB

The influence of pH and temperature on the enzymatic activity was evaluated using ONP-β-gal as a substrate. After testing the enzyme in the interval 4.0–10.0, the optimal pH was set at 5.0 (Fig. 4a). Interestingly, *Bc*GalB retained 70% of its activity from 5.0 to 7.0 whilst a sharp decrease was observed at pH 4.0 (Fig. 4a). Despite this drop at acidic pH values, the enzyme exhibited a significant stability at different pH values, ranging from acidic to alkaline ones.

As shown in Fig. 4b, the enzyme retained more than 70% of its activity up to 24 h in the pH range from 5.0 to 7.0 (Additional file 2: Figure S4). The exploitation of β-galactosidases in the dairy industry is related to the optimal pH for hydrolysis [58]. Lactose is a hygroscopic sugar characterized by low solubility that causes crystallization as well as technological issues for certain products in the dairy industry. The solubility and sweetness can be increased by lactose hydrolysis into the two glucose and galactose units [59]. Hence, the feature of stability in a wide range of pH values points to *Bc*GalB as a suitable tool for slightly acid and/or sweet hydrolysis of whey.

From an industrial point of view, the enzymes should be stable both at low (preventing the proliferation of microorganisms and nutrients in milk) and at high temperatures (pasteurization) [59]. The dependence of *Bc*GalB on temperature was studied and the maximal activity was found at 60 °C (Fig. 4C), which is quite similar to that of β-galactosidases from other *B. coagulans* strains [32, 33, 60]. Moreover, *Bc*GalB exhibited high stability at a temperature of 50 °C given that approximately 60% of its initial activity was retained after incubation for up to 24 h (Fig. 4d). Moreover, the half-life at its optimal temperature was 4 h (Fig. 4d). The loss of activity at 60 °C is counterbalanced by the high specificity activity of *Bc*GalB (i.e. about 4300 U/mg, Additional file 3: Table S2) meaning that the catalytic performance of the enzyme is still consistent for an efficient hydrolysis at high temperature by employing small quantities of protein. Interestingly, the thermophilic nature and thermal stability of *Bc*GalB is exploitable for the production of lactose-free dairy products by coupling the thermization to the hydrolysis of lactose preventing microbial contamination, decreasing viscosities of the substrate solution and reducing the cost of the whole process [59].

Finally, enzymes employed in the preparation of lactose-free products are positively selected for their relatively high activity at neutral pH and stability at low temperature [59]. In this regard, the high specific activity of *Bc*GalB at neutral pH and its stability at 4 °C for up to several months match the chemical physical requirements of this biotechnological application.

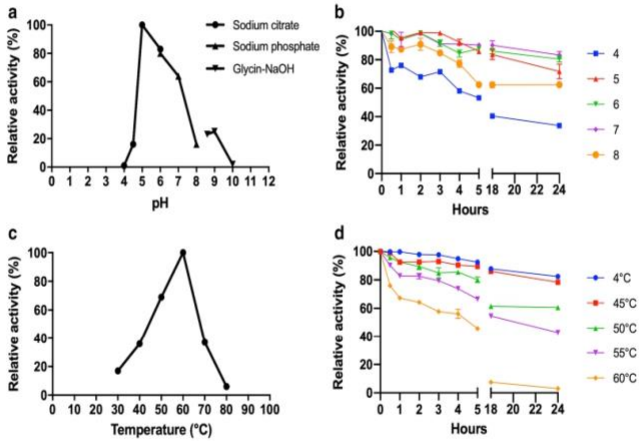

Fig. 4 Effect of pH and temperature on the enzymatic activity. **a** The pH dependence was evaluated in different buffers ranging from pH 4.0 to pH 10.0. **b** The pH stability was studied by incubating *Bc*GalB in different buffers ranging from pH 4.0 to 8.0 up to 24 h. **c** Temperature optimum was determined by testing the enzyme in the range 30–80 °C. **d** For thermostability studies, the recombinant enzyme was incubated at different temperatures ranging from 4 to 60 °C up to 24 h

Effects of metal ions and monosaccharides on *Bc*GalB activity

It is well known that ions affect the catalytic performance of β-galactosidases. For instance, the activity of yeast enzymes isolated from *Kluyveromyces lactis* and *K. fragilis* depends on the presence of Mn^{2+} or Na^{+}, and Mn^{2+}, Mg^{2+}, K^{+}, respectively [61]. Moreover, some metal ions such as Ca^{2+}, Mg^{2+} and Mn^{2+} can act as cofactors for β-galactosidases and their presence might significantly enhance their activities. Finally, it has been reported that Ca^{2+} and heavy metals inhibit the enzyme activity of several β-galactosidases. For the examination of the metal ion requirements, *Bc*GalB was assayed in the presence of 1 mM mono- and divalent ions after dialysis of the enzyme in 10 mM EDTA. Results from this study were overall in agreement with former analyses conducted on other *B. coagulans* β-galactosidases (Additional file 2: figure S5) [32, 33, 60]. Since Ca^{2+} is one of the prime elements in milk, dairy-industries processes would benefit from enzymatic activities not affected by Ca^{2+}. In this regard the negligible effect of this ion up to 2 mM on the hydrolytic capability of *Bc*GalB, makes this enzyme an attractive candidate in these applications. Cu^{2+} is the only ion affecting the enzyme activity (60% reduction), as reported for other β-galactosidases. Indeed, some metal ions, such as Fe^{3+} and Cu^{2+}, could inactivate the enzyme by inducing structural changes upon interaction with the protein [62, 63].

In order to foresee the employment of *Bc*GalB in the manufacturing of lactose-free products, the effect of galactose and glucose on enzyme activity was also studied. The inhibitory effect exerted by the lactose hydrolysis products on *Bc*GalB activity seems different from previous studies since glucose affected the *Bc*GalB enzymatic activity more than galactose (Fig. 5). Moreover, since lactose hydrolysis produces equimolar amounts of the two sugar units, we resolved to investigate the combined influence of galactose and glucose. A stronger decrease of the enzymatic activity was observed especially at high concentration of the sugars although the effect is not additive. Furthermore, xylose and arabinose were included in these experiments since the former is an acceptor of transgalactosylation reactions whereas the latter is one of the substrates of *Bc*GalB (see below). These two monosaccharides had a minor effect on the enzymatic activity compared to galactose and glucose, since *Bc*GalB retained at least 66% of the activity at the highest concentrations tested (Fig. 5). Finally, as part of the general biochemical characterisation of *Bc*GalB, the effect of surfactants (SDS and Tween 20), reducing (DTT and β-mercaptoethanol) and chaotropic (urea and guanidine chloride) agents, was studied. The enzyme

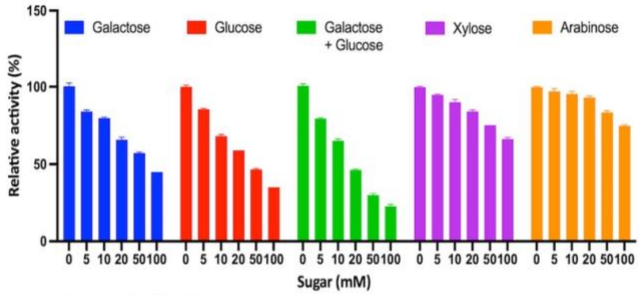

Fig. 5 Inhibitory effect of sugars on *Bc*GalB hydrolytic activity

Table 2 Substrate specificity of *Bc*GalB

Substrate	Specific activity (U/mg)
ONP-β-gal	4373.4 ± 77.6
PNP-β-gal	795.9 ± 3.9
PNP-α-ara	328.5 ± 11.6
PNP-β-xyl	N.D
PNP-β-glu	N.D
D-lactose	1283.0 ± 24.7

The highest activity of *Bc*GalB is toward ONP-β-gal, whilst the enzyme is not active on the arylic compounds PNP-β-xyl and PNP-β-glu. These latter two substrates are shown since they have been used in transglycosylation reactions (see below). *N.D.* not detected

Table 3 Kinetic parameters of *Bc*GalB

Substrate	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M (mM^{-1} s^{-1})
ONP-β-gal	0.723	5466.7	756.2
D-lactose	136.2	1603.7	11.8

K_M, k_{cat} and k_{cat}/K_M values towards the natural and artificial substrates are reported. Standard deviations were lower than 2% of the calculated values

activity significantly decreased only in the presence of SDS whereas it retained at least 65% of the relative activity when tested with all the other agents (Additional file 3: Table S3).

Catalytic properties of *Bc*GalB

The hydrolytic activity of *Bc*GalB was tested on different *ortho*- or *para*-nitrophenyl synthetic glycosides as well as on natural polysaccharide substrates and specificity of the enzyme was determined by carrying out individual reactions with each of the compounds as indicated in Material and Methods section. As shown in Table 2, the highest specific activity was recorded on ONP-β-gal, whereas the enzyme performed less efficiently on *para*-substituted substrates. As shown in Fig. 1 analysis of the intracellular cell extract revealed the presence of enzyme(s) able to hydrolyse PNP-α-ara. Interestingly, a lower but still significant activity of *Bc*GalB was found on this substrate suggesting that the enzyme is endowed with an ancillary activity on PNP-β-ara. Then, the observed enzymatic activity in the cell extract can be traced back, at least in part, to *Bc*GalB (Fig. 1; Table 2). This accessory activity is surprising, since it has never been described for other thermophilic GH 42 β-galactosidases [64] and it will be a matter of further investigation. Some β-galactosidases can support the growth of environmental microorganisms from hot springs, soils and hypersaline sites where lactose is not present, but rather plant biomasses are preferential carbon and energy sources. Since *B. coagulans* MA-13 was isolated from beans processing waste, it is conceivable that *Bc*GalB may be also involved in the hydrolysis of arabino-derived oligosaccharides in vivo (Table 2).

