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**DEPOLIMERIZACIJA MATRIKSA MEDICINSKI
ZNAČAJNIH BIOFILMOVA GLIKOZID-
HIDROLAZAMA POREKLOM IZ MIKROBIOTE
ŠKRGU DEVERIKE (*ABRAMIS BRAMA*)**

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**DEPOLYMERIZATION OF THE BIOFILM
MATRIX OF MEDICALLY RELEVANT
BIOFILMS BY GLYCOSIDE HYDROLASES
DERIVED FROM THE COMMON BREEM
(*ABRAMIS BRAMA*) GILL MICROBIOTA**

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Depolimerizacija matriksa medicinski značajnih biofilmova glikozid-hidrolazama poreklom iz mikrobiote škrge deverike (*Abramis brama*)

SAŽETAK

Biofilmovi predstavljaju dominantan način života mikroorganizama. Ekstracelularni matriks biofilma, izgrađen od polisaharida, proteina, lipida i DNK, predstavlja zaštitnu barijeru koja ograničava prodor antimikrobnih agenasa do bakterija i smanjuje njihovu efikasnost. Jedan od perspektivnih pristupa za kontrolu biofilmova se zasniva na primeni glikozid-hidrolaza koje ciljano depolimerizuju polisaharide, glavnu strukturnu komponentu matriksa. Cilj ove doktorske disertacije bio je identifikacija, produkcija i karakterizacija glikozid-hidrolaza poreklom iz mikrobiote škrge deverike sa potencijalom za depolimerizaciju matriksa medicinski značajnih biofilmova. U okviru disertacije okarakterisana su tri enzima - β -glukozidaza BglB-BG28, poligalakturonaza PG-BG31 i β -1,2-manozidaza GH130-BG31. BglB-BG28 je inhibirala formiranje biofilma bakterije *Salmonella* Enteritidis, ciljajući celulozu, glavnu polisaharidnu komponentu matriksa. Antibiofilm aktivnost poligalakturonaze PG-BG31 je pokazana na biofilmovima kliničkih izolata *Escherichia coli*. Pretpostavljena meta ovog enzima su polisaharidi koji sadrže glukuronsku kiselinu, kao što je kolanska kiselina, komponenta biofilmova *E. coli*. β -1,2-manozidaza GH130-BG31, koja pokazuje aktivnost prema mananskim strukturama, je ispoljila sposobnost inhibicije i delimične degradacije biofilmova bakterija povezanih sa bolničkim infekcijama, kao što su *Klebsiella pneumoniae*, *E. coli* i *Staphylococcus epidermidis*. Ovi enzimi ne ispoljavaju direktno baktericidno delovanje, što smanjuje verovatnoću za razvoj rezistencije. Takođe, nisu citotoksični prema ćelijama u kulturi i ne utiču na preživljavanje nematode *Caenorhabditis elegans*, što ukazuje na njihov povoljan bezbednosni profil i potencijal za bezbednu primenu u antibiofilm strategijama.

Ključne reči: bakterijski biofilm, infekcija, ekstracelularni matriks biofilma, egzopolisaharidi, enzimi, glikozid-hidrolaze, β -glukozidaza, poligalakturonaza, β -1,2-manozidaza

Naučna oblast: Biologija

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Depolymerization of the biofilm matrix of medically relevant biofilms by glycoside hydrolases derived from the common bream (*Abramis brama*) gill microbiota

ABSTRACT

Biofilms represent the dominant lifestyle of microorganisms. The extracellular biofilm matrix, composed of polysaccharides, proteins, lipids and DNA, acts as a protective barrier that limits the penetration of antimicrobial agents to bacterial cells and reduces their effectiveness. One of the promising approaches for biofilm control is using the glycoside hydrolases that selectively depolymerize polysaccharides, the main structural components of the matrix. The aim of this dissertation was identification, production and characterization of glycoside hydrolases originating from the common bream gill microbiota, with the potential to depolymerize the matrix of medically relevant biofilms. Three enzymes were characterized within the dissertation, β -glucosidase BglB-BG28, polygalacturonase PG-BG31 and β -1,2-mannosidase GH130-BG31. BglB-BG28 inhibited biofilm formation of *Salmonella* Enteritidis by targeting cellulose, the main polysaccharide component of its matrix. The antibiofilm activity of the PG-BG31 was demonstrated on biofilms formed by clinical isolates of *Escherichia coli*. The presumed target of this enzyme are glucuronic acid-containing polysaccharides, such as colanic acid, a component of *E. coli* biofilms. The GH130-BG31, which exhibits activity toward mannan structures, inhibited and partially degraded biofilms formed by bacteria associated with hospital-acquired infections, such as *Klebsiella pneumoniae*, *E. coli*, and *Staphylococcus epidermidis*. These enzymes do not exhibit direct bactericidal activity, which reduces the likelihood of resistance development. Furthermore, they are not cytotoxic and do not affect the survival of the nematode *Caenorhabditis elegans*, indicating their favorable safety profile and potential for safe application in antibiofilm strategies.

Key words: bacterial biofilms, infection, biofilm matrix, exopolysaccharides, enzymes, glycoside hydrolase, β -glucosidase, polygalacturonase, β -1,2-mannosidase

Scientific field: biology

Scientific subfield: microbiology

Spisak skraćenica i oznaka korišćenih u radu:

AI	autoinducer
AU	medijum koji imitira urin (eng. <i>Artificial Urine</i>)
BglB-BG28	β -glukozidaza BglB-BG28
BDAR	braon, suv i hrapav morfolip (eng. <i>Brown, Dry and Rough</i>)
CAUTI	infekcije urinarnog trakta povezana sa kateterom (eng. <i>Catheter Associated Urinary Tract Infection</i>)
c-di-GMP	ciklični di-guanozin-monofosfat
CF	cistična fibroza
CFTR	gen čoveka koji kodira transmembranski jonski kanala (eng. <i>Cystic Fibrosis Transmembrane Conductance Regulator</i>)
CIP	sistem za čišćenje bez demontaže (eng. <i>Clean In Place</i>)
DNKaza	deoksiribonukleaza
eDNK	ekstracelularna DNK
GH130-BG31	β -1,2-manozidaza GH130-BG31
HPAEC-PAD	anjonsko-izmenjivačka hromatografija visokih performansi sa pulsnom amperometrijskom detekcijom (eng. <i>High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection</i>)
MBK	minimalna baktericidna koncentracija
MIK	minimalna inhibitorna koncentracija
MRSA	meticilin rezistentni <i>Staphylococcus aureus</i>
NTS	netifoidni sojevi roda <i>Salmonella</i>
PG-BG31	poligalakturonaza PG-BG31
PNAG	poli- β -1,6-N-acetil-D-glukozamin
QQ	ometanje međućelijske komunikacije bakterija (eng. <i>Quorum Quenching</i>)
QS	međućelijska komunikacija zasnovana na gustini populacije (eng. <i>Quorum Sensing</i>)
RDAR	crven, suv i hrapav morfolip (eng. <i>Red, Dry and Rough</i>)
sRNK	male nekodirajuće regulatorne RNK
STEC	sojevi <i>Escherichia coli</i> koji proizvode šiga toksin
TLC	tankoslojna hromatografija (eng. <i>Thin Layer Chromatography</i>)

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1. UVOD

Jedan od najvećih savremenih izazova u oblasti javnog zdravlja jeste antimikrobna rezistencija. Prema procenama, infekcije uzrokovane patogenima rezistentnim na antibiotike bile su povezane sa oko 4,95 miliona smrtnih ishoda širom sveta tokom 2019. godine. Sa druge strane, aktuelne projekcije pokazuju da bi se, ukoliko se trenutni trend nastavi, ovaj broj do 2050. godine mogao popeti na čak 10 miliona godišnje (Walsh et al., 2023). Razvoju i širenju antimikrobne rezistencije značajno doprinosi sposobnost mikroorganizama da formiraju biofilme, koji predstavljaju dominantan način života većine bakterija. U okviru biofilma bakterije ispoljavaju niz fenotipskih karakteristika koje im omogućavaju znatno veću toleranciju na antimikrobne agense u poređenju sa planktonskim ćelijama. Zahvaljujući tome, kao i zaštiti koju pruža ekstracelularni matriks, infekcije povezane sa bakterijskim biofilmovima su posebno problematične i često zahtevaju produženu terapiju antibioticima i karakteriše ih ponovno javljanje nakon završetka tretmana (Lebeaux et al., 2014). Biofilmovi su značajan uzrok brojnih bakterijskih infekcija, naročito hroničnih stanja, pri čemu njihovo dugotrajno prisustvo može izazvati adaptivni inflamatorni odgovor bez uspešne eliminacije patogena. Intenzivne interakcije između ćelija u biofilmovima stvaraju pogodno okruženje za horizontalni transfer gena, što dodatno ubrzava evoluciju i širenje rezistencije (H. Y. Liu et al., 2024). Ovi izazovi ukazuju na hitnu potrebu za razvojem novih i efikasnijih terapijskih strategija usmerenih na prevenciju formiranja i eliminaciju biofilмова, kako bi se unapredila uspešnost lečenja infekcija i usporilo dalje širenje antimikrobne rezistencije.

1.1 Bakterijski biofilmovi

Bakterijski biofilmovi su agregati mikroorganizama uronjenih u ekstracelularni matriks koji se sastoji od ekstracelularnih polimernih supstanci koje bakterije same proizvode. Ti polimeri im omogućavaju pričvršćivanje za različite površine ili udruživanje u agregate koji ne moraju biti vezani za podlogu (Flemming et al., 2016). Biofilmovi predstavljaju dominantan oblik života mikroorganizama i mogu se naći u gotovo svim sredinama (Flemming & Wuertz, 2019). Formiraju se na stenama, u zemljištu, tkivima biljaka i životinja, u ekstremnim uslovima kao što su termalni izvori, kao i na brojnim antropogenim površinama poput cevi i radnih površina. Iako u nekim industrijskim procesima mogu biti korisni (Philipp et al., 2024), biofilmovi predstavljaju veliki izazov za zdravlje ljudi i životinja, bezbednost hrane i snabdevanje vodom (Cámara et al., 2022; Sentenac et al., 2022). Medicinski relevantni biofilmovi se mogu razvijati kako na biotičkim, tako i na abiotičkim površinama. Oni se formiraju na bolničkim površinama, medicinskim uređajima, tkivima pacijenata i implantatima (Atanaskovic & Senerovic, 2024; Percival, Suleman, et al., 2015) (Slika 1). Pored toga, biofilmovi mogu postojati i u vidu nezvanih agregata i kontaminirati medicinsku opremu, industrijska postrojenja i pogone za preradu hrane. U prehrambenoj industriji, biofilmovi mogu biti rezervoari patogenih mikroorganizama koji kontaminiraju prehrambene proizvode i predstavljaju ozbiljan rizik po bezbednost hrane i javno zdravlje (X. Liu et al., 2023).



Slika 1. Rasprostranjenost biofilmova u bolničkom okruženju i prehrambenoj industriji. Preuzeto i modifikovano iz Atanaskovic & Senerovic, 2024.

Ključni problem u terapiji infekcija povezanih sa bakterijskim biofilmovima predstavlja njihova izuzetna tolerancija i otpornost na delovanje antimikrobnih agenasa. Infekcije povezane sa biofilmovima često su perzistentne i teško se leče, te zahtevaju dugotrajne i agresivne terapijske pristupe. I *in vitro* i *in vivo* studije pokazuju da su minimalna inhibitorna koncentracija (MIK) i minimalna baktericidna koncentracija (MBK) kliničkih antibiotika uglavnom 10 do 1.000 puta veće za bakterije u biofilmu nego za planktonske bakterije (Høiby et al., 2011). Postizanje efektivne MBK za eradikaciju biofilma *in vivo* predstavlja veliki izazov zbog toksičnosti i neželjenih efekata primene antibiotika u visokim dozama. Ova tolerancija potiče od različitih faktora (Ciofu et al., 2022). Matriks koji se sastoji od polisaharida, proteina, lipida i ekstracelularne DNK (eDNK) (Hobley et al., 2015) inhibira difuziju antimikrobnih agenasa i time smanjuje njihovu efektivnu koncentraciju unutar biofilma (Powell et al., 2021). Pored toga, matriks može hemijski inaktivirati pojedine antimikrobne agense ili sadržati enzime koji mogu da ih razgrade (S. Singh et al., 2017). Sam matriks nije jedini faktor, i spor rast ćelija u biofilmu takođe doprinosi njihovoj toleranciji. Ćelije u biofilmovima često ulaze u stacionarnu fazu rasta ili u stanje kada su bakterije vijabilne ali ne i kultivabilne, što ih čini manje osetljivim na antibiotike koji deluju na ćelije koje se aktivno dele (Ayrapetyan et al., 2018; Stewart & Franklin, 2008). Okruženje biofilma pogoduje i horizontalnom transferu gena, procesu u kojem bakterije razmenjuju genetički materijal, između ostalog i gene za rezistenciju na antibiotike (Metzger et al., 2022). Ovaj proces je naročito efikasan u bakterijskim biofilmovima zbog velike gustine ćelija i njihove bliske prostorne povezanosti (Mah, 2012).

Zbog niske osetljivosti bakterija u biofilmu na antibiotike, lečenje infekcija koje uključuju formiranje biofilmova često je otežano i neretko neefikasno. Dok se mladi biofilmovi lakše uklanjaju, zreli biofilmovi su posebno otporni na terapiju, zbog čega je rana dijagnostika ključna za uspešnu intervenciju (Hengzhuang et al., 2011). Međutim, većina kliničkih infekcija povezanih sa biofilmom dijagnostikuje se u zreloj fazi njegovog razvoja. To značajno otežava njihovu eradikaciju primenom antibiotika, te je često neophodno sprovesti dugotrajnu supresivnu terapiju ili kontrolu recidiva (Høiby et al., 2011; H. Wu et al., 2015).

1.2 Bakterijski biofilmovi u medicinskom okruženju

Mikroorganizmi koji formiraju biofilm odgovorni su za 65–80% infekcija kod ljudi (Jamal et al., 2018). Formiranje biofilma na bolničkim površinama predstavlja značajnu pretnju po bezbednost pacijenata i ishod lečenja. Biofilmovi se mogu formirati na površinama medicinskih uređaja, često dodirivanim mestima i medicinskim instrumentima (Assefa & Amare, 2022). Ovi biofilmovi su poznati po tome što ih je teško ukloniti i čest su uzrok bolničkih infekcija, čineći više od 65% ovih slučajeva (Preda & Săndulescu, 2019). Prisustvo biofilma naglašava hitnu potrebu za

efikasnim protokolima čišćenja i dezinfekcije kako bi se smanjio rizik od prenosa infekcija u zdravstvenim ustanovama. Kontaminacija vodovodnih linija takođe predstavlja značajan rizik od prenosa mikroorganizama na bolničke površine, što doprinosi potencijalnom širenju infekcija među pacijentima i zdravstvenim radnicima (Suleyman et al., 2018).

Biofilmovi nisu ograničeni samo na kolonizaciju abiotičkih površina, već se mogu formirati i na biotičkim površinama. Ove mikrobne zajednice mogu se razviti u tkivima pacijenata i dovesti do hroničnih infekcija koje je teško lečiti. Neke od najčešćih infekcija praćenih formiranjem bakterijskih biofilmova se javljaju kod pacijenata sa hroničnim ranama, u disajnim putevima pacijenata sa cističnom fibrozom (CF) i kod pacijenata koji poseduju ugrađene medicinske implantate (Del Pozo, 2018).

Oštećenje kože omogućava prodor mikroorganizama iz mikrobiote pacijenta ili spoljašnje sredine. Iako imunski sistem u početku može kontrolisati ove mikroorganizme, vezivanje bakterija za površinu rane i njihovo razmnožavanje može dovesti do formiranja biofilma, koji tokom sazrevanja postaje sve otporniji na imunski odgovor i antimikrobne terapije (Percival, McCarty, et al., 2015). S obzirom na to da pacijenti sa hroničnim ranama često imaju pridružena medicinska stanja koja kompromituju njihov imunski sistem (Falanga et al., 2022), većina ovih rana (~80%) postaje kolonizovana biofilmovima (Malone et al., 2017), što značajno otežava zarastanje i povećava rizik od ozbiljnih komplikacija, uključujući sepsu i amputacije (Eriksson et al., 2022). Većina biofilmova u hroničnim ranama je polimikrobna, ali kada se javljaju monospecijjski biofilmovi, oni su najčešće izazvani bakterijama iz rodova *Pseudomonas* ili *Staphylococcus* (Wolcott et al., 2016).

Još jedan tip povrede koji pruža povoljno okruženje za razvoj biofilma je opekotina. Ona čini pacijenta imunokompromitovanim i podložnim oportunističkim patogenima poput *Pseudomonas aeruginosa*, *Acinetobacter baumannii* i *Staphylococcus aureus* (Maslova et al., 2021). Većina smrtnih ishoda kod pacijenata sa opekotinama (oko 75%) posledica je infekcije rana (Maslova et al., 2021).

CF je genetički poremećaj koji primarno pogađa pluća i digestivni sistem, a karakteriše ga proizvodnja guste, lepljive sluzi koja opstruira disajne puteve, dovodeći do ozbiljnih respiratornih komplikacija. CF nastaje usled mutacija u *CFTR* genu (eng. *Cystic Fibrosis Transmembrane Conductance Regulator*), što narušava transport jona i dovodi do proizvodnje dehidrirane sluzi (Cutting, 2015). Ovo nakupljanje sluzi stvara okruženje koje pogoduje hroničnim bakterijskim infekcijama, posebno onim uzrokovanih bakterijama *P. aeruginosa* i *S. aureus*, i dovodi do upale i oštećenja pluća (Cohen & Prince, 2012).