Lactose, which is the natural substrate for most β-galactosidases, is translocated inside cells through specific lactose-transporters [65]. Therefore, the hydrolytic performance of *Bc*GalB on this substrate was also studied and the specific activity was found to be 1283 U/mg, which is a quite high value compared to β-galactosidases from other *B.coagulans* strains [32, 33, 60].

The kinetic parameters of *Bc*GalB were evaluated using both the preferred artificial substrate and lactose under standard reaction conditions (Table 3). Results of this analysis highlighted that *Bc*GalB showed the highest affinity towards ONP-β-gal (K_M = 0.72 mM) and interestingly this value is among the lowest determined so far among mesophilic and thermophilic β-galactosidases [14, 52, 66]. Moreover, even among closely related β-galactosidases from other *B. coagulans* strains, *Bc*GalB displays the highest affinity towards this substrate [32, 33, 60].

Interestingly, the enzymatic activity on lactose was not affected by Ca^{2+} and even a slight increase (114%) was recorded (data not shown). The K_M was found to be higher than for the artificial substrate; however, previous studies have revealed that most GH42 β-galactosidases prefer to hydrolyse chromogenic substrates while showing weaker lactose hydrolysis activity. Although GH2 β-galactosidases perform better than GH42 representatives on lactose hydrolysis, *Bc*GalB exhibits a significant specific activity toward this substrate [32, 33, 60, 67]. Accordingly, *B. coagulans* MA-13 is able to grow on lactose by over-producing *Bc*GalB (Additional file 2: figure S1), whereas several prokaryotes possessing a GH42 gene are unable to utilize this substrate [48]. This indicates that *Bc*GalB can sustain the host metabolism through hydrolysis of either lactose or more complex oligosaccharides.

Transgalactosylation activity of *Bc*GalB

The transgalactosylation activity of *Bc*GalB was evaluated using ONP-β-gal substrate in either auto- or hetero condensation reactions, in this latter case, with different acceptors.

When ONP-β-gal was used as a donor and acceptor, TLC analysis revealed the synthesis of products of homo-galactosylation products already after 10 min of reaction (Fig. 6a, lane S 10). Moreover, additional signals were clearly visible after 20 min (Fig. 6a, lane S 20), demonstrating that in the early stages of the reaction the donor was promptly consumed in favor of the synthesis of transgalactosylation products (lower red circles).

More importantly, these compounds were not hydrolyzed by *Bc*GalB up to 18 h (Fig. 6a, lane S ON) although their complete hydrolysis was observed after the addition of fresh *Bc*GalB to the transgalactosylation mixture (data not shown). Hence, the persistence of the transgalactosylation products up to 18 h may be due to the combined effect of the partial inactivation of *Bc*GalB occurring after 4 h at 60 °C (Fig. 4d), with the inhibitory effect on the enzymatic activity due to D-galactose accumulation (Fig. 5).

*Bc*GalB is also able to synthetise hetero-oligosaccharides with PNP-β-glu and PNP-β-xyl as acceptors and ONP-β-gal as a donor. Indeed, signals that can be traced back to the formation of hetero-oligosaccharides

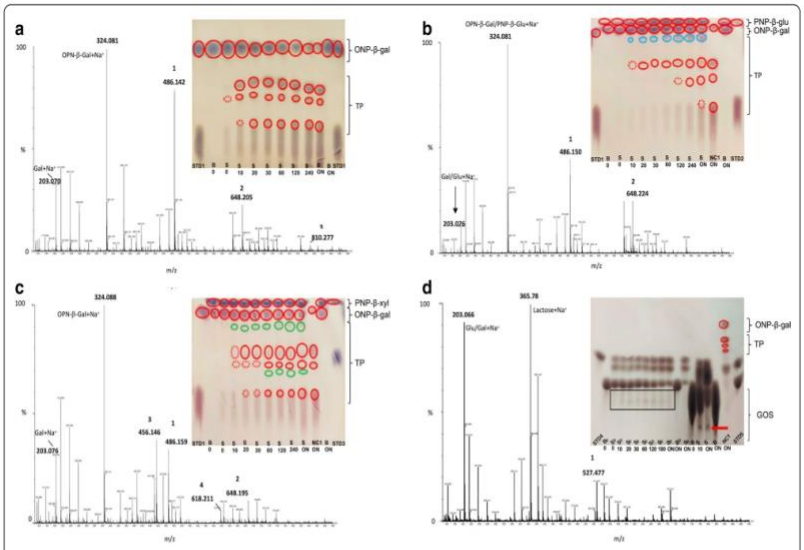

Fig. 6 Time course of transgalactosylation reaction detected by TLC analysis. Homo-condensation reactions using ONP-β-gal as donor and acceptor (**a**). Hetero-condensation reactions performed with PNP-β-glu (**b**) and PNP-β-xyl (**c**) as acceptors and ONP-β-gal as donor. Transgalactosylation reactions using D-lactose as substrate (**d**). Red, blue and green circles show the UV signals obtained from the arylic group of ONP-β-gal, PNP-β-glu, and PNP-β-xyl respectively. *STD1*: Standard with ONP-β-gal and D-galactose, *STD2*: Standard with PNP-β-glu and D-glucose, *STD3*: Standard with PNP-β-xyl and D-xylose, *STD4*: Standard with D-glucose, *STD5*: Standard with D-galactose and D-lactose, *S*: Samples collected at different times (min), *B*: Blank at different times, S_D: Sample diluted, B_D: Blank diluted, *TP*: Transgalactosylation products

(highlighted in blue, Fig. 6b and in green Fig. 6c), were identified togheter along with transgalactosylation products with migration properties similar to those found in homo-condensation reactions (Fig. 6b, c).

The transgalactosylation products were analysed by ESI–MS (Table 4) after carrying out all the reaction for 18 h. In all spectra, the galactose as the product of the hydrolytic activity of *Bc*GalB was detected together with the substrate(s) (ONP-β-gal, ONP-β-glu, PNP-β-xyl). For homo-condensation reactions, m/z values of 486.142, 648.205 and 810.277 corresponding to the sodium adducts of the disaccharide, trisaccharide and tetrasaccharide, were observed (Fig. 6a; Table 4). These trangalactosylation products contained galactose unit(s) (m/z = 162) and the galactose residue of ONP-β-gal (m/z = 324).

In the presence of glucose as acceptor, ESI–MS analysis revealed the formation of two transgalactosylation products (Fig. 6b, blue circles) counting for an increase in mass of one or two hexoses (Gal/Glu) (Fig. 6b; Table 4). These reaction products could result from both homo- and hetero-condensation of glucose and galactose molecules, although they were not distinguishable by ESI–MS analysis because of their identical molecular weight (Table 4).

In the hetero-condensation reactions containing ONP-β-Gal/PNP-β-Xyl as donor and acceptor respectively (Fig. 6c), four different products were detected by ESI–MS. Interestingly, both di-and trisaccharides deriving from homo-and hetero-condensation reactions were identified. The former contained one or two Gal molecules added to the ONP-β-Gal (m/z = 486.159 and 648.195 respectively, Table 4), whereas the latter were made up of xylose and one or two Gal units (m/z = 456.146 and 618.211 respectively, Table 4). It is noteworthy that hetero-condensation products were synthetised when either xylose or glucose were used, thus demonstrating that *Bc*GalB displays a broad acceptor specificity in transgalactosylation reactions.

Since from a biotechnological perspective, the ONP-β-gal is useless as a donor in industrial processes, the natural, plentiful and inexpensive substrate lactose was employed as the glycosyl donor and acceptor in the synthesis of glycoconjugates. A high initial lactose concentration of 160 mM was chosen to enhance GOS synthesis over hydrolysis. TLC analysis revealed the presence of hydrolysis products as well as of several GOS signals already after 10 min of incubation (Fig. 6d, lanes S_D0 to S_D ON, S10 and S ON). This result indicates that *Bc*GalB is able to produce GOS at the expenses of lactose hydrolysis in a short time range. As the reaction proceeded, lactose was consumed, and glucose and galactose were formed following lactose hydrolysis. However, concurrent increase of GOS amount was not observed, as revealed by the intensity of the spots (Fig. 6d, lanes S10-S ON). This indicates that the two reactions were in a dynamic equilibrium in which GOS production reached a plateau before lactose was completely hydrolysed. ESI–MS analysis revealed a transgalactosylation product (m/z value of 527.2, Table 4) consisting of a lactose molecule increased by one galactose unit (S ON, Fig. 6d) along with the corresponding signal to the D-lactose substrate.