Medicinski implantati se ugrađuju u telo pacijenata u dijagnostičke, terapijske ili rehabilitacione svrhe. Najčešće korišćeni medicinski uređaji uključuju neurološke, dentalne, kardiovaskularne, gastrointestinalne, urološke, intravaskularne i ortopedske implantate, kontaktne sočiva, silikonske implantate dojke, intrauterine implantate i penilne implantate (Caldara et al., 2022). Infekcije povezane sa ovim uređajima često su praćene formiranjem biofilma i predstavljaju značajan globalni zdravstveni problem sa ozbiljnim kliničkim i ekonomskim posledicama (Percival, Suleyman, et al., 2015). Vrste *Staphylococcus* roda su najčešće izolovane bakterije sa većine medicinskih uređaja (Caldara et al., 2022). Najveći diverzitet izolata potiče sa gastrointestinalnih uređaja, gde se pored *Staphylococcus* spp. često izoluju enterokoke, *Enterobacteriaceae*, *Bacillus* spp. i streptokoke (Dautle et al., 2003). Dentalni implantati sadrže najrazličitije mikrobne zajednice, uključujući *Fusobacterium* spp., *Porphyromonas gingivalis*, *Prevotella* spp., *Selenomonas* spp., *Staphylococcus* spp. i *Streptococcus* spp. (Caldara et al., 2022). Urološki implantati i intravaskularni uređaji često su kolonizovani bakterijama *Escherichia coli*, *Proteus mirabilis*, *Enterobacter* spp., *Enterococcus faecalis*, *P. aeruginosa* i *Klebsiella pneumoniae* (Holá et al., 2010; Schulze et al., 2021). Infekcije urinarnog trakta povezane sa kateterom (CAUTIs) čine preko 40% bolničkih infekcija i više od 80% svih urinarnih infekcija, zbog čega predstavljaju globalni

zdravstveni problem (Milo et al., 2019), a najčešći uzročnici su *E. coli* i *K. pneumoniae* (Broughton et al., 2025; X. Liu et al., 2020).

1.3 Bakterijski biofilmovi u prehrambenoj industriji

Formiranje bakterijskih biofilmova u prehrambenoj industriji ima višestruke negativne posledice kako za bezbednost hrane, tako i za proizvodne procese. Mnoge bakterijske vrste sposobne za formiranje biofilma u pogonima prehrambene industrije jesu humani patogeni koji mogu kolonizovati različite materijale u proizvodnim postrojenjima, uključujući nerđajući čelik, polietilen, drvo, staklo, polipropilen i gumu (Abdallah et al., 2014). Biofilmovi na opremi i u kontaktu sa hranom mogu biti izvor trovanja ili infekcija, jer bakterije unutar biofilmova luče toksine koji kontaminiraju hranu i izazivaju pojedinačne ili grupne epizode trovanja. Lokacije na kojima se najčešće formiraju biofilmovi su vodovodni sistemi i mlekovodi, pasterizatori, membrane za reverznu osmozu, radne površine, rukavice zaposlenih, ali i trupovi životinja, skladišta sirovina i linije za pakovanje. Prisustvo biofilma na ovim mestima značajno povećava rizik po zdravlje potrošača i dodatno usložnjava higijenske procedure (Galié et al., 2018). Najčešći patogeni iz hrane sposobni za formiranje biofilma uključuju pripadnike roda *Salmonella*, *E. coli*, *Bacillus cereus*, *Listeria monocytogenes* i *S. aureus*, koji predstavljaju značajan zdravstveni rizik u prehrambenim postrojenjima (Galié et al., 2018).

Dijareja je najčešća zdravstvena posledica konzumiranja kontaminirane hrane, a *Salmonella* se smatra jednim od četiri najčešća uzročnika, pri čemu je *S. enterica* subsp. *enterica* serovar Enteritidis bio najčešće prijavljivani uzročnik u evropskim zemljama od 2021. do 2023. godine (EFSA & ECDC, 2022, 2023, 2024). Netifoidni sojevi iz roda *Salmonella* (NTS) obično izazivaju inflamatorni gastroenteritis sa relativno blagim simptomima kod imunokompetentnih osoba, dok su deca, starije osobe i imunokompromitovani pacijenti podložniji ozbiljnim komplikacijama, uključujući dehidraciju, sistemske infekcije sa bakterijemijom, meningitis, osteomijelitis, septički artritis, infekcije dubokih mekih tkiva i pneumoniju (Wen et al., 2017). Iako imunokompetentni pacijenti obično ne zahtevaju specifične terapije, ponovljena izloženost NTS, čak i bez simptoma, može predstavljati faktor rizika za razvoj ozbiljnijih bolesti, poput kolorektalnog karcinoma (Van Elsland et al., 2022).

E. coli, posebno sojevi koji proizvode šiga toksin (STEC), predstavljaju značajan problem zbog sposobnosti da izazovu enterohemoragični gastroenteritis. Konzumacija hrane kontaminirane STEC sojevima može izazvati ozbiljne zdravstvene poremećaje koji u najtežim slučajevima rezultiraju smrtnim ishodom (Yang et al., 2017). U težim slučajevima bolest može napredovati do hemolitičko-uremičkog sindroma, koji karakterišu akutno oštećenje bubrega i trombocitopenija (Bowen & Coward, 2018). Posebno zabrinjava visoka infektivna sposobnost sojeva poput *E. coli* O157:H7, kod kojih je potrebno manje od 50 bakterijskih ćelija da bi izazvale infekciju, što znači da čak i niska kontaminacija biofilmom ove bakterije u prehrambenim pogonima predstavlja zdravstveni rizik i zahteva stroge kontrolne mere (Marouani-Gadri et al., 2010).

B. cereus je sporogena bakterija sposobna za rast u širokom temperaturnom opsegu i preživljavanje nepovoljnih uslova, uključujući termičke i hemijske tretmane, što je čini značajnim patogenom u prehrambenoj industriji (Bottone, 2010). Njena sposobnost formiranja endospora omogućava preživljavanje industrijskih procesa pasterizacije i doprinosi perzistenciji biofilmova na površinama za preradu hrane, naročito u postrojenjima za preradu mleka (Auger et al., 2009). Pojedini sojevi proizvode dijarealne enterotoksine ili emetički toksin cereulid, koji izazivaju trovanje hranom sa simptomima dijareje, abdominalnog bola ili povraćanja, a u težim slučajevima i ozbiljnu dehidraciju i smrt (López et al., 2015; Soni et al., 2016; Tschiedel et al., 2015).

L. monocytogenes je široko rasprostranjena bakterija i jedan od značajnih patogena prenosivih hranom. Nije otporna na pasterizaciju, ali se ipak često prenosi putem različitih prehrambenih proizvoda (Milillo et al., 2012; Rothrock et al., 2017). Ova bakterijska vrsta lako

formira biofilmove, pretežno sastavljene od teihoičnih kiselina, na različitim industrijskim površinama, odakle se može preneti na prehrambene proizvode i umnožavati čak i na temperaturama hlađenja (Silva et al., 2008). Sposobnost rasta na niskim temperaturama, uz povećanu hidrofilnost i indukciju formiranja biofilma, kao odgovor na hladnoću, doprinosi njenoj povećanoj adheziji na površine i otpornosti na procedure čišćenja u prehrambenim postrojenjima (B.-H. Lee et al., 2017). Kod zdravih osoba ova bakterija najčešće izaziva gastroenteritis, dok kod trudnica, novorođenčadi, starijih i imunokompromitovanih osoba može dovesti do listerioze, teške bolesti praćene sepsom i meningitisom, a u trudnoći i do spontanog pobačaja ili oštećenja ploda (V. Ferreira et al., 2014; Silk et al., 2012).

S. aureus je značajan oportunistički patogen u prehrambenoj industriji, prvenstveno zbog svoje sposobnosti produkcije enterotoksina u širokom temperaturnom opsegu (Hennekinne et al., 2012). Ova bakterija se često nalazi na koži i sluzokoži radnika, što predstavlja važan izvor kontaminacije hrane, naročito imajući u vidu da su stafilokokni enterotoksini termostabilni i mogu se akumulirati u hrani tokom rasta bakterije (Giaouris et al., 2015). Ovi toksini izazivaju akutne gastrointestinalne simptome poput dijareje i povraćanja. Poseban problem predstavlja pojava meticilin-rezistentnih sojeva (MRSA) u stočarstvu, jer hrana životinjskog porekla može biti značajan rezervoar ovog rezistentnog patogena (Vergara et al., 2017). *S. aureus* je sposoban da formira biofilmove na biotičkim i abiotičkim površinama duž čitavog lanca proizvodnje hrane, što otežava njegovo uklanjanje i ima značajne ekonomske posledice.

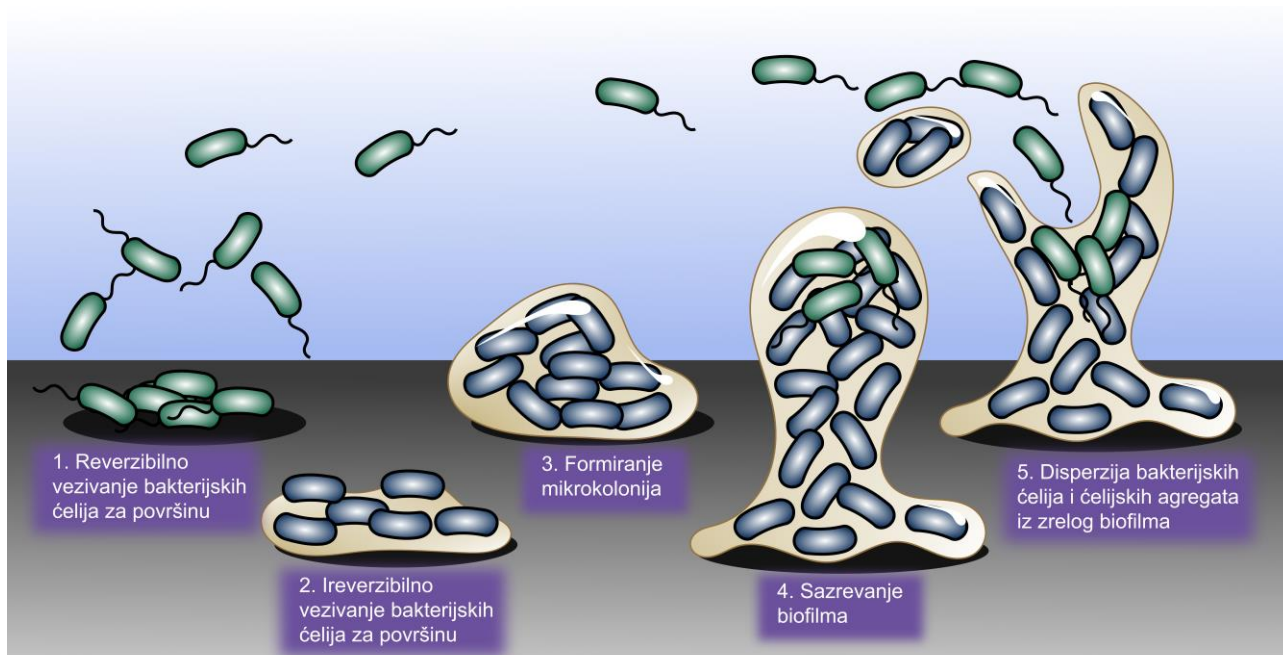
Svi ovi patogeni predstavljaju veliki izazov u prehrambenoj industriji, jer se lako vezuju za površine i formiraju biofilmove koji odolevaju standardnim procedurama čišćenja i dezinfekcije što dovodi do perzistentne kontaminacije i ozbiljnog narušavanja bezbednosti hrane (Galié et al., 2018). Prisustvo biofilмова ne samo da povećava verovatnoću izbijanja bolesti prenosivih hranom, već i otežava čišćenje i higijenske mere zbog povećane otpornosti biofilмова na antimikrobne agense i fizičke metode uklanjanja (X. Liu et al., 2023).

1.4 Formiranje biofilмова

Formiranje biofilмова je cikličan proces. Započinje adhezijom planktonskih ćelija za podlogu, a završava se kada pojedinačne ćelije počnu da napuštaju zreo biofilm u cilju rasejavanja (Sauer et al., 2022) (Slika 2). U fazi adhezije, planktonske ćelije dolaze u kontakt sa površinom, koja može biti biotička (npr. koža ili kost) ili abiotička (npr. medicinski uređaji poput urinarnih katetera ili veštačkih zglobova). Ova početna adhezija je slaba i reverzibilna i zavisi od osobina površine i uslova okoline. Uz pomoć pilusa i fimbrija, vezivanje bakterija za površinu postaje znatno jače. Kada bakterije započnu proizvodnju ekstracelularnih polimernih supstanci, koje omogućavaju snažno međusobno povezivanje ćelija i njihovo vezivanje za površinu, adhezija postaje ireverzibilna (Sauer et al., 2022). Tokom faze maturacije, vezane bakterije počinju da se razmnožavaju i formiraju mikrokolonije. Proizvodnja ekstracelularnih polimernih supstanci se povećava, čime se formira potporni matriks koja drži rastuću zajednicu na okupu i razvija se u trodimenzionalnu arhitekturu biofilma. Kako biofilm nastavlja da raste i sazreva, postaje sve kompleksniji i organizovaniji sistem, stvarajući mikrookruženje koje karakterišu gradijenti nutrijenata i kiseonika. To dovodi do fiziološke heterogenosti, sa brzorastućim ćelijama na površini i spororastućim, tolerantnim ćelijama u dubljim slojevima. Kao posledica toga, biofilm u ovoj fazi postaje tolerantniji na antibiotike i stresne uslove sredine (Sauer et al., 2022). Na kraju, u fazi disperzije, pojedinačne ćelije ili ćelijski klasteri se odvajaju od biofilma i kolonizuju nove površine. Ovaj proces se pokreće promenama u okruženju ili nedostatkom hranljivih materija (Sauer et al., 2022).

Istraživanja formiranja, strukture i funkcije biofilмова intenzivno napreduju i usmerena su ka razvoju inovativnih strategija za borbu protiv biofilмова. Kako bi se razvili efikasni pristupi za eliminaciju biofilмова, savremena istraživanja fokusirana su na otkrivanje novih antimikrobnih agenasa koji mogu sprečiti formiranje biofilмова i razgrađivati prethodno formirane biofilmove. Za

prevenciju formiranja biofilмова od presudnog je značaja sprečiti adheziju mikroorganizama na površine, jer je adhezija prvi korak u razvoju biofilma i trenutak kada je biofilm najosetljiviji (P. Gupta et al., 2016).



Slika 2. Shematski prikaz procesa formiranja biofilma. Planktonske bakterije i bakterije na površini zrelog biofilma (zeleno) koje se aktivno disperguju su metabolički aktivnije u odnosu na bakterije u dubljim slojevima biofilma (plavo).

1.4.1 Regulacija formiranja biofilмова

Formiranje biofilma zavisi od različitih regulatornih signala, uključujući glasnike kao što su ciklični di-guanozin-monofosfat (c-di-GMP), male nekodirajuće regulatorne RNK (sRNK, eng. *small regulatory RNAs*, sRNAs) i mali molekuli poznati kao autoinduceri (AI) koji posreduju u međućelijskoj komunikaciji (eng. *quorum sensing*, QS).

Prelazak između planktonskog načina života i biofilma kontroliše c-di-GMP (Römling et al., 2013). Visoke koncentracije c-di-GMP molekula podstiču proizvodnju adhezina i polisaharida matriksa i inhibiraju različite tipove pokretljivosti, čime se favorizuje formiranje biofilma. Suprotno tome, niski nivoi c-di-GMP molekula smanjuju proizvodnju adhezina i egzopolisaharida i povećavaju pokretljivost bakterija, što dovodi do disperzije biofilma (Valentini & Filloux, 2016).

sRNK regulišu formiranje biofilma modulacijom ekspresije gena, često na posttranskripcionom nivou (Taylor et al., 2017; Thomason et al., 2012). One pomažu bakterijama da se prilagode stresnim uslovima sredine, poput nedostatka hranljivih materija i prisustva antimikrobnih agenasa, regulacijom ekspresije gena uključenih u odgovor na stres, čime se poboljšavaju stabilnost i otpornost biofilмова. Pored toga, sRNK utiču na sintezu ekstracelularnih polimernih supstanci i regulišu ekspresiju gena uključenih u disperziju biofilma, omogućavajući bakterijama povratak u planktonsko stanje kada se uslovi promene. Na ovaj način, sRNK kontrolišu složene procese koji leže u osnovi formiranja, održavanja i disperzije biofilмова (Mitra & Mukhopadhyay, 2023).