Collectively, these data indicate that the thermophilic *Bc*GalB is effective in the production of GOS from lactose. Moreover, lactose solubility in water is rather low in comparison to other carbohydrates; therefore, achieving a lactose concentration high enough to promote transgalactosylation reactions is a difficult task. Since lactose solubility increases exponentially with temperature,

Table 4 Transgalactosylation products identified by ESI–MS

Acceptor:Donor	Transgalactosylation products	MNa^+ Theoretical (Da)	MNa^+ observed (Da)
(ONP-β-Gal: ONP-β-Gal)	1. (ONP-β-Gal + Gal) Na^+	486.408	486.142
	2. (ONP-β-Gal + 2 Gal) Na^+	648.565	648.205
	3. (ONP-β-Gal + 3Gal) Na^+	810.722	810.277
(ONP-β-Gal/PNP-β-Glu: ONP-β-Gal)	1. (ONP-β-Gal/PNP-β-Glu + Gal/Glu) Na^+	486.408	486.150
	2. (ONP-β-Gal/PNP-β-Glu + 2Gal/2Glu) Na^+	648.565	648.224
(PNP-β-Xyl:ONP-β-Gal)	1. (ONP-β-Gal + Gal) Na^+	486.408	486.159
	2. (ONP-β-Gal + 2Gal) Na^+	648.565	648.195
	3. (PNP-β-Xyl + Gal) Na^+	456.382	456.146
	4. (PNP-β-Xyl + Gal + Gal) Na^+	618.539	618.211
(D-Lactose:D-Lactose)	1. (Lactose + Gal) Na^+	527.477	527.202

All components were detected as adducts with Na^+. The observed and theoretical molecular weights are reported. The ESI–MS analysis cannot distinguish between the epimer Gal and Glu, which are reported as alternatives in the interpretation of MS spectra obtained with the couple ONP-β-Gal/PNP-β-Glu: ONP-β-Gal as acceptor and donor, respectively

GOS synthesis can benefit from carrying out reactions with thermostable enzymes and thermophilic microorganisms.

Conclusions

B. coagulans MA-13 is a versatile strain with the potential to be employed in industrial processes aimed not only at the production of value-added chemicals from lignocellulose but also of products/enzymes suitable for various industrial food applications. Indeed, this work shows the capability of this microorganism to over-produce under standard growth conditions α-and β-galactosidases that are key enzymes for improving the nutritional value of RFO- and lactose containing food. Moreover, the expression of these two enzymes can be simultaneously increased in the presence of a natural and inexpensive substrate such as lactose which is abundant in dairy wastes (i.e whey). Interestingly enough, *Bc*GalB is able to produce GOS from artificial and natural (lactose) substrates as well as to perform homo- and hetero-condensation reactions. All together these features point to *B. coagulans* MA-13 as a good candidate for the valorization of dairy waste products and for an eco-friendly and sustainable production of GOS by using whole cells.

Abbreviations

LAB: Lactic acid bacteria; RFO: Raffinose family oligosaccharides; GOS: Galacto-oligosaccharides; CAZy: Carbohydrate-Active enZymes; GH: Glycoside hydrolase; g: Gravity; IPTG: Isopropyl-β-D-1-thiogalactopyranoside; ONP-β-gal: *ortho*-Nitrophenyl-β-D-galactopyranoside; PNP-α-gal: *para*-Nitrophenyl-α-D-galactopyranoside; k_{cat}: Catalytic constant; U: Hydrolytic Units; K_M: Michaelis–Menten constant; V_{max}: Maximal velocity; s: Second(s); SDS-PAGE: Sodium dodecyl sulphate–polyacrylamide gel electrophoresis; DTT: Dithiothreitol; EDTA: Ethylenediaminetetraacetic acid; TLC: Thin-Layer Chromatography; *Bc*GalB: *B. coagulans* MA-13 β-galactosidase expressed in *E.coli*; ESI–MS: Electro-Spray Mass Spectrometry; LC–MS/MS: Liquid Chromatography—Tandem Mass Spectrometry.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12934-021-01553-y.

Additional file 1: **Table S1**. Intra- and extra- cellular proteins active on ONP-b-gal, PNP-b-gal and PNP-a-gal as revealed by gel zymography, were identified through LC-MS/MS method (XLSX 275 KB)

Additional file 2: **Figure S1**. Detection of intracellular enzymatic activities on ONP-β-gal and PNP-α-gal from *B. coagulans* MA-13 cells grown in the presence of lactose. **Figure S2**. Genetic organization of EcsA and EcsB cluster in *B. coagulans* MA-13 genome. **Figure S3**. SDS-PAGE analysis of *Bc*GalB. M. Molecular mass markers; 1 *E. coli* BL21 (DE3) Rosetta cellular extract not transformed; 2. *E. coli* BL21 (DE3) Rosetta pET28B/BcGalB cellular extract not-induced; 3. *E. coli* BL21 (DE3) Rosetta pET28B/BcGalB cellular extract induced with 0,5 mM IPTG over-night; 4. His-Trap affinity chromatography. **Figure S4**. Relative activity of *Bc*GalB after 5, 18 and 24 hours of incubation at different pH values. **Figure S5**. Effect of metal ions on the enzymatic activity of *Bc*GalB (PPTX 1530 KB)

Additional file 3: **Table S2**. Purification table of *Bc*GalB. **Table S3**. Relative activity of *Bc*GalB in presence of chemicals (PPTX 43 KB)

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Authors' contributions

MA, FS and FC performed experiments. MA, SB, GF, DL, PC, AS, Moracci M and Monti M supervised the project. MA and PC drafted the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

Recombinant strains described in this work are made available upon request to the corresponding author. Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

Declarations

Competing interests

The authors declare that they have no competing interests.

Author details

[1] Department of Biology, University of Naples Federico II, 80126 Naples, Italy. [2] Division of Biological Systems and Engineering, Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA. [3] Task Force On Microbiome Studies, University of Naples Federico II, Naples, Italy. [4] Department of Chemical Sciences, University of Naples Federico II, 80126 Naples, Italy. [5] CEINGE Advanced Biotechnologies, University of Naples Federico II, 80145 Naples, Italy. [6] Institute of Biosciences and BioResources—National Research Council of Italy, Naples, Italy. [7] BAT Center—Interuniversity Center for Studies On Bioinspired Agro-Environmental Technology, University of Napoli Federico II, Portici, NA, Italy.

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References:

1. Cao J, Yu Z, Liu W, Zhao J, Zhang H, Zhai Q, et al. Probiotic characteristics of Bacillus coagulans and associated implications for human health and diseases. J Funct Foods. 2019;2020(64):103643. https://doi.org/10.1016/j.jff.2019.103643.
2. Lee NK, Kim WS, Paik HD. Bacillus strains as human probiotics: characterization, safety, microbiome, and probiotic carrier. Food Sci Biotechnol. 2019;28:1297–305. https://doi.org/10.1007/s10068-019-00691-9.
3. Burgess SA, Lindsay D, Flint SH. Thermophilic bacilli and their importance in dairy processing. Int J Food Microbiol. 2010;144:215–25. https://doi.org/10.1016/j.ijfoodmicro.2010.09.027.
4. Aulitto M, Tom LM, Ceja-navarro JA, Simmons BA. Whole-genome sequence of Brevibacillus borstelensis SDM, isolated from a sorghum-adapted microbial community. Microbiol Resour Announc. 2020. https://doi.org/10.1128/MRA.01046-20.
5. Aulitto M, Fusco S, Bartolucci S, Franzén CJ, Contursi P. Bacillus coagulans MA-13: a promising thermophilic and cellulolytic strain for the production of lactic acid from lignocellulosic hydrolysate. Biotechnol Biofuels. 2017;10:1–15.
6. Aulitto M, Fusco S, Nickel DB, Bartolucci S, Contursi P, Franzén CJ. Seed culture pre-adaptation of Bacillus coagulans MA-13 improves lactic acid production in simultaneous saccharification and fermentation. Biotechnol Biofuels. 2019. https://doi.org/10.1186/s13068-019-1382-2.
7. Ferreira-Lazarte A, Moreno FJ, Villamiel M. Bringing the digestibility of prebiotics into focus: update of carbohydrate digestion models. Crit Rev Food Sci Nutr. 2020. https://doi.org/10.1080/10408398.2020.1798344.

8. Aulitto M, Fusco S, Limauro D, Fiorentino G, Bartolucci S, Contursi P. Galactomannan degradation by thermophilic enzymes: a hot topic for biotechnological applications. World J Microbiol Biotechnol. 2019;35:1–13.
9. Teixeira JS, McNeill V, Gänzle MG. Levansucrase and sucrose phoshorylase contribute to raffinose, stachyose, and verbascose metabolism by lactobacilli. Food Microbiol. 2012;31:278–84. https://doi.org/10.1016/j.fm.2012.03.003.
10. Carević M, Banjanac K, Ćorović M, Jakovetić S, Milivojević A, Vukašinović-Sekulić M, et al. Selection of lactic acid bacteria strain for simultaneous production of α- and β-galactosidases. Zast Mater. 2016;57:265–73.
11. Han YR, Youn SY, Ji GE, Park MS. Production of α- and β-galactosidases from Bifidobacterium longum subsp longum RD47. J Microbiol Biotechnol. 2014;24:675–82.
12. Ibrahim SA, Alazzeh AY, Awaisheh SS, Song D, Shahbazi A, Abughazaleh AA. Enhancement of α- And β-galactosidase activity in lactobacillus reuteri by different metal ions. Biol Trace Elem Res. 2010;136:106–16.
13. Van Beilen JB, Li Z. Enzyme technology: an overview. Curr Opin Biotechnol. 2002;13:338–44.
14. Xavier JR, Ramana KV, Sharma RK. β-galactosidase: Biotechnological applications in food processing. J Food Biochem. 2018;42:1–15.
15. Botvynko A, Bednářová A, Henke S, Shakhno N, Čurda L. Production of galactooligosaccharides using various combinations of the commercial β-galactosidases. Biochem Biophys Res Commun. 2019;517(4):762–6.
16. Fusco S, Aulitto M, Iacobucci I, Crocamo G, Pucci P, Bartolucci S, et al. The interaction between the F55 virus-encoded transcription regulator and the RadA host recombinase reveals a common strategy in Archaea and Bacteria to sense the UV-induced damage to the host DNA. Biochim Biophys Acta Gene Regul Mech. 2020. https://doi.org/10.1016/j.bbagrm.2020.194493.
17. Fusco S, Aulitto M, Bartolucci S, Contursi P. A standardized protocol for the UV induction of Sulfolobus spindle-shaped virus 1. Extremophiles. 2015;19:539–46. https://doi.org/10.1007/s00792-014-0717-y.
18. Contursi P, D'Ambrosio K, Pirone L, Pedone E, Aucelli T, She Q, et al. C68 from the Sulfolobus islandicus plasmid-virus pSSVx is a novel member of the AbrB-like transcription factor family. Biochem J. 2011;435:157–66.
19. Contursi P, Farina B, Pirone L, Fusco S, Russo L, Bartolucci S, et al. Structural and functional studies of Stf76 from the Sulfolobus islandicus plasmid-virus pSSVx: a novel peculiar member of the winged helix-turn-helix transcription factor family. Nucleic Acids Res. 2014;42:5993–6011.
20. Bartolucci S, Contursi P, Fiorentino G, Limauro D, Pedone E. Responding to toxic compounds: a genomic and functional overview of Archaea (383 views visite). Front Biosci. 2013;18:165–89.
21. Contursi P, Cannio R, Prato S, She Q, Rossi M, Bartolucci S. Transcriptional analysis of the genetic element pSSVx: Differential and temporal regulation of gene expression reveals correlation between transcription and replication. J Bacteriol. 2007;189:6339–50.
22. Prato S, Vitale RM, Contursi P, Lipps G, Saviano M, Rossi M, et al. Molecular modeling and functional characterization of the monomeric primase-polymerase domain from the Sulfolobus solfataricus plasmid pIT3. FEBS J. 2008;275:4389–402.
23. Contursi P, Pisani FM, Grigoriev A, Cannio R, Bartolucci S, Rossi M. Identification and autonomous replication capability of a chromosomal replication origin from the archaeon Sulfolobus solfataricus. Extremophiles. 2004;8:385–91.
24. Contursi P, Fusco S, Limauro D, Fiorentino G. Host and viral transcriptional regulators in Sulfolobus: an overview. Extremophiles. 2013;17:881–95.
25. Gaglione R, Pirone L, Farina B, Fusco S, Smaldone G, Aulitto M, et al. Insights into the anticancer properties of the first antimicrobial peptide from Archaea. Biochim Biophys Acta Gen Subj. 2017;1861:2155–64. https://doi.org/10.1016/j.bbagen.2017.06.009.
26. Notomista E, Falanga A, Fusco S, Pirone L, Zanfardino A, Galdiero S, et al. The identification of a novel Sulfolobus islandicus CAMP-like peptide points to archaeal microorganisms as cell factories for the production of antimicrobial molecules. Microb Cell Fact. 2015;14:1–11.
27. Roscetto E, Contursi P, Vollaro A, Fusco S, Notomista E, Catania MR. Anti-fungal and anti-biofilm activity of the first cryptic antimicrobial peptide from an archaeal protein against Candida spp clinical isolates. Sci Rep. 2018;8:1–11.
28. Strazzulli A, Fusco S, Cobucci-Ponzano B, Moracci M, Contursi P. Metagenomics of microbial and viral life in terrestrial geothermal environments. Rev Environ Sci Biotechnol. 2017;16:425–54.
29. Kapse NG, Engineer AS, Gowdaman V, Wagh S, Dhakephalkar PK. Functional annotation of the genome unravels probiotic potential of Bacillus coagulans HS243. Genomics. 2019;111:921–9. https://doi.org/10.1016/j.ygeno.2018.05.022.
30. Su F, Xu P. Genomic analysis of thermophilic Bacillus coagulans strains: Efficient producers for platform bio-chemicals. Sci Rep. 2014. https://doi.org/10.1038/srep03926.
31. Zhao R, Zhao R, Tu Y, Zhang X, Deng L, Chen X. A novel α-galactosidase from the thermophilic probiotic Bacillus coagulans with remarkable protease-resistance and high hydrolytic activity. PLoS ONE. 2018. https://doi.org/10.1371/journal.pone.0197067.
32. Batra N, Singh J, Banerjee UC, Patnaik PR, Sobti RC. Production and characterization of a thermostable β-galactosidase from Bacillus coagulans RCS3. Biotechnol Appl Biochem. 2002;36:1.
33. Liu P, Xie J, Liu J, Ouyang J. A novel thermostable β-galactosidase from Bacillus coagulans with excellent hydrolysis ability for lactose in whey. J Dairy Sci. 2019;102:9740–8.
34. Aulitto M, Fusco S, Franzén CJ, Strazzulli A, Moracci M, Bartolucci S, et al. Draft genome sequence of bacillus coagulans MA-13, a thermophilic lactic acid producer from lignocellulose. Microbiol Resour Announc. 2019;8:341–60. https://doi.org/10.1128/mra.00341-19.
35. Yin Y, Mao X, Yang J, Chen X, Mao F, Xu Y. DbCAN: A web resource for automated carbohydrate-active enzyme annotation. Nucleic Acids Res. 2012. https://doi.org/10.1093/nar/gks479.
36. Aulitto M, Fusco S, Fiorentino G, Limauro D, Pedone E, Bartolucci S, et al. Thermus thermophilus as source of thermozymes for biotechnological applications: Homologous expression and biochemical characterization of an α-galactosidase. Microb Cell Fact. 2017;16:1–10.
37. Iaconis D, Monti M, Renda M, Van Koppen A, Tammaro R, Chiaravalli M, et al. The centrosomal OFD1 protein interacts with the translation machinery and regulates the synthesis of specific targets. Sci Rep. 2017;7:1–15. https://doi.org/10.1038/s41598-017-01156-x.
38. Fusco S, Liguori R, Limauro D, Bartolucci S, She Q, Contursi P. Transcriptome analysis of Sulfolobus solfataricus infected with two related fuselloviruses reveals novel insights into the regulation of CRISPR-Cas system. Biochimie. 2015;118:322–32. https://doi.org/10.1016/j.biochi.2015.04.006.
39. Punia S, Bala S, Purewal SS, Sandhu KS. Kidney bean (Phaseolus vulgaris) starch: a review. Legum Sci. 2020. https://doi.org/10.1002/leg3.52.
40. Khedr MA, Desouky SE, Badr UM, Elboudy SS, Khlil KM. Overproduction of β-galactosidase enzyme from Escherichia coli through genetic improvement. J Appl Sci Res. 2013;9:4809–22.
41. Bidart GN, Rodríguez-Díaz J, Pérez-Martínez G, Yebra MJ. The lactose operon from Lactobacillus casei is involved in the transport and metabolism of the human milk oligosaccharide core-2 N-acetyllactosamine. Sci Rep. 2018;8:1–12.
42. Leskelä S, Wahlström E, Hyyryläinen HL, Jacobs M, Palva A, Sarvas M, et al. Ecs, an ABC transporter of Bacillus subtilis: Dual signal transduction functions affecting expression of secreted proteins as well as their secretion. Mol Microbiol. 1999;31:533–43.
43. Jonsson IM, Juuti JT, François P, AlMajidi R, Pietiäinen M, Girard M, et al. Inactivation of the Ecs ABC transporter of Staphylococcus aureus attenuates virulence by altering composition and function of bacterial wall. PLoS ONE. 2010. https://doi.org/10.1371/journal.pone.0014209.
44. Chen YH, Anderson M, Hendrickx AP, Missiakas D. Characterization of EssB, a protein required for secretion of ESAT-6 like proteins in Staphylococcus aureus. BMC Microbiol. 2012;12(1):1–11.
45. Nam KH, Jang MS, Park HY, Koneva E. Biochemical characterization of α-galactosidase-producing thermophilic Bacillus coagulans KM-1. Korean J Fish Aquat Sci. 2014;47:516–21.
46. Lokuge MA, Deepal MC. Isolation from soil of bacteria producing extracellular alpha galactosidase. J Natl Sci Found Sri Lanka. 2000;28:243–52.
47. Patil AGG, Praveen Kumar SK, Mulimani VH, Veeranagouda Y, Lee K. α-galactosidase from Bacillus megaterium VHM1 and its application in removal of flatulence-causing factors from soymilk. J Microbiol Biotechnol. 2010;20:1546–54.
48. Shipkowski S, Brenchley JE. Bioinformatic, genetic, and biochemical evidence that some glycoside hydrolase family 42 β-galactosidases are arabinogalactan type I oligomer hydrolases. Appl Environ Microbiol. 2006;72:7730–8.
49. De Vries RP, Van Den Broeck HC, Dekkers E, Manzanares P, De Graaff LH, Visser J. Differential expression of three α-galactosidase genes and a