QS je sistem komunikacije između ćelija koji omogućava bakterijama da donose kolektivne odluke u zavisnosti od gustine populacije i da se ponašaju poput višćelijskog organizma. QS sistem se sastoji od AI, sintaza koje proizvode AI i receptora/transkripcionih regulatora koji prepoznaje signal. Vezivanjem AI za receptor dolazi do aktivacije transkripcije gena, uključujući i biosintezu samog AI, čime se uspostavlja pozitivna povratna sprega. Pri niskoj gustini ćelija, nivoi

AI su niski i receptor je samo slabo aktivan. Sa porastom gustine ćelija, koncentracija AI dostiže prag koji dovodi do potpune aktivacije receptora i pojačane ekspresije ciljnih gena (Senerovic et al., 2020). QS signalni putevi regulišu gene koji su najčešće povezani sa patogenošću i utiču na proizvodnju faktora virulencije, formiranje biofilma i različite tipove pokretljivosti (Rutherford & Bassler, 2012).

1.5 Sastav matriksa biofilmova

Matriks biofilmova predstavlja visoko organizovanu, dinamičnu ekstracelularnu strukturu koja čini dominantnu komponentu biofilma i ključni je faktor njegove stabilnosti i funkcionalnosti. To je kompleksna mešavina polisaharida, proteina, eDNK, lipida i drugih biopolimera, uz visok sadržaj vode koja omogućava difuziju hranljivih materija i signalnih molekula (Flemming et al., 2016). Sastav i relativni udeo pojedinih komponenti matriksa variraju u zavisnosti od bakterijske vrste, uslova okoline i faze razvoja biofilma, predstavljajući adaptivni sistem koji omogućava biofilmu da funkcioniše kao koordinisana, višćelijska zajednica (Flemming et al., 2016).

Proteini učestvuju u strukturnoj organizaciji kroz vezivanje za polisaharide i eDNK i adheziju za različite površine (Flemming et al., 2023). Učestvuju i u održavanju i regulaciji metaboličkih procesa, pri čemu ekstracelularni enzimi omogućavaju razgradnju složenih supstrata i lokalnu dostupnost hranljivih materija, dok pojedini proteini učestvuju u redoks procesima i vezivanju metabolita i jona, čime se povećava metabolička efikasnost biofilma (Flemming et al., 2023).

eDNK doprinosi stabilnosti matriksa i formiranju mikrogradijenata vezivanjem jona i molekula, čime indirektno utiče na dostupnost nutrijenata i metabolita (Okshevsky et al., 2015). Takođe, eDNK može služiti kao lokalni rezervoar genetičkog materijala, omogućavajući horizontalni transfer gena povezanih sa metaboličkom adaptacijom i rezistencijom, što doprinosi funkcionalnoj plastičnosti biofilma (Madsen et al., 2012).

Lipidi u matriksu bakterijskih biofilmova doprinose funkcionalnoj organizaciji i metaboličkoj efikasnosti zajednice mikroorganizama u biofilmu (Constantino-Teles et al., 2022). Kroz povećanje hidrofobnosti matriksa i formiranje membranskih vezikula, lipidi utiču na zadržavanje i distribuciju metabolita, enzima i signalnih molekula unutar biofilma (Flemming et al., 2023).

Polisaharidi se smatraju glavnom i najvažnijom komponentom matriksa biofilma (Flemming et al., 2023). Oni obezbeđuju mehaničku stabilnost, adheziju za površine i međusobno povezivanje ćelija. Pored toga, zadržavaju vodu čime stvaraju povoljno mikrokruženje za međubakterijsku komunikaciju i difuziju hranljivih materija. Međutim, njihovo proučavanje je složeno, jer su polisaharidi izuzetno raznovrsni po sastavu, tipu glikozidnih veza, stepenu grananja i prisustvu različitih hemijskih supstituenata i naelektrisanja.

1.5.1 Struktura i uloga egzopolisaharida

Različite bakterijske vrste proizvode različite polisaharide, a čak i jedan isti soj može sintetisati više polisaharida u zavisnosti od faze razvoja biofilma i uslova sredine. Dodatno, polisaharidi matriksa mogu biti naknadno modifikovani nakon eksporta, što dodatno utiče na njihovu funkciju i stabilnost biofilma (Flemming et al., 2023). Polisaharidi imaju ključnu ulogu u adheziji, formiranju mikrokolonija, strukturi i arhitekturi biofilmova. Njihov sastav značajno doprinosi kolektivnim osobinama biofilma, uključujući zaštitu od nepovoljnih uslova okoline i imunskog odgovora domaćina (Limoli et al., 2015). Iako sastav matriksa biofilma pokazuje veliku varijabilnost među vrstama i sojevima, u nastavku su opisani egzopolisaharidi koji su do sada najtemeljnije okarakterisani i za koje postoji najviše eksperimentalnih dokaza o njihovoj ulozi u formiranju patogenih bakterijskih biofilmova (Slika 3).

Bakterije *P. aeruginosa* proizvode tri hemijski različita egzopolisaharida koji čine glavne polisaharidne komponente njihovih biofilmova: alginat, Pel i Psl. Svaki od ovih polisaharida ima jedinstvenu hemijsku strukturu i funkcionalnu ulogu u formiranju, stabilnosti i otpornosti biofilma (Gheorghita et al., 2023).

Alginat je anjonski, acetilovani polimer koji se sastoji od neponavljajućih monomera L-guluronske i D-manuronske kiseline povezanih β -1,4 glikozidnim vezama, koji se naročito prekomerno proizvodi u mukoidnim sojevima izolovanim iz pluća pacijenata sa CF i doprinosi hidriranosti biofilma, njegovoj arhitekturi i toleranciji na imunski odgovor i antibiotike (Mann & Wozniak, 2012).

Psl je neutralni polisaharid koji se sastoji od ponavljajućih pentasaharidnih jedinica D-manoze, D-glukoze i L-ramnoze, u odnosu 3:1:1. Učestvuje u adheziji na površine, međusobnom vezivanju ćelija i ranim fazama formiranja biofilma. Značajno doprinosi otpornosti biofilma na antibiotike i učestvuje u interakcijama sa drugim komponentama matriksa kao što su eDNK i proteinski adhezini (Mann & Wozniak, 2012).

Pel je katjonski polisaharid čija je slobodna forma uglavnom sastavljena od ponavljajućih jedinica α -1,4-galaktozamina i N-acetilgalaktozamina, a ključan je za međućelijske interakcije, agregaciju i formiranje struktura biofilma, često interagujući sa eDNK radi povećanja mehaničke kohezije matriksa (Le Mauff et al., 2022).

Alginat

$-\beta$ -D-ManUA-(1 $\rightarrow$ 4)- β -D-ManUAAc-(1 $\rightarrow$ 4)- β -D-ManUAAc-(1 $\rightarrow$ 4)- β -L-GulUA-(1 $\rightarrow$ 4)- β -D-ManUA-

Psl

$[\rightarrow 3)-\beta$ -D-Manp-(1 $\rightarrow$ 3)- β -D-Manp-(1 $\rightarrow$ 3)- α -L-Rhap-(1 $\rightarrow$ 3)- β -D-Glcp-(1 $\rightarrow$)]_n
 $\begin{array}{c} 2 \\ \uparrow \\ 1 \\ \alpha\text{-D-Manp} \end{array}$

Pel

$-\alpha$ -D-GalNAc-(1 $\rightarrow$ 4)- α -D-GalN-(1 $\rightarrow$ 4)- α -D-GalNAc-(1 $\rightarrow$ 4)- α -D-GalN-

PNAG

$-\beta$ -D-GlcNAc-(1 $\rightarrow$ 6)- β -D-GlcNAc-(1 $\rightarrow$ 6)- β -D-GlcN-(1 $\rightarrow$ 6)- β -D-GlcNAc-

Celuloza

$-\beta$ -D-Glcp-(1 $\rightarrow$ 4)- β -D-Glcp-(1 $\rightarrow$ 4)- β -D-Glcp-(1 $\rightarrow$ 4)- β -D-Glcp-

Kolanska kiselina

$[\rightarrow 3)-\beta$ -D-GlcpAc-(1 $\rightarrow$ 3)- β -L-Fucp-(1 $\rightarrow$ 4)- α -L-Fucp-(1 $\rightarrow$)]_n
 $\begin{array}{c} 4 \\ \uparrow \\ 1 \\ \beta\text{-D-Galp} \\ 3 \\ \uparrow \\ 1 \\ \beta\text{-D-GlcUA} \\ 4 \\ \uparrow \\ 1 \\ \beta\text{-D-GalpPyr} \end{array}$

ManUA – manuronska kiselina
ManUAAc – acetilovana manuronska kiselina
GulUA – guluronska kiselina
Manp – manopiranoza
Rhap – ramnopiranoza
Glcp – glukopiranoza
GalNAc – N-acetilgalaktozamin
GalN – galaktozamin
GlcNAc – N-acetilglukozamin
GlcN – glukozamin
GlcpAc – acetilovana glukopiranoza
Fucp – fukopiranoza
Galp – galaktopiranoza
GlcUA – glukuronska kiselina
GalpPyr – piruvilisana galaktopiranoza

Slika 3. Shematski prikaz strukture bakterijskih egzopolisaharida.

U biofilmovima *E. coli* identifikovana su tri glavna egzopolisaharida: poli- β -1,6-N-acetil-D-glukozamin (PNAG), celuloza i kolanska kiselina, za koje je pokazano da imaju ključnu ulogu u formiranju i stabilnosti biofilma (Beloin et al., 2008).

PNAG je katjonski polisaharid sastavljen od β -1,6-povezanih jedinica N-acetilglukozamina, pri čemu je deo acetil grupa uklonjen, što rezultuje pozitivnim neto naelektrisanjem. Predstavlja važan adhezivni element koji učestvuje u međusobnom povezivanju ćelija i vezivanju za površine. PNAG je karakterističan i za biofilmove *S. aureus*, *Staphylococcus epidermidis*, *Actinobacillus actinomycetemcomitans*, *Actinobacillus pleuropneumoniae* i *A. baumannii* (Beloin et al., 2008; Choi et al., 2009).

Celuloza, linearni neutralni homopolisaharid građen od β -1,4-povezanih D-glukopiranoznih jedinica, predstavlja važnu strukturnu komponentu biofilma i povezana je sa formiranjem rigidnih struktura biofilma, naročito na granici vazduh-tečnost. Proizvodnja celuloze je široko rasprostranjen fenomen u porodici Enterobacteriaceae, uključujući *Salmonella enterica*, *K. pneumoniae*, *Citrobacter* spp. i *Enterobacter* spp (Beckman et al., 2024; Beloin et al., 2008).

Kolanska kiselina je anjonski heteropolisaharid sastavljen od glukoze, galaktoze, fukoze i glukuronske kiseline i može formirati kapsulu ili biti oslobođena u ekstracelularni prostor (Tsvetkova & Koshel, 2024). Iako kolanska kiselina može inhibirati početnu adheziju bakterija, njena sinteza je pojačana u zrelih biofilmovima, gde doprinosi razvoju trodimenzionalne arhitekture i zaštiti ćelija od stresa (Beloin et al., 2008). Kolanska kiselina ulazi i u sastav biofilma *S. enterica* (Sande & Whitfield, 2021).

Pored ovih slobodnih egzopolisaharida, ulogu u održavanju strukture biofilma imaju i brojni kapsularni polisaharidi i lipopolisaharidi koji pokazuju izraženu strukturnu raznovrsnost i mogu značajno varirati između različitih izolata, čak i unutar iste bakterijske vrste (Beloin et al., 2008; Mann & Wozniak, 2012; Sande & Whitfield, 2021).

1.6 Strategije za inhibiciju formiranja biofilma i razgradnju prethodno formiranih biofilma

Strategije za inhibiciju formiranja biofilma i razgradnju prethodno formiranih biofilma zasnovane su na primeni hemijskih agenasa i fizičkih metoda sa različitim načinima delovanja:

1) Baktericidni agensi: Ovi agensi sprečavaju formiranje biofilma inhibicijom rasta ili ubijanjem bakterija. Uobičajeni baktericidni inhibitori formiranja biofilma uključuju male molekule i strukturne analoge kliničkih antibiotika (Blasco et al., 2024; Kokot et al., 2023), metalne komplekse (Glišić et al., 2016; Rinehart et al., 2023; Savić et al., 2016; Sovari et al., 2021), nanočestice (A. Gupta et al., 2018), biljne ekstrakte i eterična ulja (Gómez-Sequeda et al., 2020; Sánchez et al., 2016; Zizovic et al., 2018), antimikrobne peptide (Klubthawee et al., 2023), bakteriofage (Vukotic et al., 2020) i probiotike koji proizvode antimikrobne metabolite (Al-Shamiri et al., 2023; J.-E. Lee et al., 2021). Dok su efikasni u ranim fazama infekcije, dugotrajna upotreba baktericidnih agenasa može dovesti do razvoja rezistencije, što kompromituje efikasnost terapije. Pored ovih farmakoloških agenasa, u ovu grupu spadaju i baktericidna sredstva koja se široko koriste u postupcima čišćenja i dezinfekcije u medicinskim, prehrambenim i industrijskim sistemima, kao što su hlor, vodonik-peroksid, jod, izotiazolinoni, kiseline, biocidi na bazi aldehida, fenoli, bigvanidi, surfaktanti, halogeni i kvartenarna amonijumova jedinjenja (Nahar et al., 2018).

2) Antivirulentni agensi: Za razliku od baktericidnih agenasa, ovi agensi ciljano deluju na virulentne osobine patogena, uključujući formiranje biofilma, bez ometanja rasta bakterija. Ovo čini antivirulentne strategije posebno korisnim, jer nose manji rizik od razvoja rezistencije na tretman. Efikasna prevencija formiranja biofilma antivirulentnim agensima može se postići ciljanjem različitih faza procesa formiranja biofilma.

Prekid adhezije bakterija na površine može se postići upotrebom biosurfaktanata (Aleksic et al., 2017; Amirinejad et al., 2022; Sambanthamoorthy et al., 2014) kao što su glikolipidi, ramnolipidi, lipopeptidi, polisaharid-protein kompleksi, fosfolipidi ili masne kiseline, antitela koja ciljaju adhezinske proteine poput pilusa ili fimbrija (de Freitas et al., 2021; D. Sun et al., 2005), ili helatori koji mogu ukloniti katjone (Fe , Ca^{2+} i Mg^{2+}) uključene u mikrobnu adheziju i formiranje biofilma (Abraham et al., 2012; O'May et al., 2009).

QS se može ometati na više nivoa signalnih puteva. Pristupi uključuju inhibiciju sinteze AI pomoću malih molekula koji ciljaju sintaze (Chang et al., 2014), upotrebu enzima koji hidrolizuju AI smanjujući njihovu koncentraciju (Djokic et al., 2022; Koch et al., 2014; Malešević et al., 2020; Pustelny et al., 2009) i blokiranje interakcije AI sa receptorima kompetitivnom inhibicijom pomoću prirodnih, sintetičkih ili polusintetičkih molekula sličnih prirodnim AI (Aleksic et al., 2017, 2018, 2019; Pekmezovic et al., 2016).

Signalizacija posredovana c-di-GMP molekulom se može modulirati malim molekulskim inhibitorima sinteze c-di-GMP molekula (Andersen et al., 2021; Chua et al., 2015) ili direktnim smanjenjem nivoa c-di-GMP molekula pomoću peptida koji ga vezuju (Hee et al., 2020).

Inhibicija maturacije biofilma može se postići blokiranjem biosintetskih puteva ekstracelularnih polimernih supstanci malim molekulima (Razvi et al., 2023) ili hidrolizom komponenti ekstracelularnih polimernih supstanci pomoću specifičnih enzima (Atanaskovic & Senerovic, 2024; Baker et al., 2016; Banar et al., 2016; Lim et al., 2019).

3) Fizičke metode: Hirurško uklanjanje biofilma podrazumeva mehaničko odstranjivanje inficiranog tkiva, nekrotičnih delova ili uklanjanje/zamenu kontaminiranih medicinskih implantata kako bi se redukovalo prisustvo bakterija i omogućilo efikasnije delovanje antimikrobne terapije (Wilkins et al., 2014). Ovaj pristup se primenjuje kada konzervativno lečenje, uključujući primenu antibiotika i antiseptika, nije dovoljno efikasno, naročito zbog visoke tolerancije bakterija unutar biofilma. Hirurška intervencija je često neophodna kod hroničnih rana, dubokih infekcija mekih tkiva, infekcija povezanih sa implantatima ili protezama, kao i u slučajevima prisustva nekrotičnog tkiva koje sprečava zarastanje rane i predstavlja rezervoar patogena.

Ostale fizičke metode za prevenciju ili uklanjanje bakterijskih biofilмова obuhvataju mehaničke, termičke i energetske zasnovane tehnike koje narušavaju integritet matriksa, adheziju ili vijabilnost ćelija bez primene hemijskih ili bioloških agenasa (Otto et al., 2011). *Mehanička razgradnja* uključuje pranje pod visokim pritiskom, ribanje, četkanje, što fizički uklanja biofilmove sa površina kao što su cevi ili zubi (Barry & Kanematsu, 2015; Sahni et al., 2016). *Termički pristupi* podrazumevaju primenu povišenih temperatura, koje dovode do denaturacije proteina ekstracelularnog polimernog matriksa i ubijanja ćelija (O'Toole et al., 2015). *Metode zasnovane na primeni energije* najčešće koriste zračenje (Jahid et al., 2014; Jung et al., 2018), ultrazvuk i zvučne talase za eroziju biofilma (Hazan et al., 2006; H. Yu et al., 2020). Postoje i napredniji pristupi, poput primene nanomehurića, koji formiraju hidrofobni sloj na površini koji sprečava adheziju bakterija i formiraju reaktivne kiseonične vrste koje ubijaju bakterije. Hladna plazma proizvodi reaktivne kiseonične i azotne vrste, UV zračenje i naelektrisane čestice koje prodiru u matriks i razgrađuju i matriks i ćelijske membrane. Naizmenična elektromagnetna polja mehanički erodiraju biofilmove, dok električne struje mogu indukovati elektroporaciju ćelijskih membrana i dodatno doprineti destabilizaciji biofilma (Gilmore et al., 2018; Gu et al., 2020; H. Wang & Ren, 2017).

1.6.1 Strategije zasnovane na enzimima

Strategije zasnovane na enzimima predstavljaju jedan od perspektivnijih pristupa u borbi protiv biofilмова, jer omogućavaju ciljano delovanje na specifične komponente matriksa biofilma ili na signalne molekule uključene u regulaciju formiranja biofilma. U tu svrhu proučavani su različiti tipovi enzima, uključujući enzime koji ometaju međućelijsku komunikaciju bakterija (engl. *quorum quenching*, QQ) kao i enzime koji razgrađuju egzopolisaharide matriksa, proteolitičke i

lipolitičke enzime, oksidativne enzime, kao i deoksiribonukleaze (DNKaze) (Al-Madboly et al., 2024; Nahar et al., 2018). Za razliku od konvencionalnih antibiotika, koji uglavnom deluju direktno na rast i preživljavanje bakterijskih ćelija, enzimi prvenstveno narušavaju strukturu i funkcionalnost biofilma. Na taj način dolazi do razgradnje ekstracelularnog matriksa, smanjenja adhezije bakterija za površine ili ometanja bakterijske komunikacije, čime se biofilm destabilizuje i postaje podložniji delovanju drugih antimikrobnih agenasa. Zbog ovakvog mehanizma delovanja, primena enzima je povezana sa manjim selektivnim pritiskom na bakterije i samim tim manjim rizikom od razvoja rezistencije u poređenju sa klasičnim antibioticima (Al-Madboly et al., 2024).

Međutim, pošto većina enzima nema izražen baktericidni efekat, njihova primena kao samostalne terapije često nije dovoljna za potpunu eliminaciju bakterija. Iz tog razloga, enzimi se najčešće koriste u kombinaciji sa antibioticima ili drugim antimikrobnim agensima (Borges et al., 2020).

Tretmani zasnovani na enzimima se suočavaju sa izazovima koji mogu ograničiti njihovu širu primenu u kliničkoj i industrijskoj praksi. Jedan od glavnih problema predstavlja nestabilnost enzima pod određenim uslovima sredine, kao što su promene temperature, pH vrednosti ili prisustvo proteolitičkih enzima, što može dovesti do smanjenja njihove aktivnosti i efikasnosti (Rigoldi et al., 2018). Pored toga, proizvodnja i prečišćavanje enzima često zahtevaju složene biotehnološke procese, zbog čega su troškovi njihove proizvodnje relativno visoki (R. D. G. Ferreira et al., 2018). Takođe, postoji rizik od imunskih reakcija organizma na primenjene enzime. Imunski sistem može prepoznati enzime kao strane molekule i pokrenuti imunski odgovor, što dovodi do smanjenja terapijske efikasnosti usled neutralizacije enzima, ali i do pojave neželjenih reakcija, poput lokalne inflamacije ili alergijskih reakcija (de la Fuente et al., 2021). Zbog toga je prilikom razvoja enzimskih terapija neophodno pažljivo proceniti njihovu biokompatibilnost, stabilnost i bezbednost primene.

Jedini enzim koji ima sposobnost razgradnje biofilмова, a koji se već primenjuje u lečenju je rekombinantna humana DNKaza poznata kao Dornaza alfa. Primenjuje se u lečenju pacijenata sa CF i uglavnom se koristi za smanjenje viskoznosti sluzi u disajnim putevima i poboljšanje funkcije pluća. Ovaj enzim razgrađuje DNK koja se oslobađa iz neutrofila (Shak, 1995), ali i onu koja ulazi u sastav matriksa bakterijskog biofilma (Z. Wang et al., 2024). Iako su kliničke studije do 2006. godine pokazale da dosledna upotreba DNKaze može značajno smanjiti učestalost plućnih egzacerbacija, usporiti pogoršanje funkcije pluća i sprečiti bakterijske infekcije (Frederiksen et al., 2006), do danas nije korišćen nijedan drugi enzim sa uporedivim svojstvima.

Sa druge strane, enzimi se široko primenjuju u čišćenju medicinskih instrumenata zbog svoje sposobnosti da efikasno razgrađuju organske nečistoće koje ostaju nakon kliničke upotrebe, poput krvi, proteina, lipida i polisaharida, što poboljšava uklanjanje ostataka čak i sa teško dostupnih površina i složenih instrumenata (Cortés et al., 2017). Preparati zasnovani na enzimima, kao što su Lysonox Enzy4Clean, Lysonox Instruments DR, Gigazyme® Enzymatic Cleaner, CIDEZYME™, MEDAZYM, NOSOZYM i Instruton E, koriste proteaze, lipaze i amilaze kako bi ciljano razložili proteine, masnoće i skrob, omogućavajući temeljno čišćenje pri nižim temperaturama i neutralnom pH, uz smanjen rizik od oštećenja instrumenata ili fiksiranja organskih ostataka. Enzimski tretmani su posebno pogodni za fazu pred-čišćenja, jer putem uklanjanja matriksa biofilma i organskih depozita omogućavaju da kasniji koraci dezinfekcije i sterilizacije budu efikasniji, značajno doprinoseći kontroli i prevenciji bolničkih infekcija (Cortés et al., 2017).

Zbog svoje sposobnosti da efikasno razgrađuju organske nečistoće i komponente biofilмова, kao što su proteini, lipidi i polisaharidi, enzimi se sve češće primenjuju i u čišćenju i održavanju higijene u prehrambenoj industriji (Siddikey et al., 2025). Enzimski preparati koji sadrže kombinacije proteaza, lipaza i amilaza, omogućavaju uklanjanje tvrdokornih naslaga sa površina i iz CIP (eng. *Clean In Place*) sistema pri nižim temperaturama i uz manju upotrebu agresivnih hemikalija. U tu svrhu koriste se proizvodi kao što su MIDA ENZY 1004, MIDA ENZY

1005 i MIDA ENZY 1006, namenjeni čišćenju otvorenih površina i CIP sistema u prehrambenim pogonima (Blasi et al., 2025). Upotrebom ovakvih proizvoda postiže se efikasnije uklanjanje biofilмова, smanjuje rizik od perzistentne kontaminacije i poboljšava bezbednost hrane, uz istovremeno manji negativni uticaj na opremu i životnu sredinu.

1.6.1.1 Glikozid-hidrolaze u razgradnji egzopolisaharida iz bakterijskih biofilмова

Potpuna enzimska razgradnja polisaharida uključuje različite enzime, među kojima glikozid-hidrolaze imaju ključnu ulogu, jer katalizuju raskidanje glikozidnih veza u polisaharidima i oligosaharidima, oslobađajući kraće, metabolički iskoristive proizvode (Berlemont & Martiny, 2016). Glikozid-hidrolaze se klasifikuju u različite familije na osnovu strukturnih karakteristika, pri čemu mnoge pokazuju visoku specifičnost prema određenim supstratima, dok pojedine imaju širu supstratnu specifičnost.

S obzirom na to da ciljano deluju na polisaharidne komponente ekstracelularnog polimernog matriksa, koje imaju centralnu ulogu u obezbeđivanju strukturnog integriteta, mehaničke stabilnosti i zaštitne funkcije biofilma, glikozid-hidrolaze predstavljaju perspektivnu strategiju zasnovanu na enzimima u borbi protiv patogenih biofilмова. Razgradnjom glikozidnih veza unutar ovih polisaharida, glikozid-hidrolaze dovode do destabilizacije matriksa, povećanja njegove poroznosti i narušavanja kohezije biofilma, čime se značajno olakšava prodor antibiotika, dezinfekcionih sredstava i efektorskih molekula imunskog sistema (S. Wang et al., 2023).

Poseban značaj ima kombinovana primena glikozid-hidrolaza sa antibioticima ili hemijskim dezinfekcionim sredstvima, pri čemu je u brojnim studijama opisan izražen sinergistički efekat (Donelli et al., 2007; Fleming et al., 2016; Kaplan et al., 2025). Ovakav pristup omogućava smanjenje efektivnih doza antimikrobnih agenasa, skraćanje trajanja terapije i potencijalno smanjenje neželjenih efekata, ali i umanjeње selektivnog pritiska koji doprinosi razvoju i širenju antimikrobne rezistencije. Zbog svoje specifičnosti, biorazgradivosti i relativno niskog rizika od indukovanja rezistencije, glikozid-hidrolaze se sve češće razmatraju kao inovativna biološka sredstva za prevenciju i kontrolu biofilмова u različitim medicinskim, prehrambenim i industrijskim sistemima (S. Wang et al., 2023).

U tom kontekstu, brojne komercijalno dostupne glikozid-hidrolaze ispitivane su kao potencijalni antibiofilm agensi, uključujući celulaze, α -amilaze, alginat-lijaze, pektinaze, amiloglukozidaze, inulinaze i ksilanaze, koje se odlikuju širokom supstratnom specifičnošću i sposobnošću degradacije različitih polisaharidnih komponenti matriksa (Redman et al., 2021). Iako ovi enzimi često deluju nespecifično, njihova primena dovodi do značajnog narušavanja strukture biofilma. Pored toga, identifikovane su i geni za specifične glikozid-hidrolaze koje predstavljaju sastavne delove operona uključenih u biosintezu egzopolisaharida kod pojedinih patogenih bakterija, a čija je antibiofilm aktivnost detaljno proučavana. Među najznačajnijim primerima ovakvih enzima su Disperzin B, PslG i PelA, koji učestvuju u kontrolisanom remodelovanju matriksa bakterijskih biofilмова. Kada se primene egzogeno, u dozama višim od fizioloških, oni ciljano razgrađuju ključne strukturne polisaharide biofilмова i time direktno doprinose njegovoj dezintegraciji (Baker et al., 2015; Dobrynina et al., 2015; Szymańska et al., 2020).

Iako se već više od deset godina proučavaju enzimi sa sposobnošću razgradnje odnosno uklanjanja biofilмова, do danas samo Dornaza alfa ima kliničku primenu. Razlog tome nije samo smanjena efikasnost enzima u *in vivo* model sistemima u poređenju sa *in vitro* esejima koji se uobičajeno koriste u laboratorijskim uslovima, već u česte probleme spadaju i niska stabilnost ovih enzima i široka specifičnost što za posledicu neretko ima pojavu toksičnosti. Sa druge strane, previše uska specifičnost takođe predstavlja izazov, jer takvi enzimi deluju samo na ograničen broj bakterijskih vrsta, čime se značajno sužava mogućnost njihove šire primene, naročito u prevenciji i tretmanima biofilмова čiji bakterijski sastav nije poznat. Iz ovih razloga i dalje postoji potreba za identifikacijom novih glikozid-hidrolaza koje bi istovremeno imale dovoljno selektivnu aktivnost da

ne ispoljavaju toksičnost prema humanim ćelijama niti baktericidno dejstvo, ali i dovoljno širok spektar delovanja da obuhvate različite patogene vrste, čime bi bile pogodne za primenu u prevenciji i tretmanu biofilмова nepoznatog ili mešovitog bakterijskog sastava.

1.7 Mikrobiota škrga deverike (*Abramis brama*) kao izvor novih glikozid-hidrolaza

Mikrobiota škrga ima važnu ulogu u imunskom sistemu riba i ispoljava izražena antimikrobna svojstva, te predstavlja prvu liniju odbrane od patogena (Ortiz et al., 2023). Zbog stalne izloženosti spoljašnjoj sredini, promenama fizičko-hemijskih uslova i prisustvu potencijalno patogenih mikroorganizama, bakterije koje naseljavaju škrge izložene su specifičnim selektivnim pritiscima (Merrifield & Rodiles, 2015). Škrge deverike predstavljaju posebno interesantnu ekološku nišu za potragu za novim glikozid-hidrolazama. Deverika je bentivorna slatkovodna vrsta koja najveći deo vremena provodi u priobalnim i sporotekućim zonama dna, bogatim sedimentom i organskom materijom. Tokom ishrane i ventilacije, velika količina vode i suspendovanih čestica prolazi preko škrga, izlažući ih mikroorganizmima i organskim supstratima poreklom iz sedimenta (Hoogenboezem & Boogaart, 1993). Mikrobiota škrga često odražava diverzitet spoljašnje sredine i pokazuje sličnosti sa mikrobim zajednicama prisutnim u okolnoj vodi i sedimentu. Sediment dna, posebno u eutrofnim slatkovodnim ekosistemima koje deverika nastanjuje je bogat algama i detritusom koji predstavljaju značajan rezervoar strukturno raznovrsnih polisaharida (Merrifield & Rodiles, 2015). Algalni polisaharidi, kao i drugi ugljeno-hidratni polimeri prisutni u sedimentu, mogu predstavljati izvor ugljenika i energije za pridružene mikroorganizme, favorizujući selekciju bakterija sposobnih za sintezu glikozid-hidrolaza.

Dodatni faktor koji podržava hipotezu da je mikrobiota škrga riba dobar izvor glikozid-hidrolaza je prisustvo mukusa na površini škrga. Mukus škrga predstavlja hidrogelnu zaštitnu barijeru čiju dominantnu komponentu čine mucini, visoko glikozilovani proteini bogati različitim ugljeno-hidratnim strukturama (Reverter et al., 2018). Ovakvo glikanima bogato okruženje predstavlja potencijalni nutritivni supstrat i selektivni pritisak za mikroorganizme sposobne da sintetišu enzime za razgradnju ili modifikaciju glikanskih i polisaharidnih komponenti. Iako se mukus škrga strukturno razlikuje od ekstracelularnog matriksa bakterijskih biofilмова, oba sistema karakteriše prisustvo kompleksnih ugljeno-hidratnih mreža, zbog čega mikrobiota škrga može predstavljati perspektivan izvor novih glikozid-hidrolaza sa potencijalom za degradaciju egzopolisaharidnih komponenti matriksa bakterijskih biofilмова.

Glikozid-hidrolaze poreklom iz ovakvog ekološkog okruženja potencijalno poseduju visok afinitet prema polisaharidnim komponentama ekstracelularnog matriksa biofilma, kao i povećanu stabilnost i aktivnost u uslovima promenljive temperature, saliniteta i pH vrednosti. Zbog svega navedenog, mikrobiota škrga ribe predstavlja obećavajući i do sada nedovoljno istražen rezervoar novih glikozid-hidrolaza sa potencijalnom primenom u kontroli i suzbijanju infekcija uzrokovanih biofilmovima.

2. CILJ ISTRAŽIVANJA

Glavni cilj ovog istraživanja je da se identifikuju nove glikozid-hidrolaze specifične za polisaharide iz matriksa biofilmova odabranih medicinski značajnih patogenih bakterija.

Specifični ciljevi istraživanja:

1. Evaluacija glikozid-hidrolazne aktivnosti kolekcije bakterijskih izolata izolovanih iz škrga deverike,
2. Identifikacija, rekombinantna ekspresija i biohemijska karakterizacija novih glikozid-hidrolaza koje imaju potencijal da razgrade matriks biofilmova različitih patogenih bakterija,
3. Uspostavljanje modela biofilmova koji simuliraju uslove u prehrambenoj industriji ili medicinskom okruženju koristeći patogene bakterije okarakterisanog egzopolisaharidnog sastava, njihove kliničke izolate ili generisane delecione mutante za pojedinačne egzopolisaharide,
4. Ispitivanje antibiofilm aktivnosti glikozid-hidrolaza u monotretmanu i u kombinaciji sa antibioticima ili dezinfekcionim sredstvima na odgovarajućim modelima biofilmova,
5. Ispitivanje bezbednosnog profila odabranih enzima određivanjem njihovog potencijalnog baktericidnog dejstva na ispitivane patogene bakterije, citotoksičnosti na kulturi humanih fibroblasta i uticaja na vijabilnost model-organizma *Caenorhabditis elegans*.

3. NAUČNI RADOVI PROIZAŠLI IZ DOKTORSKE DISERTACIJE

3.1 Inhibition of *Salmonella* Enteritidis adhesion and biofilm formation by β -glucosidase B from *Microbacterium* sp. BG28



Inhibition of *Salmonella* Enteritidis adhesion and biofilm formation by β -glucosidase B from *Microbacterium* sp. BG28

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ABSTRACT

Salmonella: Enteritidis is the most commonly reported pathogen for foodborne illness outbreaks in both under-developed and developed regions. *S. Enteritidis* biofilms, which form on various food contact surfaces, are resistant to conventional physical and chemical cleaning and disinfection procedures routinely used in food processing. The aim of this study was to identify novel, industrially applicable enzymes that are active against *S. Enteritidis* biofilms. We describe the properties and anti-biofilm activity of heterologously expressed β -glucosidase B derived from the environmental strain *Microbacterium* sp. BG28 (BgIB-BG28) collected from gills of bream fish. The enzyme inhibited adhesion and the early stages of biofilm formation in clinical isolates of *S. Enteritidis*. At a concentration of 200 μ g/mL, BgIB-BG28 effectively reduced biofilm formation, by decreasing biofilm biomass by 50% and metabolic activity within biofilms by 80%. The enzyme reduced the formation of air-liquid biofilms on various surfaces, including plastic, glass and metal, as observed by fluorescence microscopy. BgIB-BG28 inhibited biofilm formation in *Escherichia coli*, another important food pathogen that also forms cellulose-rich biofilms. Using o-NPG as substrate, the enzyme showed activity at temperatures up to 50 °C and in a pH range between 4 and 8, high tolerance to sodium chloride and glucose, and compatibility with nonionic detergents. Importantly, no toxicity was observed in the model system *Caenorhabditis elegans* even at an enzyme concentration of 1 mg/mL. These results suggest that the β -glucosidase BgIB-BG28 is a promising candidate for the development of a new enzyme-based disinfection protocol that can be used in food processing facilities.