single β- galactosidase gene from Aspergillus niger. Appl Environ Microbiol. 1999;65:2453–60.
50. Kuhlman T, Zhang Z, Saier MH†, Hwa T. Combinatorial transcriptional control of the lactose operon of Escherichia coli. 2007
51. Campuzano S, Serra B, Llull D, García JL, Garcí P. Cloning, expression, and characterization of a peculiar choline-binding β-galactosidase from Streptococcus mitis. Appl Environ Microbiol. 2009;75:5972–80.
52. Godoy AS, Camilo CM, Kadowaki MA, Muniz H dos S, Espirito Santo M, Murakami MT, et al. Crystal structure of β1→6-galactosidase from Bifidobacterium bifidum S17: trimeric architecture, molecular determinants of the enzymatic activity and its inhibition by α-galactose. FEBS J. 2016;283:4097–112.
53. Holmes ML, Scopes RK, Moritz RL, Simpson RJ, Englert C, Pfeifer F, et al. Purification and analysis of an extremely halophilic β-galactosidase from Haloferax alicantei. Biochim Biophys Acta Protein Struct Mol Enzymol. 1997;1337:276–86.
54. Solomon HV, Tabachnikov O, Lansky S, Salama R, Feinberg H, Shoham Y, et al. Structure-function relationships in Gan42B, an intracellular GH42 β-galactosidase from Geobacillus stearothermophilus. Acta Crystallogr Sect D Biol Crystallogr. 2015;71:2433–48.
55. Pisani FM, Rella R, Raia CA, Rozzo C, Nucci R, Gambacorta A, et al. Thermostable β-galactosidase from the archaebacterium Sulfolobus solfataricus Purification and properties. Eur J Biochem. 1990;187:321–8.
56. Marín-Navarro J, Talens-Perales D, Oude-Vrielink A, Cañada FJ, Polaina J. Immobilization of thermostable β-galactosidase on epoxy support and its use for lactose hydrolysis and galactooligosaccharides biosynthesis. World J Microbiol Biotechnol. 2014;30:989–98.
57. Aulitto M, Fusco FA, Fiorentino G, Bartolucci S, Contursi P, Limauro D. A thermophilic enzymatic cocktail for galactomannans degradation. Enzyme Microb Technol. 2017;2018(111):7–11. https://doi.org/10.1016/j.enzmictec.2017.12.008.
58. Vera C, Guerrero C, Aburto C, Cordova A, Illanes A. Conventional and non-conventional applications of β-galactosidases. Biochim Biophys Acta Proteins Proteomics. 2020;1868:140271. https://doi.org/10.1016/j.bbapap.2019.140271.
59. Dekker PJT, Koenders D, Bruins MJ. Lactose-free dairy products: Market developments, production, nutrition and health benefits. Nutrients. 2019;11:1–14.
60. Levin RE, Mahoney RR. Purification and characterization of β-galactosidase from a strain of Bacillus coagulans. Antonie van Leeuwenhoek J Microbiol. 1981;47:53–64.
61. Mlichová Z, Rosenberg M. Current trends of β-galactosidase application in food technology. J Food Nutr Res. 2006;45:47–54.
62. Adalberto P, Massabni A, Carmona E, Goulart A, Marques D, Monti R. Effect of divalent metal ions on the activity and stability of Beta-galactosidase isolated from Kluyveromyces lactis. Rev Ciências Farm Básica e Apl. 2010;31:143–50.
63. Sun J, Yao C, Wang W, Zhuang Z, Liu J, Dai F, et al. Cloning, expression and characterization of a novel cold-adapted β-galactosidase from the deep-sea bacterium Alteromonas sp ML52. Mar Drugs. 2018;16(12):469.
64. Di Lauro B, Strazzulli A, Perugino G, La Cara F, Bedini E, Corsaro MM, et al. Isolation and characterization of a new family 42 β-galactosidase from the thermoacidophilic bacterium Alicyclobacillus acidocaldarius: Identification of the active site residues. Biochim Biophys Acta Proteins Proteomics. 2008;1784:292–301.
65. Juers DH, Matthews BW, Huber RE. LacZ β-galactosidase: Structure and function of an enzyme of historical and molecular biological importance. Protein Sci. 2012;21:1792–807.
66. Martarello RD, Cunha L, Cardoso SL, de Freitas MM, Silveira D, Fonseca-Bazzo YM, et al. Optimization and partial purification of beta-galactosidase production by Aspergillus niger isolated from Brazilian soils using soybean residue. AMB Express. 2019. https://doi.org/10.1186/s13568-019-0805-6.
67. Strazzulli A, Cobucci-Ponzano B, Carillo S, Bedini E, Corsaro MM, Pocsfalvi G, et al. Introducing transgalactosylation activity into a family 42 β-galactosidase. Glycobiology. 2017;27:425–37.

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REFERENCES

1. Corona-Hernandez RI, Álvarez-Parrilla E, Lizardi-Mendoza J, Islas-Rubio AR, de la Rosa LA, Wall-Medrano A. **Structural stability and viability of microencapsulated probiotic bacteria: A review.** Compr Rev Food Sci Food Saf. 2013; **12:614–28**.

2. Mazzoli R, Bosco F, Mizrahi I, Bayer EA, Pessione E. **Towards lactic acid bacteria-based biorefineries.** Biotechnol Adv. Elsevier Inc.; 2014; **32:1216–36**.

3. Wu C, Huang J, Zhou R. **Genomics of lactic acid bacteria: Current status and potential applications.** Crit Rev Microbiol. Informa Healthcare USA, Inc; 2017; **43:393–404**.

4. Abdel-Rahman MA, Tashiro Y, Sonomoto K. **Recent advances in lactic acid production by microbial fermentation processes.** Biotechnol Adv. Elsevier Inc.; 2013; **31:877–902**.

5. Alvarez-Sieiro P, Montalbán-López M, Mu D, Kuipers OP. **Bacteriocins of lactic acid bacteria: extending the family.** Appl Microbiol Biotechnol. 2016; **100:2939–51**.

6. Tarraran L, Mazzoli R. **Alternative strategies for lignocellulose fermentation through lactic acid bacteria: The state of the art and perspectives.** FEMS Microbiol Lett. 2018; **365**.

7. Michon C, Langella P, Eijsink VGH, Mathiesen G, Chatel JM. **Display of recombinant proteins at the surface of lactic acid bacteria: Strategies and applications.** Microb Cell Fact. BioMed Central; 2016; **15:1–16**.

8. Derkx PMF, Janzen T, Sørensen KI, Christensen JE, Stuer-Lauridsen B, Johansen E. **The art of strain improvement of industrial lactic acid bacteria without the use of recombinant DNA technology.** Microb Cell Fact. BioMed Central Ltd; 2014; **13:S5**.

9. Cao J, Yu Z, Liu W, Zhao J, Zhang H, Zhai Q, et al. **Probiotic characteristics of Bacillus coagulans and associated implications for human health and diseases.** J Funct Foods. Elsevier; 2020; **64:103643**.

10. Aulitto M, Fusco S, Bartolucci S, Franzén CJ, Contursi P. ***Bacillus coagulans*** **MA-13: A promising thermophilic and cellulolytic strain for the production of lactic acid from lignocellulosic hydrolysate.** Biotechnol Biofuels. BioMed Central; 2017; **10:1–15**.

11. Aulitto M, Fusco S, Nickel DB, Bartolucci S, Contursi P, Franzén CJ. **Seed culture pre-adaptation of *Bacillus coagulans* MA-13 improves lactic acid production in simultaneous saccharification and fermentation.** Biotechnol Biofuels. BioMed Central; 2019; **12:1–11**.

12. Aulitto M, Fusco S, Franzén CJ, Strazzulli A, Moracci M, Bartolucci S, et al. **Draft Genome Sequence of *Bacillus coagulans* MA-13, a Thermophilic Lactic Acid Producer from Lignocellulose.** Microbiol Resour Announc. 2019; **8:341–60**.

13. Zhang Z, Lv J, Pan L, Zhang Y. **Roles and applications of probiotic *Lactobacillus* strains.** Appl Microbiol Biotechnol. Applied Microbiology and Biotechnology; 2018; **102:8135–43**.

14. Paulova L, Patakova P, Branska B, Rychtera M, Melzoch K. **Lignocellulosic ethanol: Technology design and its impact on process efficiency.** Biotechnol Adv. Elsevier Inc.; 2015; **33:1091–107**.

15. Sun Z, Harris HMB, McCann A, Guo C, Argimón S, Zhang W, et al. **Expanding the biotechnology potential of lactobacilli through comparative genomics of 213 strains and associated genera.** Nat Commun. 2015; **6**.

16. Burgess SA, Lindsay D, Flint SH. **Thermophilic bacilli and their importance in dairy processing.** Int J Food Microbiol. Elsevier B.V.; 2010; **144:215–25**.

17. Aulitto M, Tom LM, Ceja-Navarro JA, Simmons BA, Singer SW. **Whole-Genome Sequence of *Brevibacillus borstelensis* SDM, Isolated from a Sorghum-Adapted Microbial Community**. Microbiol Resour Announc. 2020; **9:8–9**.

18. Koutsoumanis K, Allende A, Alvarez-Ordóñez A, Bolton D, Bover-Cid S, Chemaly M, et al. **Scientific Opinion on the update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA (2017–2019).** EFSA J. 2020; **18**.

19. Mu Y, Cong Y. ***Bacillus coagulans* and its applications in medicine.** Benef Microbes. 2019; **10:679–88**.

20. Majeed M, Nagabhushanam K, Natarajan S, Sivakumar A, Ali F, Pande A, et al. ***Bacillus coagulans*** **MTCC 5856 supplementation in the management of diarrhea predominant Irritable Bowel Syndrome: A double blind randomized placebo controlled pilot clinical study.** Nutr J. Nutrition Journal; 2016; **15:1–10**.