1. Introduction

Foodborne diseases impose a significant burden on global health. According to the World Health Organisation (WHO), approximately one in ten people contract a foodborne illness within a year. Diarrheal disease is the most common health consequence of consuming contaminated food and *Salmonella* is considered one of the four most common causes (World Health Organisation, 2018), with *S. Enteritidis* being the most frequently reported causative agent in European countries in 2021 (Authority & European Centre for Disease Prevention and Control, 2022).

The non-typhoidal *Salmonella* serovars (NTS), including *S. Enteritidis*, usually cause inflammatory gastroenteritis with relatively mild symptoms in immunocompetent individuals. Children, the elderly and other immunocompromised individuals are more likely to develop fatal complications such as dehydration, systemic infections with bacteremia,

meningitis, osteomyelitis, septic arthritis, deep soft tissue infections, and pneumonia (Wen et al., 2017). Although immunocompetent individuals recover in most cases without specific treatment, repeated exposure to NTS, even without symptoms, has recently been shown to be an environmental risk factor for the development of serious diseases such as colorectal cancer (Van Elsland et al., 2022). In Europe, about 90,000 human cases of salmonellosis are reported each year, but it is estimated that for every reported case, there are on average about 57 *Salmonella* infections that go unreported because they have few or no symptoms (Mughini-Gras et al., 2018). Therefore, the priority is to prevent outbreaks of infection by targeting their origins, such as food processing facilities. NTS outbreaks are usually associated with food of animal origin, but recently more cases have been associated with the fresh-cut fruit and vegetable industry (White et al., 2021).

NTS biofilms are commonly found on surfaces in food processing facilities and can serve as reservoirs for recurrent food contamination.

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Bacteria embedded in biofilms are less susceptible to various types of environmental stresses compared to their planktonic counterparts. This is due to several mechanisms such as reduced penetration of disinfectants into the biofilm, altered physiology of biofilm cells, protection in mixed-species biofilms, and the appearance of persister cells (Yuan et al., 2021). Chlorine, hydrogen peroxide, iodine, isothiazolinones, acidic compounds, aldehyde-based biocides, phenols, biguanides, surfactants, halogens, and quaternary ammonium compounds are commonly used chemicals in the food industry, and many of them are unable to completely eliminate biofilms, while producing toxins and promoting the corrosion of the surfaces (Nahar et al., 2018). Enzymatic treatments have been proposed as an efficient and environmentally-friendly way to degrade biofilm components and facilitate the penetration of detergents and disinfectants (Nahar et al., 2018).

Apart from inadequate cleaning and sanitation practises, persistence of NTS in food processing environments may be caused by the acquisition of resistance to disinfectants (Møretro et al., 2012). Exposure of NTS to disinfectants may lead to cross-resistance to other antimicrobials (Donaghy et al., 2019) and increased biofilm production (Mangalappalli-Illathu et al., 2008). New strategies for biofilm removal in the food industry are emerging, including surface modification, pulsed light, acoustic pressure, ultrasound, various chemical disinfectants and natural extracts, quorum sensing inhibitors, bacteriophages, antimicrobial peptides, and enzymes (Gao et al., 2023; Ling et al., 2020; Pelyuntha & Vongkamjan, 2022; Toushik et al., 2020; Wason & Subbiah, 2023; Yu et al., 2021; Yuan et al., 2020). Given the challenges associated with effective removal of mature biofilms, emphasis has been placed on developing strategies that prevent biofilm formation (Nahar et al., 2018). Among these strategies, those focused on inhibiting initial stages of biofilm development, are of great interest.

Even though some pathogenic *Salmonella* strains lost the ability of biofilm formation due to the host-adaptation, many of them kept this ability and are expressing a rdar (red, dry, and rough) morphotype which enables them better survival in non-host environment (MacKenzie et al., 2019). The rdar morphotype primarily enables survival outside the host under nutrient-limited conditions through the overproduction of curli fimbriae and cellulose, the major components of *Salmonella* biofilms (Lamas et al., 2018). Cellulose does not play a critical role in the virulence of *Salmonella*, but allows survival in environmental surface habitats (Solano et al., 2002). Cellulase has been shown to be efficient in disintegrating *Salmonella* biofilms, implying that cellulase is a suitable target for anti-biofilm agents (M.-J. Kim & Kim, 2022; H. Wang et al., 2016). However, cellulases have wide application in various food processing industries, they are known as macerating enzymes used in the extraction of fruit and vegetable juices (Soares et al., 2016), so it is important to use them with caution. Pretreatment of food contact surfaces with these enzymes has the potential to alter the surface properties of fresh produce, potentially accelerating their spoilage. With this in mind, we searched for enzymes that specifically target the mechanism of cellulose synthesis used by *Salmonella* in biofilm formation without affecting the cellulose present in the cell structure of fruits and vegetables.

β -Glucosidases are enzymes that play a central role in the hydrolysis of cellulose-derived oligosaccharides, thus they have the potential to alter cellulose synthesis without degrading mature cellulose polymers. Moreover, β -glucosidase from almonds has shown significant anti-biofilm effect on *Pseudomonas aeruginosa* biofilms (Banar et al., 2019) although their polysaccharides are not specific substrates for β -glucosidases.

We searched for anti-biofilm enzymes within bacterial isolates obtained from fresh water fish gills. The gill microbiota plays a critical role in the immune system of fish (Ortiz et al., 2023) and acts as the first line of defense against pathogens, thus exhibiting antimicrobial properties (Merrifield & Rodiles, 2015). These bacteria face specific selection pressures that drive them to synthesize robust extracellular enzymes

with a wide range of functions and improved stability (Merrifield & Rodiles, 2015). In this, study we describe the anti-biofilm activity of β -glucosidase B from *Microbacterium* sp. BG28 isolated from the gills of bream. The enzyme was heterologously expressed and characterized, and its activity against *S. Enteritidis* and other foodborne pathogens was evaluated.

2. Materials and methods

2.1. Microbial strains and growth conditions

Clinical *Salmonella* Enteritidis strains were isolated from samples of human feces at National Health Institution "Zavod za javno zdravlje Subotica", the Republic of Serbia. *S. Enteritidis* ATCC 13076 was used as the referent strain. All *S. Enteritidis* strains were cultured in LB-NaCl (1% tripton (Biolife Italiana, Milan, Italy); 0.5% yeast extract (Biolife Italiana) at 37 °C. *S. Typhimurium* ATCC 14028, *S. Infantis* ATCC 51741, *S. Infantis* isolates from poultry samples obtained from Specialistic veterinary Institute "Subotica", Republic of Serbia (Kiskároly et al., 2017) and *Escherichia coli* IMD989 (Novovic et al., 2016) were cultured in LB-NaCl, while *P. aeruginosa* PAO1 NCTC 10332, and *Staphylococcus aureus* ATCC 25923 were cultured in LB (1% tripton (Biolife Italiana); 1% NaCl (HiMedia Laboratories, Mumbai, India); 0.5% yeast extract (Biolife Italiana srl) and BHI (HiMedia) respectively at 37 °C.

2.2. Isolation and identification of cellulase producing bacteria from fresh water fish

2.2.1. Sample collection, isolation of environmental bacteria and screening for their cellulolytic activity

From freshly caught bream fish in the Danube River (vicinity of the city of Belgrade, Serbia), samples for bacterial isolation were taken by swabbing different fish body parts, including gills. Swabs were put on ice and immediately transported to the laboratory. After adding 1 mL of phosphate buffered saline (PBS) (0.8% NaCl (HiMedia); 0.02% KCl (SIGMA-ALDRICH Co., St. Luis, MO, USA); 0.024% KH_2PO_4 (SIGMA-ALDRICH Co.); 0.287% $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ (Thermo Fisher Scientific Inc., Waltham, MA, USA)) and vortexing, the serial dilutions of the samples were plated on four microbiological media suitable for growth of different bacterial genera. Media were supplemented with 100 $\mu\text{g}/\text{mL}$ cycloheximide (SIGMA-ALDRICH Co.) and 25 $\mu\text{g}/\text{mL}$ nystatin (SIGMA-ALDRICH Co.) to prevent growth of fungi and 25 $\mu\text{g}/\text{mL}$ nalidixic acid (SIGMA-ALDRICH Co.) to inhibit the growth of fast-growing bacterial species. Following media were used: BHI and 1/10 BHI, R2A (0.05% yeast extract; 0.05% proteose peptone (SIGMA-ALDRICH Co.); 0.05% casamino acids (SIGMA-ALDRICH Co.); 0.05% glucose (Torlak, Belgrade, Serbia); 0.05% soluble starch (Thermo Fisher Scientific Inc.); 0.03% K_2HPO_4 (SIGMA-ALDRICH Co.); 0.005% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (SIGMA-ALDRICH Co.); 0.03% sodium pyruvate (Thermo Fisher Scientific Inc.); 1.5% agar (Torlak, Belgrade, Serbia) (Massa et al., 1998), ISP2 (0.4% glucose; 0.4% yeast extract; 1% malt extract (Difco Laboratories, Detroit, MI, USA); 2% agar) (Hamaki et al., 2005), and CDM (0.5% sodium lactate (SIGMA-ALDRICH Co.); 0.001% K_2HPO_4 ; 0.2% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 0.0003% $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (SIGMA-ALDRICH Co.); 0.1% NH_4Cl (Thermo Fisher Scientific Inc.); 0.1% Na_2SO_4 (SIGMA-ALDRICH Co.); 0.007% $\text{CaCl}_2 \cdot 7\text{H}_2\text{O}$ (SIGMA-ALDRICH Co.); 0.08% NaHCO_3 (SIGMA-ALDRICH Co.); 1.5% agar) (Weeger et al., 1999). Plates were incubated at 30 °C and bacterial growth was monitored for up to five days. Colonies were separated based on different morphology. BG (bream gills) collection of 75 bacterial isolates was screened for cellulase-degrading activity on carboxymethylcellulose (CMC) agar plates (0.2% NaNO_3 (SIGMA-ALDRICH Co.); 0.1% K_2HPO_4 ; 0.05% MgSO_4 (SIGMA-ALDRICH Co.); 0.05% KCl; 0.2% CMC Sodium salt (SIGMA-ALDRICH Co.); 0.02% peptone (SIGMA-ALDRICH Co.); 1.7% agar) 24 h at 30 °C. Cellulolytic activity of tested isolates was observed as zone of clearance around bacteria after the plates were flooded with

Gram's Iodine (SIGMA-ALDRICH Co.) (Kasana et al., 2008).

2.2.2. 16S rDNA sequencing

The genomic DNA (gDNA) was extracted using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Bacteria-specific 27F and 1492R primers (Invitrogen, Waltham, MA, USA) were used for amplification of the gene encoding 16S rRNA, by FastGene Taq DNA Polymerase (Nippon Genetics, Duren, Germany). The reaction (50 µL) contained 1U of Taq DNA Polymerase, 1X Buffer B dilution, 200 µM dNTP, 0.4 µM primers and 150–200 ng of genomic DNA. The sequence was amplified in 35 cycles with annealing temperature of 50 °C and 1.4 min of elongation. The PCR product was purified with GeneJET PCR Purification Kit (Thermo Fisher Scientific Inc.), sequenced from both ends using Big-Dye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems®, Waltham, MA, USA) and run on Applied Biosystems® 3130 Genetic Analyzer (USA). Sequences were analyzed and assembled with the SeqMan tool from DNASTAR Lasergene Software (DNASTAR, Madison, WI, USA). A similarity search was performed by the BLASTn program in the NCBI GenBank database (Altschul et al., 1990). The 16S rDNA sequence was deposited in the GeneBank under accession number OR517285.

2.2.3. Whole-genome sequencing and analysis

Microbacterium sp. BG28 gDNA was extracted using the QIAamp DNA Mini Kit (Qiagen) and sequenced on Illumina HiSeq 2500 platform in MicrobesNG (Birmingham Research Park, Birmingham, United Kingdom). MicrobesNG provides 30X depth of coverage for Illumina sequencing. Genome assembly was performed using De Bruijn Graph methods and contigs shorter than 200 base pairs (bp) were eliminated. Burrows-Wheeler Aligner (BWA) was used for genome mapping while, Rapid Annotations using Subsystems Technology (RAST) server was used for genome annotation. The closest available reference genome was identified using Kraken 1, and reads were mapped to this using BWA-MEM to assess the quality of the data. The draft genome sequence was deposited in the GenBank under accession number JAXCHA000000000.

2.3. β -glucosidase recombinant expression and purification

Expresso™ T7 Cloning and Expression System was used for recombinant expression of BglB-BG28. The sequence was amplified by Phusion™ High-Fidelity DNA Polymerase (Thermo Fisher Scientific Inc.) using gene-specific primers including 15–18 nt of overlap with the expression vector (F: 5' GAAGGAGATATACATATGAGCCGACGCTCTGCGCGCGTTC 3'; R: 5' GTGATGGTGGTGATGATGCCTCTGCAGGACGGCGCCCTC 3'). Reaction (20 µL) contained 1U of Phusion DNA Polymerase, 1X Phusion HF Buffer dilution, 200 µM dNTP, 1 µM primers and 150–200 ng of *Microbacterium* sp. BG28 gDNA. The sequence was amplified in 35 cycles with annealing temperature of 60 °C and 45 s of elongation. The resulting PCR product was purified and cotransformed with the provided linear form of pETite C-His Kan vector (Lucigen, Middleton, WI, USA) into the XL10Gold ultracompetent *E. coli* cells (Lucigen). After confirming BglB-BG28 positive clone by PCR using a T7 promoter forward primer and gene-specific reverse primer, plasmid DNA was transformed into HI-Control BL21(DE3) *E. coli* cells (Lucigen).

For recombinant BglB-BG28 expression, overnight culture of transformed HI-Control BL21(DE3) cells was grown in LB medium supplemented with 30 µg/mL (LB/Kan30) and 1% glucose, at 37 °C and 180 rpm. Inoculum of 2% overnight culture was grown in 200 mL LB/Kan30 medium at 37 °C and 180 rpm until reaching the optical density (OD) value of 0.4–0.6 at 600 nm and then BglB-BG28 expression was induced with 1 mM IPTG for 22 h at 25 °C and 180 rpm.

Protein purification was carried out using HisTALON™ Gravity Column Purification Kit (Takara Bio, Shiga, Japan). BglB-BG28 was eluted with an elution buffer containing 150 mM imidazole which was then exchanged with a 20 mM sodium phosphate buffer containing 300 mM NaCl pH 7.0. The protein was visualized with SDS-PAGE

electrophoresis (12.5%) after staining with Coomassie Brilliant Blue R-250. Protein concentration was determined with the Bradford method (Bradford, 1976).

The gene sequence of BglB-BG28 was deposited in the GeneBank under accession number OR526343.

2.4. Characterization of BglB-BG28 enzyme

2.4.1. Assessment of enzymatic activity

The BglB-BG28 activity was examined using 2-nitrophenyl- β -D-glucopyranoside (o-NPG) (SIGMA-ALDRICH Co.). The kinetic experiments were carried out at 37 °C and pH 7.0, with substrate concentration ranging from 0.1 mM to 4 mM and enzyme concentration maintained at 5 µg/mL. The release of 2-nitrophenol in continuous reaction was monitored every 30 s for 5 min at 405 nm using the Tecan Infinite200 plate reader (Tecan, Mannedorf, Switzerland). The amount of product was extrapolated from 2-nitrophenol standard curve (McCutcheon et al., 2010).

2.4.2. The hydrolysis of cellulose and cellobiose

The cellobiose (SIGMA-ALDRICH Co.) (5 mM) and CMC (1%) (Islam & Roy, 2018) were used to detect the enzymatic activity of BglB-BG28 on the natural substrates. Enzymatic activity was evaluated in a time span from 5 to 80 min. Reactions were terminated by adding an equal volume of DNS reagent (1.6% NaOH (SIGMA-ALDRICH Co.); 1% 3, 5-dinitrosalicylic acid (SIGMA-ALDRICH Co.); 30% KNa-tartrate (SIGMA-ALDRICH Co.)) and putting the vials in the water bath set to 96 °C for 7 min. The absorbance was measured at 540 nm using the Tecan Infinite200.

2.4.3. Determination of temperature and pH optima and stability

The optimal temperature and pH for the enzyme activity were determined using o-NPG (5 mM). Temperature conditions varied between 8 and 55 °C at pH 7.0, and pH conditions varied between 3.0 and 11.0 at 37 °C using several buffers including citrate sodium buffer (100 mM; pH 3.0, 4.0, 5.0), sodium phosphate buffer (20 mM; pH 6.0, 7.0) and Tris-HCl buffer (20 mM; pH 8.0, 9.0, 10.0). The temperature and pH stability of the purified enzyme was determined by measuring the enzymatic activity after incubation for 2 h at temperatures ranging between 8 and 55 °C and pH ranging between 3.0 and 11.0, followed by the enzymatic reactions carried out at 37 °C and pH 7.0. Reactions were terminated by Na₂CO₃ (SIGMA-ALDRICH Co.) (1 M) after 5 min and absorbance was measured at 405 nm using the Tecan Infinite200 plate reader (Nosworthy et al., 2014). The enzymatic activities were expressed relative to the highest measured activities.