21. Kircher M. **Sustainability of biofuels and renewable chemicals production from biomass.** Curr Opin Chem Biol. Elsevier Ltd; 2015; **29:26–31**.

22. Aulitto M, Fusco FA, Fiorentino G, Bartolucci S, Contursi P, Limauro D. **A thermophilic enzymatic cocktail for galactomannans degradation.** Enzyme Microb Technol. Elsevier; 2018; **111:7–11**.

23. Bindels LB, Delzenne NM, Cani PD, Walter J. **Opinion: Towards a more comprehensive concept for prebiotics.** Nat Rev Gastroenterol Hepatol. Nature Publishing Group; 2015; **12:303–10**.

24. Cantarel BI, Coutinho PM, Rancurel C, Bernard T, Lombard V, Henrissat B. **The Carbohydrate-Active EnZymes database (CAZy): An expert resource for glycogenomics.** Nucleic Acids Res. 2009; **37:233–8**.

25. Lombard V, Golaconda Ramulu H, Drula E, Coutinho PM, Henrissat B. **The carbohydrate-active enzymes database (CAZy) in 2013.** Nucleic Acids Res. 2014; **42:490–5**.

26. Koshland Jr. DE. Koshland - original. Biol Rev. 1953; **28:416–36**.

27. Davies G, Henrissat B. **Structures and mechanisms of glycosyl hydrolases.** Structure. 1995; **3:853–9**.

28. Abdul Manas NH, Md. Illias R, Mahadi NM. **Strategy in manipulating transglycosylation activity of glycosyl hydrolase for oligosaccharide production.** Crit Rev Biotechnol. Informa Healthcare USA, Inc; 2018; **38:272–93**.

29. Sinnott ML. **Catalytic Mechanisms of Enzymic Glycosyl Transfer.** Chem Rev. 1990; **90:1171–202**.

30. Mccarter JD, Withers SG. **McCarter and Withers 1994**. Curr Opin Struct Biol. 1994; **4:885–92**.

31. Davies GJ, Williams SJ. **Carbohydrate-active enzymes: Sequences, shapes, contortions and cells.** Biochem Soc Trans. 2016; **44:79–87**.

32. Dey PM, Pridham JB. **Biochemistry of α-galactosidases.** Adv Enzymol Relat Areas Mol Biol. 1972; **36:91–130**.

33. Bhatia S, Singh A, Batra N, Singh J. **Microbial production and biotechnological applications of α-galactosidase.** Int J Biol Macromol. Elsevier B.V.; 2020; **150:1294–313**.

34. Miyazaki T, Ishizaki Y, Ichikawa M, Nishikawa A, Tonozuka T. **Structural and biochemical characterization of novel bacterial α-galactosidases belonging to glycoside hydrolase family 31.** Biochem J. 2015; **444:1–28**.

35. Fernández-Leiro R, Pereira-Rodríguez Á, Cerdán ME, Becerra M, Sanz-Aparicio J. **Structural analysis of *Saccharomyces cerevisiae* α-galactosidase and its complexes with natural substrates reveals new insights into substrate specificity of GH27 glycosidases.** J Biol Chem. 2010; **285:28020–33**.

36. Wang H, Luo H, Li J, Bai Y, Huang H, Shi P, et al. **An α-galactosidase from an acidophilic *Bispora sp.* MEY-1 strain acts synergistically with β-mannanase.** Bioresour Technol. Elsevier Ltd; 2010; **101:8376–82**.

37. Simerská P, Monti D, Čechová I, Pelantová H, Macková M, Bezouška K, et al. **Induction and characterization of an unusual α-d-galactosidase from *Talaromyces flavus*.** J Biotechnol. 2007; **128:61–71**.

38. Katrolia P, Jia H, Yan Q, Song S, Jiang Z, Xu H. **Characterization of a protease-resistant α-galactosidase from the thermophilic fungus *Rhizomucor miehei* and its application in removal of raffinose family oligosaccharides.** Bioresour Technol. Elsevier Ltd; 2012; **110:578–86**.

39. Aguilera M, Rakotoarivonina H, Brutus A, Giardina T, Simon G, Fons M. **Aga1, the first alpha-galactosidase from the human bacteria *Ruminococcus gnavus* E1, efficiently transcribed in gut conditions.** Res Microbiol. Elsevier Masson SAS; 2012; **163:14–21**.

40. Aulitto M, Fusco S, Fiorentino G, Limauro D, Pedone E, Bartolucci S, et al. ***Thermus thermophilus* as source of thermozymes for biotechnological applications: Homologous expression and biochemical characterization of an α-galactosidase.** Microb Cell Fact. BioMed Central; 2017; **16:1–10**.

41. Duffaud GD, McCutchen CM, Leduc P, Parker KN, Kelly RM. **Purification and characterization of extremely thermostable β-mannanase, β-mannosidase, and α-galactosidase from the hyperthermophilic eubacterium *Thermotoga neapolitana* 5068.** Appl Environ Microbiol. 1997; **63:169–77**.

42. Jaekoo Lee. **Production and partial characterization of a-galactosidase activity from an Antarctic bacterial isolate, *Bacillus sp.* LX-1.** African J Biotechnol. 2012; **11:12396–405**.

43. Liu X, Meng K, Wang Y, Shi P, Yuan T, Yang P, et al. **Gene cloning, expression and characterization of an α-galactosidase from *Pedobacter nyackensis* MJ11 CGMCC 2503 with potential as an aquatic feed additive.** World J Microbiol Biotechnol. 2009; **25:1633–42**.

44. Di Stefano M, Miceli E, Gotti S, Missanelli A, Mazzocchi S, Corazza GR. **The effect of oral α-galactosidase on intestinal gas production and gas-related symptoms.** Dig Dis Sci. 2007; **52:78–83**.

45. Ferreira-Lazarte A, Moreno FJ, Villamiel M. **Bringing the digestibility of prebiotics into focus: update of carbohydrate digestion models.** Crit Rev Food Sci Nutr. Taylor & Francis; 2020; **0:1–12**.

46. Linden JC. **Immobilized α-D-galactosidase in the sugar beet industry.** Enzyme Microb Technol. 1982; **4:130–6**.

47. Ghazi S, Rooke JA, Galbraith H. **Improvement of the nutritive value of soybean meal by protease and α-galactosidase treatment in broiler cockerels and broiler chicks.** Br Poult Sci. 2003; **44:410–8**.

48. Malgas S, van Dyk JS, Pletschke BI. **A review of the enzymatic hydrolysis of mannans and synergistic interactions between β-mannanase, β-mannosidase and α-galactosidase.** World J Microbiol Biotechnol. Springer Netherlands; 2015; **31:1167–75**.

49. Aulitto M, Fusco S, Limauro D, Fiorentino G, Bartolucci S, Contursi P. **Galactomannan degradation by thermophilic enzymes: a hot topic for biotechnological applications.** World J Microbiol Biotechnol. Springer Netherlands; 2019; **35:1–13**.

50. Falkoski DL, Guimarães VM, De Almeida MN, Alfenas AC, Colodette JL, De Rezende ST. **Characterization of cellulolytic extract from *Pycnoporus sanguineus* PF-2 and its application in biomass saccharification.** Appl Biochem Biotechnol. 2012; **166:1586–603**.

51. Sweeney MD, Xu F. **Biomass converting enzymes as industrial biocatalysts for fuels and chemicals: Recent developments.** Catalysts. 2012; **2:244–63**.

52. Juers DH, Matthews BW, Huber RE. **LacZ β-galactosidase: Structure and function of an enzyme of historical and molecular biological importance.** Protein Sci. 2012; **21:1792–807**.

53. Aburto C, Castillo C, Cornejo F, Arenas-Salinas M, Vásquez C, Guerrero C, et al. **β-galactosidase from *Exiguobacterium acetylicum*: Cloning, expression, purification and characterization.** Bioresour Technol. 2019; **277:211–5**.

54. Oliveira C, Guimarães PMR, Domingues L. **Recombinant microbial systems for improved β-galactosidase production and biotechnological applications.** Biotechnol Adv. Elsevier B.V.; 2011; **29:600–9**.

55. Solomon H V., Tabachnikov O, Lansky S, Salama R, Feinberg H, Shoham Y, et al. **Structure-function relationships in Gan42B, an intracellular GH42 β-galactosidase from *Geobacillus stearothermophilus*.** Acta Crystallogr Sect D Biol Crystallogr. International Union of Crystallography; 2015; **71:2433–48**.

56. Mangiagalli M, Lapi M, Maione S, Orlando M, Brocca S, Pesce A, et al. **The co-existence of cold activity and thermal stability in an Antarctic GH42 β-galactosidase relies on its hexameric quaternary arrangement.** FEBS J. 2021; **288:546–65**.

57. Saqib S, Akram A, Halim SA, Tassaduq R. **Sources of β-galactosidase and its applications in food industry.** 3 Biotech. Springer Berlin Heidelberg; 2017; **7:1–7**.

58. Di Lauro B, Strazzulli A, Perugino G, La Cara F, Bedini E, Corsaro MM, et al. **Isolation and characterization of a new family 42 β-galactosidase from the thermoacidophilic bacterium *Alicyclobacillus acidocaldarius*: Identification of the active site residues.** Biochim Biophys Acta - Proteins Proteomics. 2008; **1784:292–301**.