2.4.4. Effect of detergents on BglB-BG28 activity

The o-NPG (5 mM) was added into the mixtures containing 1% SDS (SIGMA-ALDRICH Co.), Cetyltrimethylammonium bromide (CTAB) (SERVA Electrophoresis GmbH, Heidelberg, Germany), Tween 20 (USB Co., Cleveland, OH, USA) or Triton X-100 (SIGMA-ALDRICH Co.). The enzymatic reactions were carried out according to the previously described protocol (Section 2.4.3.) at optimal conditions. Reactions in the absence of detergents were considered as 100% of enzyme activity.

2.4.5. Effect of metal ions on BglB-BG28 activity

The effect of metal ions on the activity of BglB-BG28 was tested by adding FeCl₃ (SIGMA-ALDRICH Co.), CuSO₄ (SERVA Electrophoresis GmbH), CrCl₃ (SIGMA-ALDRICH Co.), ZnSO₄ (SIGMA-ALDRICH Co.), NiCl₂ (AppliChem GmbH, Darmstadt, Germany) and AlCl₃ (SIGMA-ALDRICH Co.) (0.5 mM) to the reaction mixture with o-NPG (5 mM) and BglB-BG28 (10 µg/mL). The enzymatic reactions were carried out according to the previously described protocol (section 2.4.3.) under optimal conditions. The activities obtained in reactions without added metal ions were considered as 100% of the enzyme activity.

2.4.6. Glucose and NaCl inhibition studies

The glucose (0–1 M) and NaCl (0–5 M) effect on the catalytic activity was investigated in the presence of o-NPG (5 mM) as a substrate, according to the previously described protocol (Section 2.4.3.) at the optimal conditions. Reactions in the absence of glucose or NaCl were considered as 100% of enzyme activity.

2.5. Selection of biofilm forming *S. Enteritidis* isolates

Static biofilm assay was performed in 96-well polystyrene microtiter plate (Kartell, Milan, Italy) to evaluate biofilm formation potential of 54 clinical *S. Enteritidis* isolates and ATCC 13076 strain, which was used as a reference. Overnight bacterial cultures grown in LB-NaCl (1% triptone; 0.5% yeast extract) at 37 °C, 180 rpm, were diluted at 5×10^7 cells/mL and 100 µL aliquots were placed in each well (Kartell, Milan, Italy). After 24 h incubation time at 37 °C, biofilms were washed and stained with 0.1% crystal violet (CV) (SERVA Electrophoresis GmbH) (w/v). The biofilm biomass was quantified after resolving CV in 30% acetic acid (v/v) by measuring the absorbance at 570 nm using the Tecan Infinite200. (Merritt et al., 2005).

2.6. *S. Enteritidis* morphotype analysis

S. Enteritidis isolates (SE2, SE3, SE12 and SE23) were analyzed on Congo red agar plates to examine their capabilities to produce curli fimbriae and cellulose according to previously described method (Römling et al., 2003) with a slight modification. In brief, overnight bacterial cultures were diluted to 5×10^7 cells/mL in LB-NaCl, and 10 µL of each isolate was spotted on LB-NaCl agar plates supplemented with 40 µg/mL of Congo red (SIGMA-ALDRICH Co.) and 20 µg/mL Coomassie brilliant blue (SERVA Electrophoresis GmbH). Plates were incubated at 25 °C for 48 h. The morphotypes were categorized as rdar (red, dry and rough, which express curli fimbriae and cellulose) and bdar (brown, dry and rough, expressing curli fimbriae only).

2.7. Antimicrobial activity of BglB-BG28 against planktonic cells

Overnight SE23 bacterial culture was diluted to the 5×10^5 cells/mL in LB-NaCl and subjected to BglB-BG28 (100, 200 and 400 µg/mL). Growth curve was created based on absorbance measured at 600 nm every 2 h in a time span of 18 h using Tecan Infinite200.

2.8. Evaluation of BglB-BG28 anti-biofilm activity

2.8.1. Effect on biofilm biomass

Quantification of biofilm biomass was performed as described above (Section 2.5.). Overnight bacterial cultures of *S. Enteritidis* SE2, SE3, SE12 and SE23 were diluted to 5×10^7 cells/mL in LB-NaCl, and 100 µL aliquots were added to wells of a 96-well polystyrene microtiter plate in the absence or presence of BglB-BG28 (10, 50, 100 and/or 200 µg/mL). After 24 h of incubation at 37 °C, the biofilms were washed and the biomass was quantified using the CV method (section 2.5.). The anti-biofilm effect was evaluated both at 25 °C after 24 h incubation and at 8 °C during one-week treatment. The relative anti-biofilm effect was determined in comparison to biofilm formed in the absence of enzyme. The same method was used to investigate the effect of BglB-BG28 on biofilm formation of *S. Typhimurium*, *S. Infantis*, *E. coli*, *P. aeruginosa* and *S. aureus*. The biofilms of *S. Typhimurium*, *S. Infantis* and *E. coli* were grown in LB-NaCl, while *P. aeruginosa* and *S. aureus* were grown in LB and BHI supplemented with glucose (1%), respectively.

2.8.2. Effect on biofilm metabolic activity

To measure the metabolic activity of biofilm embedded bacteria, SE23 biofilm was established as described above. After 24 h incubation at 37 °C, biofilms were washed and the medium containing resazurin (0.16 mM) (SIGMA-ALDRICH Co.), was added to the wells. Fluorescence

(λ_{ex} : 570 nm and λ_{em} : 615 nm) was measured after 30 min of incubation (37 °C, dark) using the Tecan Infinite200. Negative control with spontaneous transformation of resazurin to resorufin was considered as blank and was subtracted from the rest of the measurements. The relative anti-biofilm effect was determined in comparison to biofilm formed in the absence of the enzyme.

2.8.3. Effect on bacterial adhesion

Effect of BglB-BG28 on early stages of biofilm formation was investigated as previously described (Aleksic et al., 2017) with some modifications. An overnight culture of SE23 containing pBBR2-GFP (Passos da Silva et al., 2014) grown in LB-NaCl with gentamicin (SERVA Electrophoresis GmbH) (10 µg/mL) was subcultured in LB-NaCl at 37 °C and 180 rpm (initial OD600 = 0.02) until OD600 = 0.1 was reached. Aliquots of 200 µL with 200 µg/mL BglB-BG28 or without treatment (control) were transferred to the wells of a black polystyrene microtiter plate (Nunc™ F96 MicroWell™ Black Polystyrene Plate, Thermo Fisher Scientific Inc.). After 3 h of incubation, the supernatant was removed and the adherent cells were washed with PBS. After addition of PBS, fluorescence (λ_{ex} : 500 nm and λ_{em} : 540 nm) was measured with the Tecan Infinite200. The background signal (PBS) was subtracted from all the samples. The relative effect of BglB-BG28 on cell adhesion was determined in comparison to control (biofilms formed without the treatment).

2.8.4. Effect on pre-formed biofilms

Overnight SE23 bacterial culture grown in LB-NaCl was diluted at 5×10^7 cells/mL and 100 µL aliquots were added to 96-well polystyrene microtiter plates. After incubation time (37 °C) of 3, 6, 9, 12, 15, 18, 21 and 24 h, biofilms were washed and treated with BglB-BG28 (200 µg/mL) in LB-NaCl for 24 h. After 24 h, biofilms were washed and quantified as described.

2.8.5. Visualization of BglB-BG28 anti-biofilm effect by fluorescent microscopy

To observe biofilms under a fluorescent microscope, the overnight bacterial cultures were diluted to 5×10^7 cells/mL in LB-NaCl and were added as 30 µL drops on plastic, glass coverslips or aluminum foil in the presence (200 µg/mL) or absence of BglB-BG28. After 24 h incubation at 37 °C, biofilms were washed and stained by 2.5 µM Syto®9 green fluorescent dye and 2.5 µM propidium iodide (PI) red fluorescent dye of Live/Dead staining kit (LIVE/DEAD® BacLight™ Bacterial Viability Kit, Thermo Fisher Scientific). Bacteria were observed under a fluorescent microscope (Olympus BX51, Applied Imaging Corp., San Jose, CA, USA) under 10× magnification. Anti-biofilm effect was determined in relation to biofilm formed in the absence of an enzyme.

2.9. Effect of disinfectant on SE23 biofilm pretreated with BglB-BG28

To evaluate the efficacy of a disinfection treatment in combination with a BglB-BG28 pretreatment, we chose the disinfectant Oxicide S (Krka, Novo Mesto, Slovenia), which contains pentapotasium peroximonosulfate as the active component. An overnight bacterial culture of SE23 was diluted to 5×10^7 cells/mL in LB-NaCl, and 100 µL aliquots were added to the wells of a 96-well polystyrene microtiter plate in the absence or presence of BglB-BG28 (200 µg/mL). After 24 h of incubation at 37 °C, the biofilms were washed and 150 µL of the corresponding dilutions of Oxicide S (2%, 1%, 0.5% and 0%) were added to each well. After 5 min, the biofilms were washed and the biomass was quantified using the CV method (section 2.5.).

2.10. *Caenorhabditis elegans* toxicity assay

C. elegans AU37 obtained from the *Caenorhabditis* Genetics Center (CGC, University of Minnesota, Minneapolis, Minnesota, USA) was cultivated on a nematode growth medium (1.7% agar; 0.29% NaCl;

0.25% peptone; 0.01% CaCl_2 (SIGMA-ALDRICH Co.); 0.0005% cholesterol (SIGMA-ALDRICH Co.); 0.34% KH_2PO_4 ; 0.012% MgSO_4 , utilizing *E. coli* OP50 as a food source. The worms were kept at 15 °C and synchronized by hypochlorite bleaching. The effect of BglB-BG28 (100, 500, and 1000 µg/mL) on *C. elegans* survival rate was tested following previously established protocol (Stiernagle, 2006) with minor adjustments. The assay was performed in a 96-well microtiter plate. Each well contained 25–40 L4 stage worms in 100 µL M9 (6.4% $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$; 1.5% KH_2PO_4 ; 0.25% NaCl; 0.5% NH_4Cl):LB (95:5 v/v) solution supplemented with 5 µg/mL cholesterol. Worms were counted using the stereomicroscope (SMZ143-N2GG, Motic, Germany) at the beginning of the experiment, and after 24 and 48 h. The relative toxic effect was determined compared to the worms in the same conditions without enzyme.

2.11. Statistical analysis

GraphPad Prism 6 was used for both statistical analysis and graphical display of the enzyme kinetic results (GraphPad Software, San Diego, CA, USA). The Student's *t*-test was used to perform the statistical analyses for BglB-BG28 activity assessment using SPSS version 20 software (IBM, Armonk, NY, USA). The significance level was set at $P < 0.05$.

3. Results and discussion

3.1. Identification of cellulose-degrading strain BG28

In search of industrially applicable enzymes with anti-biofilm properties against *S. Enteritidis*, we investigated 75 morphologically diverse bacteria from our bream fish gill (BG) bacterial collection. The gill microbiota are exposed to various stressors such as water quality, temperature fluctuations, nutrient deficiencies, overcrowding, parasite infestations, and infections, resulting in valuable adaptations. Therefore, we hypothesized that bacteria isolated from fish gills would provide a rich source of enzymes with antimicrobial and antiviral properties and broad stability under various conditions.

Given that cellulose plays a critical role in the assembly of *S. Enteritidis* biofilms, especially those that are not associated with the host, we assumed that alteration of this component would lead to disruption of the overall biofilm structure. Although some studies have shown that biofilms of *S. enterica*, *E. coli*, *P. aeruginosa*, *P. fluorescens*, *P. fluorescens*, and *Burkholderia cepacia* can be controlled by cellulases (Cescutti et al., 1998; Lequette et al., 2010; Lim et al., 2019; Loisele & Anderson, 2003; Rajasekharan & Ramesh, 2013; H. Wang et al., 2016), we opted for a different approach to cellulose-mediated biofilm control. Considering the potential spoilage effect of cellulase on fresh produce, we aimed to modify the cellulose biosynthesis process rather than the final product. Since β -glucosidases are part of the cellulase complex (Singh et al., 2021) we examined the collection from BG for their cellulolytic activity. Out of the 19 isolates with the cellulose-degrading activity, isolate BG28 was selected for further study as the most active, based on the size of the zone of clearance developed on CMC agar plates (Supplementary Fig. 1). The 16S rDNA of BG28 was sequenced and the strain was identified as *Microbacterium* sp. which is most closely related to *Microbacterium binotii* NZ CP090347.1 with 99.32% identity and 100% query coverage according to phylogenetic analysis.

3.2. Heterologous expression and characterization of β -glucosidase B from *Microbacterium* sp. BG28

The genome mining of *Microbacterium* sp. BG28 revealed the presence of the gene coding for β -glucosidase B (BglB-BG28) enzyme. Sequence homology of BglB-BG28 gene is summarized in a Supplementary Table 2. The closest protein sequence homolog of BglB-BG28 with experimentally validated activity was the GH1 family β -glucosidase (GLU1) from *Microbacterium* sp. CIAB417 (WP_243232135) with 97.35% identity and 100% query coverage. GLU1 proved to be active on

cellobiose and cellooligosaccharides in a pH range from pH 4.0 to pH 9.0 and at temperatures up to 80 °C, while exhibiting great glucose tolerance and detergent compatibility (Purohit et al., 2023). The closest structural homolog is TIM barrel psychrophilic β -glucosidase BglU from *Micrococcus antarcticus* (pdb 3w53) with 54% sequence identity. β -Glucosidases of the glycoside hydrolase (GH) 1 family are known for their wide variety of substrate specificities (Ketudat Cairns & Esen, 2010), and cold adaptive enzymes are desirable for applications in the food industry (Cavicchioli et al., 2011). Considering these remarkable characteristics we proceeded with his-tagged BglB-BG28 overexpression in *E. coli* and purification by a routine metal affinity chromatography with a 100 mg per 1 g of cell pellet yield and to a purity of ~98% based on protein staining with Coomassie Brilliant Blue R-250 (Supplementary Fig. 2).

The catalytic activity of BglB-BG28 was tested using o-NPG as a substrate by measuring the release of 2-nitrophenol. BglB-BG28 showed strong affinity for o-NPG with K_m 0.4269 mM and k_{cat} 82.01547/s (Supplementary Fig. 3). To determine the optimal conditions for BglB-BG28 catalyzed reaction, we tested its activity and stability at different temperatures and pH values. The maximum activity of BglB-BG28 was obtained at 42 °C and pH 7.0 (Fig. 1A and B). The enzymatic activity with >60% of the substrate hydrolysis rate was observed in the temperature range between 25 and 42 °C, and in the pH range between 4.0 and 8.0 (Fig. 1C and D). Wide operational range of BglB-BG28 activity can ensure many opportunities for its applications across various segments of the food industry. Using the DNS reagent we confirmed that BglB-BG28 can use cellobiose as a substrate, but is not able to degrade cellulose molecules (Supplementary Fig. 4).

3.3. Selection and characterization of biofilm-forming *S. Enteritidis* isolates

In order to test the anti-biofilm efficacy of BglB-BG28 against *S. Enteritidis*, we first examined the biofilm-forming ability of strain *S. Enteritidis* ATCC 13076 and 54 clinical *S. Enteritidis* isolates (Fig. 2A) to identify the best biofilm producers. Based on the amount of biofilm biomass formed primarily at the air-liquid interface, we found that *S. Enteritidis* ATCC 13076 did not form biofilm under static conditions and therefore selected four strains with the highest biofilm-forming ability for further investigation (Fig. 2B). The identity of all four clinical isolates was confirmed as *S. Enteritidis* by PCR using serotype-specific primers (Supplementary Fig. 5). All four isolates exhibited the rdar (red, dry, and rough) morphotype (Fig. 2B) indicating cellulose and curli production. *S. Enteritidis* ATCC 13076 reference strain did not form biofilm and had a bdar (brown, dry, and rough) morphotype, showing the lack of cellulose production. Except of cellulose, *S. enterica* biofilms consist of curli fimbriae, colanic acid, biofilm-associated surface large proteins (BapA), O-antigen capsule, flagella, and extracellular DNA (eDNA) (Pradhan et al., 2023). Accordingly, DNase I, proteinase K, and subtilisin have been shown to be effective against *S. enterica* biofilms, either alone or in combination with antimicrobial compounds (M.-J. Kim & Kim, 2022; Y. Kim et al., 2023; H. Wang et al., 2016). However, DNases and proteinases are broad-spectrum enzymes with low substrate specificity, high toxicity to expressing host cells and numerous potential side effects (Linardou et al., 2000; Yang et al., 2016). To better control the process of biofilm degradation, we chose to use glycoside hydrolases, which degrade highly specific glycoside bonds, allowing for more precise biofilm degradation (Redman et al., 2021).

3.4. Assessment of anti-biofilm activity of BglB-BG28 on *S. Enteritidis*

BglB-BG28 inhibited biofilm formation to a similar extent in all four selected *S. Enteritidis* strains (Fig. 2C), so only one of them, *S. Enteritidis* SE23, was selected for further investigation. The effect of BglB-BG28 on biofilm biomass was quantified using CV staining, while the metabolic activity of cells embedded in the biofilm was measured using resazurin

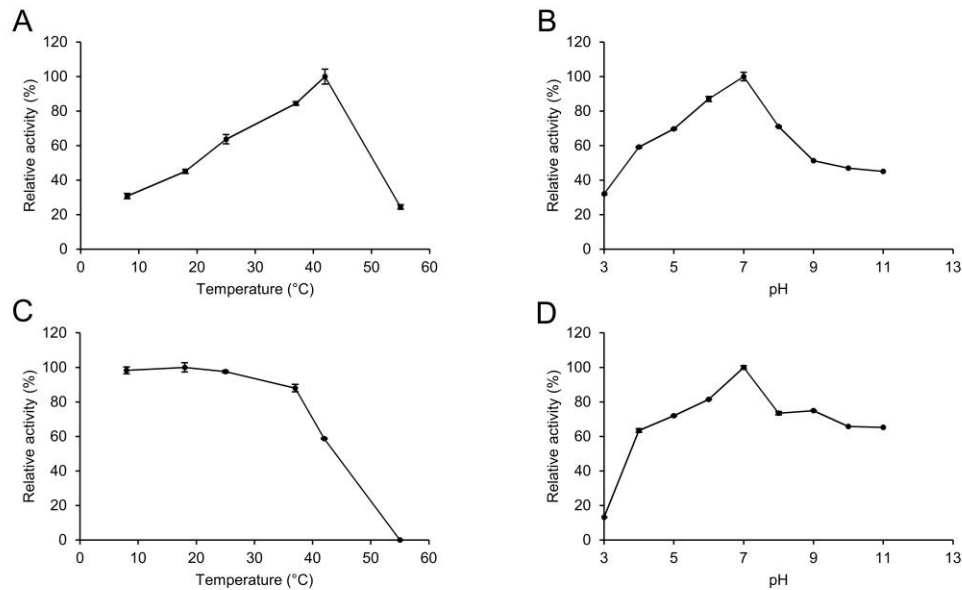


Fig. 1. Determination of optimal temperature and pH conditions for BglB-BG28. Effect of temperature (A) and pH (B) on enzyme activity. Enzyme stability after 2h pre-incubation at different temperatures (C) and pH (D). All reactions were performed using 2-nitrophenyl- β -D-glucopyranoside as a substrate. Values are presented as mean $\pm$ SD.

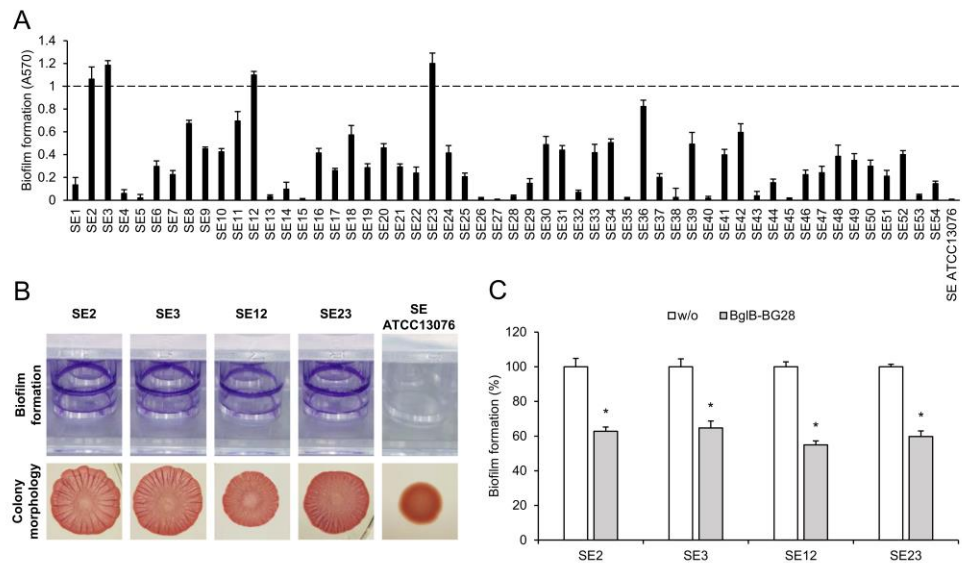


Fig. 2. Selection and characterization of biofilm-forming *Salmonella* isolates. Formation of biofilms after 24h. A dashed blue line shows selected threshold for strong biofilm formers (A). Visualization of biofilm growth at the air-liquid interface with corresponding colony morphologies on congo red agar plate (B) Effect of BglB-BG28 (100 μ g/mL) on selected *Salmonella* strains' biofilm formation (C). Biofilm formation was quantified using CV staining. w/o, control without BglB-BG28. Values are presented as mean $\pm$ SD. * p < 0.05. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

staining. BglB-BG28 inhibited biofilm formation in a dose-dependent manner, with the highest concentration tested (200 μ g/mL) inhibiting ~50% of biomass formation and ~80% of metabolic activity (Fig. 3A). Tested concentrations of BglB-BG28 had no effect on bacterial growth

(Fig. 3B), suggesting that the reduction in metabolic activity of the treated biofilms was due to the reduced number of bacteria adhering to the surface. This is the first reported bacterial β -glucosidase with anti-biofilm activity. The β -glucosidase enzyme, purified from almonds

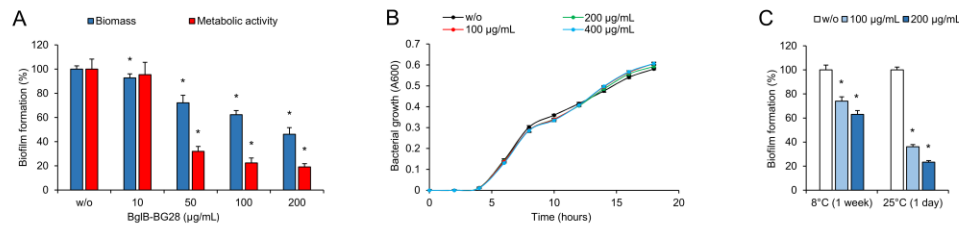


Fig. 3. Effect of BglB-BG28 on SE23 biofilm formation and bacterial growth. Dose-dependent inhibition of biofilm formation, quantified using CV (blue) or resazurin (red) staining relative to control without treatment (w/o) (A). Growth curves of SE23 in the absence (w/o) or presence of BglB-BG28 (B). BglB-BG28 effect on SE23 biofilm formation after 24h at 25 °C and 1 week at 8 °C, quantified using CV staining (C). Values are presented as mean $\pm$ SD. * $p < 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

(G4511 SIGMA) has been proposed to degrade *P. aeruginosa* biofilm by Psl polysaccharide hydrolysis at concentrations that also exhibit bactericidal activity (Banar et al., 2019). BglB-BG28 had no effect on bacterial viability, so it is not expected to lead to the development of resistance.

According to Food and Drug Administration (FDA) guidelines fresh food should be kept at 5 °C or less (FDA, 2023), but this is not always maintained throughout the supply chain. Huang et al. demonstrated that even though the growth of *S. enterica* is inhibited at 4 °C, population significantly increases over the course of 7 days at 8 °C (Huang et al., 2019). We showed that SE23 is capable to grow and form biofilm at 8 °C. Since the biofilm formation was significantly slower than at the temperatures above 25 °C, we tested the enzyme's anti-biofilm activity during one week treatment. One-week incubation of SE23 with BglB-BG28 (200 µg/mL) resulted in 40% less biofilm formation compared to the untreated control (Fig. 3C). This result implies that BglB-BG28 can be effectively used in various cooling equipment. Different studies showed that *Salmonella* formed more abundant biofilms at 20–25 °C than at 37 °C (Lamas et al., 2018), so we examined BglB-BG28 activity also at this temperature. At 25 °C, overnight incubation with the enzyme (200 µg/mL) prevented 80% of biofilm formation (Fig. 3C). The ability of *S. enterica* to form biofilms on abiotic surfaces enables them survival in non-host environments and the transmission to new hosts.

To distinguish which phase of biofilm formation BglB-BG28 affects, we tested the activity of the enzyme on bacterial adhesion using GFP-labeled SE23 (SE23-GFP). After showing that BglB-BG28 did not affect GFP expression in the bacteria (Supplementary Fig. 6), SE23-GFP was incubated with or without the enzyme for 3 h in static conditions, and after washing to remove non-adherent cells, GFP fluorescence was

measured. The enzyme reduced bacterial adhesion by 90% compared to the untreated control (Fig. 4A). Regular disinfection could prevent biofilm formation, but the rapid process of bacterial attachment to a surface makes it impossible to disinfect frequently enough to avoid this initial step (Meyer, 2003). Applying BglB-BG28 to surfaces after disinfection could help prolong the time it takes for the bacteria to adhere and bridge the time between two disinfection cycles.

The efficacy of BglB-BG28 to inhibit different stages of biofilm growth was further tested on 3, 6, 9, 12, 15, 18, 21, and 24 h old biofilms. BglB-BG28 inhibited the growth of biofilms up to 9 h old, causing a 40% reduction in biomass and up to 70% in metabolic activity (Fig. 4B). During the later stages of biofilm growth, the effectiveness of enzyme gradually decreased, with no effect observed in biofilms that were 18 h or older. These results indicate that BglB-BG28 affects biofilm formation by hydrolyzing small oligosaccharides produced during cellulose synthesis, while large cellulose polymers present in the extracellular matrix of mature biofilms cannot be degraded. BglB-BG28 inhibiting early stages of biofilm formation match the cellulose fibers crucial role in irreversible surface attachment which is a step that leads to the early development stage of biofilm formation (Lim et al., 2019).

Salmonella biofilms have been reported on plastic, rubber, cement, glass, stainless steel and packaging materials, which are commonly encountered in food industry (Steenackers et al., 2012). Biofilm cells that are not removed during standard cleaning procedures in food processing facilities can be a recurring cause of foodborne contamination that can lead to disease outbreaks (R. Wang et al., 2022). Therefore, we further investigated the efficacy of BglB-BG28 in preventing SE23 biofilm formation on different surfaces such as plastic, glass and metal. The biofilm formation was visualized using fluorescence microscopy after

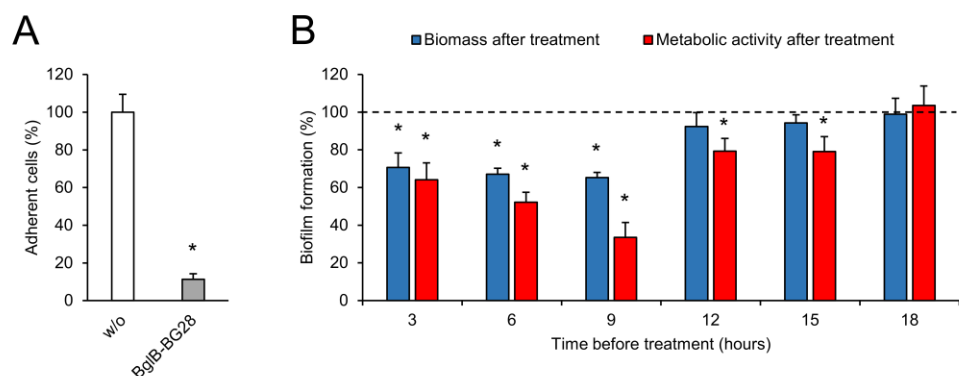


Fig. 4. Effect of BglB-BG28 on different stages of biofilm formation. Effect of BglB-BG28 (200 µg/mL) during adhesion phase (3h) quantified by fluorescence of GFP labeled SE23 (A). Effect of BglB-BG28 (200 µg/mL) against 3h, 6h, 9h, 12h, 15h, and 18h old biofilms relative to the control without treatment (dashed line) (B). Biofilm biomass and metabolic activity were quantified using CV and resazurin staining, respectively. Values are presented as mean $\pm$ SD. * $p < 0.05$.

staining of bacteria with Live/Dead® BacLight Kit which consists of Syto®9 and propidium iodide (PI) dyes that bind to nucleic acids. Syto®9 is green fluorescent dye that penetrates membrane in both viable and non-viable cells, while red fluorescent PI enters the cells with damaged membrane or non-viable cells. Incubation with BglB-BG28 caused significant reduction of air-liquid biofilm formation on all tested surfaces (Fig. 5). In the presence of treatment, the edges of the droplets appeared as thin lines, and individual bacteria were spotted within the droplet area, in contrast to the thick bacterial layers in the controls. In addition, bacterial proliferation outside the droplet area was also reduced. All bacteria within the biofilm appeared green, confirming the previous finding that BglB-BG28 does not affect the viability of Salmonella but reduces the number of attached cells.

3.5. Specificity of BglB-BG28 antibiofilm activity

In addition to *S. Enteritidis*, other *S. enterica* serotypes also pose a significant health risk. Therefore, we investigated the activity of BglB-BG28 on two other common *S. enterica* serotypes: *S. Typhimurium* and *S. Infantis*. Based on the amount of biofilm biomass formed, we chose *S. Typhimurium* ATCC 14028 and *S. Infantis* 108 (SI108) for enzymatic treatment (Supplementary Fig. 7). Similar to *S. Enteritidis*, the enzyme at a concentration of 200 µg/mL inhibited biofilm formation in both *S. Typhimurium* and *S. Infantis* by 50% and 60%, respectively. Since

several other bacterial species can cause foodborne illness, broad specificity would be a favorable characteristic for a biofilm control agent. We tested the enzyme activity against *E. coli*, *P. aeruginosa* and *S. aureus* biofilms. BglB-BG28 inhibited biofilm formation in *E. coli*, with an 80% reduction after overnight incubation with 200 µg/mL of enzyme. At the same enzyme concentration, *P. aeruginosa* biofilm formation was reduced by only 25%, while no effect was observed on *S. aureus* biofilms (Fig. 6). BglB-BG28 effectively reduced biofilm formation in *E. coli*, yet another foodborne pathogen with a similar biofilm matrix composition to *S. enterica* (Sande & Whitfield, 2021). Therefore, our results suggest that BglB-BG28 is only effective against cellulose rich biofilms.

3.6. Combination of BglB-BG28 pretreatment with disinfectant

Oxidiz S (Krka, Novo Mesto, Slovenia) is the commercially available preparation with pentapotasium peroximonosulphate as the active component, which is generally used for efficient surface disinfection in food processing facilities and animal enclosures. Its anti-biofilm activity has been previously confirmed (Oliveira et al., 2022) and here we investigated its efficiency in combination with a BglB-BG28 pretreatment. We applied the Oxidiz S solution to the mature biofilm of SE23 for 5 min and found that the lowest effective concentration was a 1% solution. When applied to the biofilms pretreated with 200 µg/mL BglB-BG28, the efficacy of Oxidiz S increased and 0.5% solution inhibited SE23 biofilms

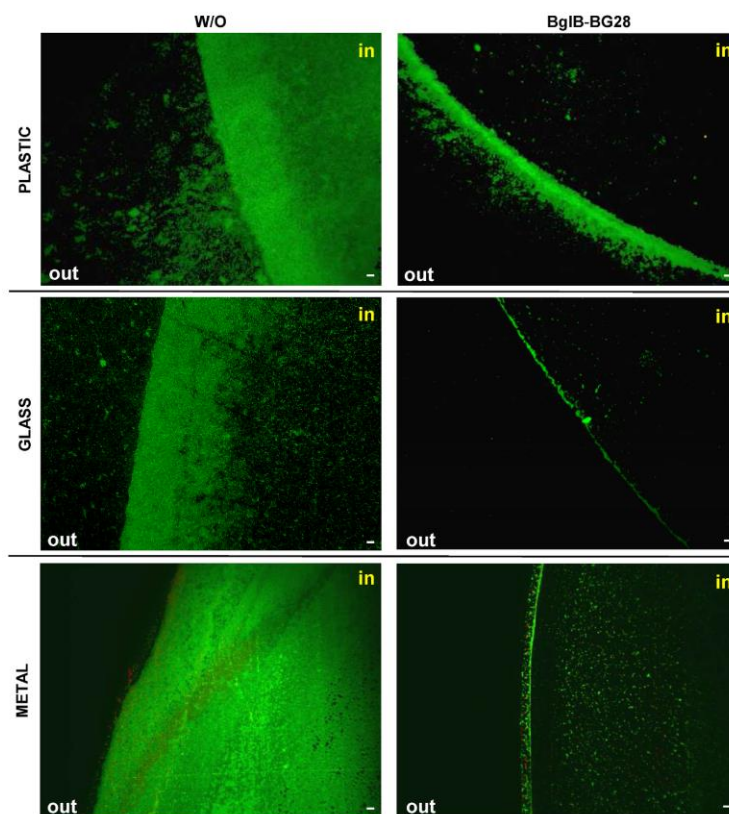


Fig. 5. Inhibition of SE23 biofilm formation on different surfaces with BglB-BG28 (200 µg/mL) treatment. Biofilms were formed for 24h on plastic and glass coverslips, and aluminum foil in the form of a liquid drop. Biofilms were analyzed by fluorescent microscopy using a Live/Dead staining kit. Live cells were stained with Syto®9 (green) and dead cells were stained with PI (red). Air-liquid interface of a liquid drop is clearly visible between the inside (in) and outside (out) areas. Scale bars represent 10 µm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

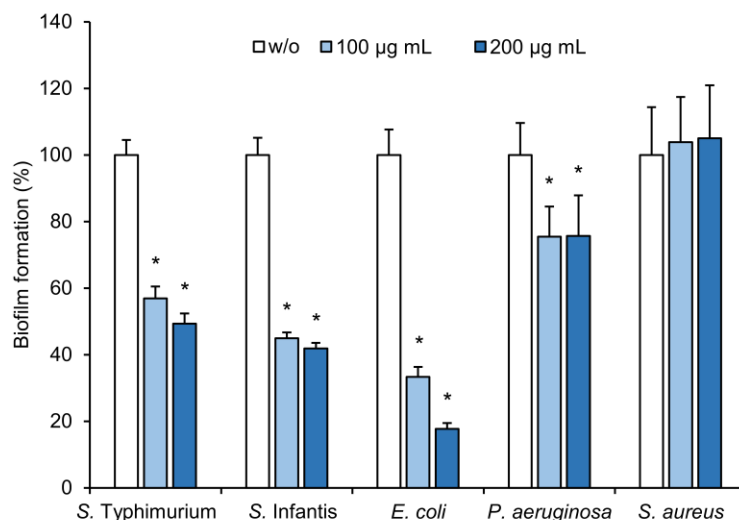


Fig. 6. Effect of BglB-BG28 on different foodborne pathogens. Biofilm formation was quantified using CV staining. w/o, control without treatment. Values are presented as mean $\pm$ SD. * $p < 0,05$.

by 70% (Fig. 7). These results suggest that pretreatment with BglB-BG28 can increase the efficacy of disinfectants so that they can be used at lower concentrations.