59. Torres DPM, Gonçalves M do PF, Teixeira JA, Rodrigues LR. **Galacto-Oligosaccharides: Production, properties, applications, and significance as prebiotics.** Compr Rev Food Sci Food Saf. 2010; **9:438–54**.

60. Gänzle MG. **Enzymatic synthesis of galacto-oligosaccharides and other lactose derivatives (hetero-oligosaccharides) from lactose.** Int Dairy J. Elsevier Ltd; 2012; **22:116–22**.

61. Searle LEJ, Cooley WA, Jones G, Nunez A, Crudgington B, Weyer U, et al. **Purified galactooligosaccharide, derived from a mixture produced by the enzymic activity of *Bifidobacterium bifidum*, reduces Salmonella enterica serovar Typhimurium adhesion and invasion in vitro and in vivo.** J Med Microbiol. 2010; **59:1428–39**.

62. Strazzulli A, Cobucci-Ponzano B, Carillo S, Bedini E, Corsaro MM, Pocsfalvi G, et al. **Introducing transgalactosylation activity into a family 42 β-galactosidase.** Glycobiology. 2017; **27:425–37**.

63. Vera C, Guerrero C, Illanes A, Conejeros R. **A pseudo steady-state model for galacto-oligosaccharides synthesis with β-galactosidase from *Aspergillus oryzae*.** Biotechnol Bioeng. 2011; **108:2270–9**.

64. Otieno DO. **Synthesis of β-galactooligosaccharides from Lactose Using Microbial β-galactosidases.** Compr Rev Food Sci Food Saf. 2010; **9:471–82**.

65. Palai T, Mitra S, Bhattacharya PK. **Kinetics and design relation for enzymatic conversion of lactose into galacto-oligosaccharides using commercial grade β-galactosidase.** J Biosci Bioeng. Elsevier Ltd; 2012; **114:418–23**.

66. Rodriguez-Colinas B, Fernandez-Arrojo L, Ballesteros AO, Plou FJ. **Galactooligosaccharides formation during enzymatic hydrolysis of lactose: Towards a prebiotic-enriched milk.** Food Chem. Elsevier Ltd; 2014; **145:388–94**.

67. Zolnere K, Ciprovica I. **The comparison of commercially available ß-Galactosidases for dairy industry: Review.** Res Rural Dev. 2017; **1:215–22**.

68. Dutra Rosolen M, Gennari A, Volpato G, Volken De Souza CF. **Lactose Hydrolysis in Milk and Dairy Whey Using Microbial β-galactosidases.** Enzyme Res. 2015; **2015**.

69. Xavier JR, Ramana KV, Sharma RK. **β-galactosidase: Biotechnological applications in food processing.** J. Food Biochem. Blackwell Publishing Ltd; 2018. **p. 1–15**.

70. Lu L, Guo L, Wang K, Liu Y, Xiao M. **β-Galactosidases: A great tool for synthesizing galactose-containing carbohydrates.** Biotechnol Adv. Elsevier; 2020; **39:107465**.

71. Beilen JB va., Li Z. **Enzyme technology: An overview.** Curr Opin Biotechnol. 2002; **13:338–44**.

72. Grand View Research. **Galacto-Oligosaccharides (GOS) Market By Application (Food & Beverage, Dietary Supplements) Is Expected To Reach USD 1.01 Billion By 2020.** 2015; **1**.

73. Panesar PS, Kaur R, Singh RS, Kennedy JF. **Biocatalytic strategies in the production of galacto-oligosaccharides and its global status.** Int J Biol Macromol. Elsevier B.V.; 2018; **111:667–79**.

74. Yin Y, Mao X, Yang J, Chen X, Mao F, Xu Y. **DbCAN: A web resource for automated carbohydrate-active enzyme annotation.** Nucleic Acids Res. 2012; **40**.

75. Iaconis D, Monti M, Renda M, Van Koppen A, Tammaro R, Chiaravalli M, et al. **The centrosomal OFD1 protein interacts with the translation machinery and regulates the synthesis of specific targets.** Sci Rep. Springer US; 2017; **7:1–15**.

76. Fusco S, Liguori R, Limauro D, Bartolucci S, She Q, Contursi P. **Transcriptome analysis of *Sulfolobus solfataricus* infected with two related fuselloviruses reveals novel insights into the regulation of CRISPR-Cas system.** Biochimie. Elsevier Ltd; 2015; **118:322–32**.

77. Punia S, Dhull SB, Sandhu KS, Kaur M, Purewal SS. **Kidney bean (*Phaseolus vulgaris*) starch: A review.** Legum Sci. 2020; **1–7**.

78. Carević M, Banjanac K, Ćorović M, Jakovetić S, Milivojević A, Vukašinović-Sekulić M, et al. **Selection of lactic acid bacteria strain for simultaneous production of α- and β-galactosidases.** Zast Mater. 2016; **57:265–73**.

79. Botvynko A, Bednářová A, Henke S, Shakhno N, Čurda L. **Production of galactooligosaccharides using various combinations of the commercial β-galactosidases.** Biochem Biophys Res Commun. 2019; **517:762–6**.

80. Khedr MA, Desouky SE, Badr UM, Elboudy SS, Khlil KM. **Overproduction of β-galactosidase enzyme from Escherichia coli through genetic improvement.** J Appl Sci Res. 2013; **9:4809–22**.

81. Bidart GN, Rodríguez-Díaz J, Pérez-Martínez G, Yebra MJ. **The lactose operon from *Lactobacillus casei* is involved in the transport and metabolism of the human milk oligosaccharide core-2 N-acetyllactosamine.** Sci Rep. 2018; **8:1–13**.

82. Leskelä S, Wahlström E, Hyyryläinen HL, Jacobs M, Palva A, Sarvas M, et al. **Ecs, an ABC transporter of *Bacillus subtilis*: Dual signal transduction functions affecting expression of secreted proteins as well as their secretion.** Mol Microbiol. 1999; **31:533–43**.

83. Jonsson IM, Juuti JT, François P, AlMajidi R, Pietiäinen M, Girard M, et al. **Inactivation of the Ecs ABC transporter of *Staphylococcus aureus* attenuates virulence by altering composition and function of bacterial wall.** PLoS One. 2010; **5**.

84. Chen YH, Anderson M, Hendrickx AP, Missiakas D. **Characterization of EssB, a protein required for secretion of ESAT-6 like proteins in *Staphylococcus aureus*.** BMC Microbiol. 2012; **12:1–11**.

85. Zhao R, Zhao R, Tu Y, Zhang X, Deng L, Chen X. **A novel α-galactosidase from the thermophilic probiotic *Bacillus coagulans* with remarkable protease-resistance and high hydrolytic activity.** PLoS One. 2018; **13:1–16**.

86. Nam KH, Jang MS, Park HY, Koneva E. **Biochemical Characterization of α-galactosidase-producing thermophilic *Bacillus coagulans* KM-1.** Korean J Fish Aquat Sci. 2014; **47:516–21**.

87. Lokuge MA, Deepal Mathew C. **Isolation from soil of bacteria producing extracellular alpha galactosidase.** J Natl Sci Found Sri Lanka. 2000; **28:243–52**.

88. Patil AGG, Praveen Kumar SK, Mulimani VH, Veeranagouda Y, Lee K. **α-galactosidase from *Bacillus megaterium* VHM1 and its application in removal of flatulence-causing factors from soymilk.** J Microbiol Biotechnol. 2010; **20:1546–54**.

89. Teixeira JS, McNeill V, Gänzle MG. **Levansucrase and sucrose phoshorylase contribute to raffinose, stachyose, and verbascose metabolism by lactobacilli.** Food Microbiol. Elsevier; 2012; **31:278–84**.

90. Shipkowski S, Brenchley JE. **Bioinformatic, genetic, and biochemical evidence that some glycoside hydrolase family 42 β-galactosidases are arabinogalactan type I oligomer hydrolases.** Appl Environ Microbiol. 2006; **72:7730–8**.

91. De Vries RP, Van Den Broeck HC, Dekkers E, Manzanares P, De Graaff LH, Visser J. **Differential expression of three α-galactosidase genes and a single β-galactosidase gene from *Aspergillus niger*.** Appl Environ Microbiol. 1999; **65:2453–60**.

92. Han YR, Youn SY, Ji GE, Park MS. **Production of α- and β-galactosidases from *Bifidobacterium longum subsp. longum* RD47.** J Microbiol Biotechnol. 2014; **24:675–82**.

93. Ibrahim SA, Alazzeh AY, Awaisheh SS, Song D, Shahbazi A, Abughazaleh AA. **Enhancement of α- And β-galactosidase activity in *Lactobacillus reuteri* by different metal ions.** Biol Trace Elem Res. 2010; **136:106–16**.

94. Kuhlman T, Zhang Z, Saier MH, Hwa T. **Combinatorial transcriptional control of the lactose operon of *Escherichia coli*.** Proc Natl Acad Sci U S A. 2007; **104:6043–8**.

95. Campuzano S, Serra B, Llull D, García JL, Garcí P. **Cloning, expression, and characterization of a peculiar choline-binding β-galactosidase from *Streptococcus mitis*.** Appl Environ Microbiol. 2009; **75:5972–80**.

96. Liu P, Xie J, Liu J, Ouyang J. **A novel thermostable β-galactosidase from *Bacillus coagulans* with excellent hydrolysis ability for lactose in whey.** J Dairy Sci. American Dairy Science Association; 2019; **102:9740–8**.

97. Godoy AS, Camilo CM, Kadowaki MA, Muniz H dos S, Espirito Santo M, Murakami MT, et al. **Crystal structure of β1→6-galactosidase from *Bifidobacterium bifidum* S17: trimeric architecture, molecular determinants of the enzymatic activity and its inhibition by α-galactose.** FEBS J. 2016; **283:4097–112**.

98. Holmes ML, Scopes RK, Moritz RL, Simpson RJ, Englert C, Pfeifer F, et al. **Purification and analysis of an extremely halophilic β-galactosidase from *Haloferax alicantei*.** Biochim Biophys Acta - Protein Struct Mol Enzymol. 1997; **1337:276–86**.

99. Pisani FM, Rella R, Raia CA, Rozzo C, Nucci R, Gambacorta A, et al. **Thermostable β-galactosidase from the archaebacterium *Sulfolobus solfataricus* Purification and properties.** Eur J Biochem. 1990; **187:321–8**.

100. Marín-Navarro J, Talens-Perales D, Oude-Vrielink A, Cañada FJ, Polaina J. **Immobilization of thermostable β-galactosidase on epoxy support and its use for lactose hydrolysis and galactooligosaccharides biosynthesis.** World J Microbiol Biotechnol. 2014; **30:989–98**.

101. Vera C, Guerrero C, Aburto C, Cordova A, Illanes A. **Conventional and non-conventional applications of β-galactosidases.** Biochim Biophys Acta - Proteins Proteomics. Elsevier; 2020; **1868:140271**.

102. Dekker PJT, Koenders D, Bruins MJ. **Lactose-free dairy products: Market developments, production, nutrition and health benefits.** Nutrients. 2019; **11:1–14**.

103. Batra N, Singh J, Banerjee UC, Patnaik PR, Sobti RC. **Production and characterization of a thermostable β-galactosidase from *Bacillus coagulans* RCS3.** Biotechnol Appl Biochem. 2002; **36:1**.

104. Levin RE, Mahoney RR. **Purification and characterization of β-galactosidase from a strain of *Bacillus coagulans*.** Antonie van LeeuwenhoekJMicrobiol. 1981; **47:53–64**.

105. Mlichová Z, Rosenberg M. **Current trends of β-galactosidase application in food technology.** J Food Nutr Res. 2006; **45:47–54**.

106. Adalberto P, Massabni A, Carmona E, Goulart A, Marques D, Monti R. **Effect of divalent metal ions on the activity and stability of Beta-galactosidase isolated from *Kluyveromyces lactis*.** Rev Ciências Farm Básica e Apl. 2010; **31:143–50**.

107. Sun J, Yao C, Wang W, Zhuang Z, Liu J, Dai F, et al. **Cloning, expression and characterization of a novel cold-adapted β-galactosidase from the deep-sea bacterium *Alteromonas sp.* ML52.** Mar Drugs. 2018; **16**.

108. Martarello RD, Cunha L, Cardoso SL, de Freitas MM, Silveira D, Fonseca-Bazzo YM, et al. **Optimization and partial purification of beta-galactosidase production by *Aspergillus niger* isolated from Brazilian soils using soybean residue.** AMB Express. Springer Berlin Heidelberg; 2019; **9**.

109. Danso D, Schmeisser C, Chow J, Zimmermann W, Wei R, Leggewie C, et al. **New insights into the function and global distribution of polyethylene terephthalate (PET)-degrading bacteria and enzymes in marine and terrestrial metagenomes.** Appl Environ Microbiol. 2018; **53:1689–99**.

110. Alimba CG, Faggio C. **Microplastics in the marine environment: Current trends in environmental pollution and mechanisms of toxicological profile.** Environ Toxicol Pharmacol. 2019; **68:61–74**.

111. Ali MI, Perveen Q, Ahmad B, Javed I, Razi-Ul-Hussnain R, Andleeb S, et al. **Studies on biodegradation of cellulose blended polyvinyl chloride films.** Int J Agric Biol. 2009; **11:577–80**.

112. Hopewell J, Dvorak R, Kosior E. **Plastics recycling: Challenges and opportunities.** Philos Trans R Soc B Biol Sci. 2009; **364:2115–26**.

113. Andrady AL, Neal MA. **Applications and societal benefits of plastics.** Philos Trans R Soc B Biol Sci. 2009; **364:1977–84**.

114. Kawai F, Kawabata T, Oda M. **Current State and Perspectives Related to the Polyethylene Terephthalate Hydrolases Available for Biorecycling.** ACS Sustain Chem Eng. 2020; **8:8894–908**.

115. Liu C, Shi C, Zhu S, Wei R, Yin CC. **Structural and functional characterization of polyethylene terephthalate hydrolase from *Ideonella sakaiensis*.** Biochem Biophys Res Commun. Elsevier Ltd; 2019; **508:289–94**.

116. Kawai F, Hu X. **Biochemistry of microbial polyvinyl alcohol degradation.** Appl Microbiol Biotechnol. 2009; **84:227–37**.

117. Flieger M, Kantorová M, Prell A, Řezanka T, Votruba J. **Biodegradable plastics from renewable sources.** Folia Microbiol (Praha). 2003; **48:27–44**.

118. Tsujiyama S, Okada A. **Biodegradation of polyvinyl alcohol by a brown-rot fungus, *Fomitopsis pinicola*.** Biotechnol Lett. 2013; **35:1907–11**.

119. Sørensen L, Groven AS, Hovsbakken IA, Del Puerto O, Krause DF, Sarno A, et al. **UV degradation of natural and synthetic microfibers causes fragmentation and release of polymer degradation products and chemical additives.** Sci Total Environ; 2021; **755:143170**.

120. Ye B, Li Y, Chen Z, Wu QY, Wang WL, Wang T, et al. **Degradation of polyvinyl alcohol (PVA) by UV/chlorine oxidation: Radical roles, influencing factors, and degradation pathway.** Water Res. Elsevier Ltd; 2017; **124:381–7**.

121. Feuilloley P, César G, Benguigui L, Grohens Y, Pillin I, Bewa H, et al. **Degradation of polyethylene designed for agricultural purposes.** J Polym Environ. 2005; **13:349–55**.

122. Restrepo-Flórez JM, Bassi A, Thompson MR. **Microbial degradation and deterioration of polyethylene - A review.** Int Biodeterior Biodegrad. Elsevier Ltd; 2014; **88:83–90**.

123. Kale SK, Deshmukh AG, Dudhare MS, Patil VB. **Microbial degradation of plastic: a review**. Swapnil. J Biochem Technol. 2015; **6:952–61**.

124. Santo M, Weitsman R, Sivan A. **The role of the copper-binding enzyme - laccase - in the biodegradation of polyethylene by the actinomycete *Rhodococcus ruber*.** Int Biodeterior Biodegrad. Elsevier Ltd; 2013; **84:204–10**.

125. Mohanan N, Montazer Z, Sharma PK, Levin DB. **Microbial and Enzymatic Degradation of Synthetic Plastics.** Front Microbiol. 2020; **11**.

126. Bardají DKR, Moretto JAS, Furlan JPR, Stehling EG. **A mini-review: current advances in polyethylene biodegradation.** World J Microbiol Biotechnol. Springer Netherlands; 2020; **36:1–10**.

127. Zhang K, Hamidian AH, Tubić A, Zhang Y, Fang JKH, Wu C, et al. **Understanding plastic degradation and microplastic formation in the environment: A review.** Environ Pollut. 2021; **274**.

128. Danso D, Chow J, Streita WR. **Plastics: Environmental and biotechnological perspectives on microbial degradation.** Appl Environ Microbiol. 2019; **85:1–14**.

129. Yoshida S, Hiraga K, Takehana T, Taniguchi I, Yamaji H, Maeda Y, et al. **A bacterium that degrades and assimilates poly(ethylene terephthalate).** 2016; **351:1–5**.

130. Taniguchi I, Yoshida S, Hiraga K, Miyamoto K, Kimura Y, Oda K. **Biodegradation of PET: Current Status and Application Aspects.** ACS Catal. 2019; **9:4089–105**.

131. Ben Halima N. **Poly(vinyl alcohol): Review of its promising applications and insights into biodegradation.** RSC Adv. Royal Society of Chemistry; 2016; **6:39823–32**.

132. Ghatge S, Yang Y, Ahn JH, Hur HG. **Biodegradation of polyethylene: a brief review.** Appl Biol Chem. Springer Singapore; 2020; **63**.

133. Finley JH. **Spectrophotometric Determination of Alcohol in Paper Coatings.** Anal Chem. 1961; **33:1925–7**.

134. Othman AR, Hasan HA, Muhamad MH, Ismail N 'Izzati, Abdullah SRS. **Microbial degradation of microplastics by enzymatic processes: a review.** Environ Chem Lett. Springer International Publishing; 2021.

135. Raddadi N, Fava F. **Biodegradation of oil-based plastics in the environment: Existing knowledge and needs of research and innovation.** Sci Total Environ. Elsevier B.V.; 2019; **679:148–58**.

136. Falkenstein P, Gräsing D, Bielytskyi P, Zimmermann W, Matysik J, Wei R, et al. **UV Pretreatment Impairs the Enzymatic Degradation of Polyethylene Terephthalate.** Front Microbiol. 2020; **11:1–10**.