3.7. Enzyme inhibition studies

Considering that anti-biofilm enzymes can be used in cleaning and disinfection processes, we investigated how various detergents influence BglB-BG28 activity. The ionic surfactants SDS and CTAB completely inhibited enzymatic activity, while the nonionic detergents Triton X-100 and Tween 20 showed a stimulatory effect (Fig. 8A). This indicates that BglB-BG28 can be effectively used with nonionic detergents, while application with ionic detergents should be sequential due to their ability to denature proteins. Enzymes are considered “green chemicals”

that do not induce resistance in pathogens, are fully biodegradable, and promote the degradation of organic matter in sewage systems (Meyer, 2003).

The release of metals from surfaces that come into contact with food is a serious problem in the food industry (Jellesen et al., 2006). We made a selection of metal ions that may be present in food processing and tested the effect of Fe^{2+} , Cu^{2+} , Cr^{3+} , Zn^{2+} , Ni^{2+} , and Al^{3+} on the activity of BglB-BG28. At the concentration that did not affect the biofilm formation of SE23 (Supplementary Fig. 8), none of the metal ions had any effect on either the enzymatic activity of BglB-BG28 on o-NPG as a substrate (Fig. 8B) or on its anti-biofilm activity (Supplementary Fig. 9).

Next, we tested whether food matrix can affect BglB-BG28 anti-biofilm efficiency and performed static biofilm assay in rich culture medium containing casein, meat extract, starch and olive oil. Presence of

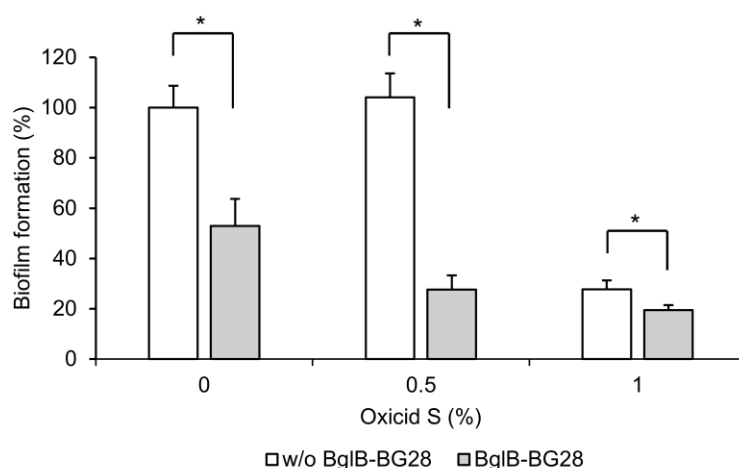


Fig. 7. Eradication of SE23 biofilm pretreated with BglB-BG28 by commercially available disinfectant Oxidic S. Biofilms were formed for 24h in 96 well microtiter plate in the presence (BglB-BG28) or absence (w/o BglB-BG28) of BglB-BG28 (200 µg/mL) and then treated with Oxidic S dilutions for 5 min. The remaining biofilm was stained with CV and quantified relative to the control without enzyme and disinfectant. Values are presented as mean $\pm$ SD. * $p < 0,05$.

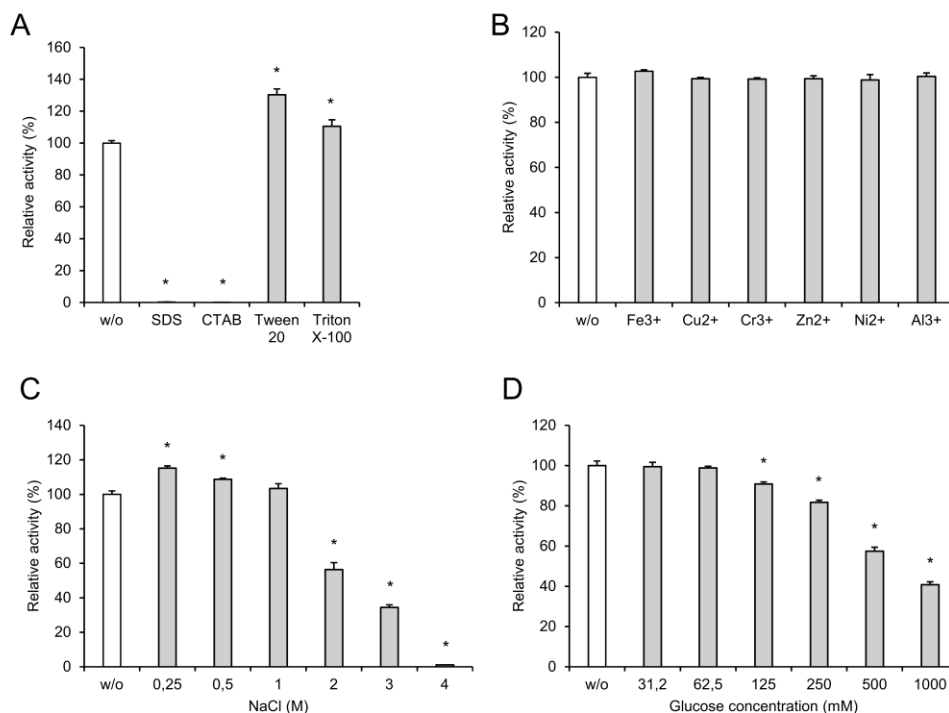


Fig. 8. Effects of detergents (A), metal ions (B), NaCl (C), and glucose (D) on BglB-BG28 activity. Reactions were performed using 2-nitrophenyl- β -glucopyranoside as a substrate. w/o, control reaction without detergents (A), metal ions (B), NaCl (C) or glucose (D). Values are presented as mean $\pm$ SD. * $p < 0,05$.

proteins, polysaccharides or lipids did not affect Bgl-BG28 anti-biofilm activity, therefore its efficiency shouldn't be affected during food processing (Supplementary Fig. 10).

In addition to surface cleaning and disinfection, β -glucosidases can also be used in other industrial applications, such as biomass degradation, where halotolerant and glucotolerant β -glucosidases are of great value. Therefore, we analyzed the effects of NaCl and glucose on BglB-BG28 activity and showed that in the presence of 1 M NaCl the enzyme remained fully active, while in the presence of 0.5 M glucose residual activity was at 60% (Fig. 8C and D).

3.8. Toxicity assessment

Finally, we tested the safety of the enzyme in a *C. elegans* AU37 model system. While it is a simple organism, it possesses many conserved biological processes that are relevant to food safety (Reddy et al., 2023). BglB-BG28 had no effect on the survival rate of *C. elegans* exposed to enzyme concentrations up to 1 mg/mL for 48 h (Fig. 9). To surpass the lengthy food safety evaluations, Nahar et al. suggested that the search for anti-biofilm enzymes should be conducted among the already approved food additives. This was the case with Flavourzyme (Novozymes, Bagsværd, Denmark), a mixture of exo- and endo-peptidase, a popular food additive proved to be efficient in *S. enterica* biofilm prevention (Nahar et al., 2021). β -Glucosidases are used as flavor enhancers in fruit juices, and particularly in wine processing. Their potential is yet to be explored, as plants have been shown to accumulate important flavor compounds as non-volatile and flavor-neutral glycoconjugates, forming a flavor reserve that needs to be exploited (Sarry & Günata, 2004). This indicates that the transition of BglB-BG28 towards food industry applications would be relatively easy regarding food safety measures.

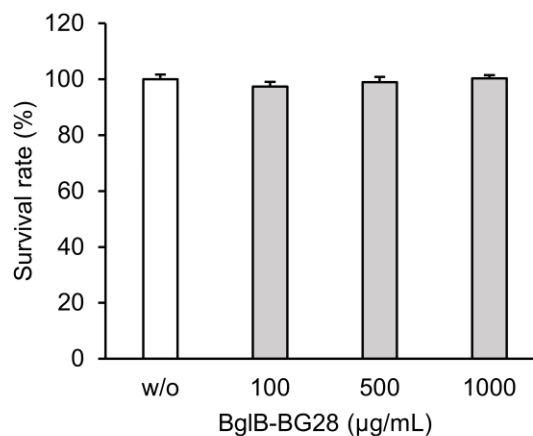


Fig. 9. Evaluation of BglB-BG28 *in vivo* toxicity using *Caenorhabditis elegans* model. The effect of enzyme on *C. elegans* survival rate was determined compared to the control without treatment (w/o). Values are presented as means $\pm$ SD.

4. Conclusion

In the present study, we showed for the first time that bacterial β -glucosidase exhibits biofilm inhibitory activity. We describe highly efficient β -glucosidase B in terms of preventing bacterial adhesion and

inhibiting biofilm formation of *S. Enteritidis* in nutrient-rich medium. BglB-BG28 exhibits activity in a wide range of temperature and pH, which makes it suitable for harsh and diverse industrial settings. Moreover, BglB-BG28 is compatible with nonionic detergents, highly tolerant to NaCl and glucose, and effectively inhibits biofilm formation on various surfaces such as plastic, glass, and metal. This versatility suggests a wide range of potential applications. The lack of toxicity gives BglB-BG28 additional advantage over conventional disinfectants in food industry. Therefore, β -glucosidases, including BglB-BG28 should be further examined for utilization in food safety protocols for *Salmonella* biofilm control.

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CRediT authorship contribution statement

Marija Atanaskovic: Writing – original draft, Methodology, Investigation. **Ivana Moric:** Writing – review & editing, Investigation. **Milos B. Rokic:** Methodology. **Andjela Djokic:** Investigation. **Jelena Pantovic:** Investigation. **Dragana Despotovic:** Writing – review & editing, Formal analysis. **Lidija Senerovic:** Writing – review & editing, Visualization, Supervision, Methodology, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fbio.2023.103543>.

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3.2 Novel polygalacturonase PG-BG31 prevents biofilm formation and increases antibiotic efficacy against catheter-associated *Escherichia coli*



Novel polygalacturonase PG-BG31 prevents biofilm formation and increases antibiotic efficacy against catheter-associated *Escherichia coli*

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ABSTRACT

Escherichia coli is a leading cause of urinary tract infections, which are particularly problematic in catheterized patients due to the formation of biofilms that are resistant to standard treatments. Here we investigated a novel polygalacturonase (PG-BG31) from *Pedobacter* sp. BG31 and its anti-biofilm properties against *E. coli* isolates originating from the urine of UTI patients. The enzyme was heterologously expressed and biochemically characterized using polygalacturonic acid as substrate. The enzyme showed strong biofilm inhibition with BIC50 values between 2 and 5 µg/mL, which varied depending on the *E. coli* strain. It outperformed enzymes commonly used in *E. coli* biofilm studies, including proteinase K, DNase and cellulase. When used as a pretreatment, it significantly improved the efficacy of ciprofloxacin and trimethoprim by reducing biofilm formation by 4 log (99.99 %) at lower antibiotic concentrations. Polygalacturonase PG-BG31 works optimally at temperatures of 25 °C - 42 °C and a slightly acidic pH value (pH 5.0 – 6.0), which corresponds to the environment in urine. In addition, the enzyme showed no toxicity to cells in culture or *C. elegans*. These results suggest that polygalacturonase has the potential for the development of anti-biofilm strategies for catheter-related urinary tract infections.

1. Introduction

Escherichia coli is a Gram-negative, facultatively anaerobic bacterium commonly found in the gut of humans and animals, where most strains are harmless and play a beneficial role in the gut microbiome. However, pathogenic strains of *E. coli* are an important cause of disease in humans (Braz et al., 2020). These pathogenic strains possess virulence factors that allow them to attach to host tissues, evade immune responses, and produce toxins, leading to various clinical consequences ranging from mild gastrointestinal discomfort to severe conditions such as hemolytic uremic syndrome (HUS), urinary tract infections (UTIs), and sepsis (Kaper et al., 2004).

E. coli is a well-known biofilm-forming bacterium, particularly in its pathogenic forms, and this characteristic contributes significantly to its role in persistent infections (Ballén et al., 2022). In healthcare settings, *E. coli* biofilms are a major cause of catheter-associated urinary tract infections (CAUTIs), ventilator-associated pneumonia, and other device-related infections, as biofilms can easily form on indwelling medical devices (Hamdy Mohamed et al., 2024). Catheter-associated urinary tract infections account for more than 40 % of

hospital-acquired infections and more than 80 % of all urinary tract infections (Milo et al., 2019). Uropathogenic *E. coli* (UPEC), which are frequently responsible for UTIs, can also invade bladder epithelial cells and form intracellular biofilm-like communities, that lead to persistent infections and frequent relapses after antibiotic treatment (Goller and Seed, 2010).

The biofilm matrix of *E. coli* is a complex and dynamic structure composed of extracellular polymeric substances (EPS), which include polysaccharides, proteins, lipids, and extracellular DNA (eDNA). Major constituents include polysaccharides such as cellulose, poly-β-1,6-N-acetyl-D-glucosamine (PGA) and colanic acid, a capsular heteropolysaccharide with hexasaccharide repeating units, consisting of glucose, fucose, galactose and glucuronic acid (Sande and Whitfield, 2021). Polysaccharides, such as the capsular polysaccharides (CPS) and the lipopolysaccharide (LPS) layer found on the outer membrane of *E. coli*, serve as a protective barrier against the host's immune defenses and protect the bacteria from phagocytosis and complement-mediated lysis (Arredondo-Alonso et al., 2023; Liu et al., 2019). Polysaccharides and oligosaccharides are thus key factors in the pathogenesis of *E. coli*, promoting bacterial survival, colonization and formation of biofilms,

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which are often resistant to conventional therapies. Targeting polysaccharides can disrupt the protective barriers, including the biofilm, that help the bacteria resist host defenses and make them more susceptible to clearance by the immune system and antibiotics (Lim et al., 2019; Park and Park, 2021; Wu et al., 2019).

An effective approach to combat *E. coli* biofilms is the use of enzymes that target the major biofilm components. Glycoside hydrolases (e.g., cellulase) degrade polysaccharides, proteases (e.g., proteinase K, subtilisin) degrade structural proteins, and DNases (e.g., DNase I) target extracellular DNA, weaken the biofilm structure and improve the efficacy of antibiotics (Lim et al., 2019; Van Nederveen and Melton-Celsa, 2024; Villa et al., 2015). The disintegration of the biofilm matrix supports antibiotic and immune responses and helps in the treatment of persistent *E. coli* infections.

Polygalacturonases belong to the group of pectinolytic enzymes that are widely used in various biotechnological industries (Jayani et al., 2005). The commercially available pectinase from *Aspergillus niger*, which is known for its polygalacturonase activity, has previously been successfully used to prevent biofilm formation in the laboratory strain *E. coli* K-12 (Villa et al., 2015). Since polygalacturonases can utilize glucuronic acid oligosaccharides as substrates (Tan et al., 2020), LPS, colanic acid and other CPS containing glucuronic acid are possible targets in the *E. coli* biofilm (Klein et al., 2013; Sande and Whitfield, 2021).

The role of CPS in biofilm formation is complex and not fully understood due to the variability of CPS production between different bacterial strains and stages of infection. It is generally believed that CPS inhibit bacterial adherence and biofilm formation (Beloin et al., 2008), but they play an important role also in biofilm maintenance (Limoli et al., 2015). In uropathogenic *E. coli*, CPS is involved in the formation of intracellular biofilm-like communities in bladder cells (Goller and Seed, 2010). In addition, LPS contributes to the adhesive properties of the biofilm by interacting directly with adhesion molecules on the cell surface, which helps the bacteria to adhere more effectively to surfaces and initiate the process of biofilm formation (Sharma et al., 2016). Colanic acid primarily contributes to biofilm maturation and matrix thickening (Prigent-Combaret et al., 2000). Recent studies have shown that inhibition of colanic acid biosynthesis effectively prevents biofilm formation, demonstrating its important role even in the early stages of biofilm formation (Zhang and Poh, 2018).

In this study, we describe a novel polygalacturonase named PG-BG31 from *Pedobacter* sp. BG31 that effectively inhibits *E. coli* biofilm formation. The recombinantly expressed enzyme was purified, characterized and its ability to inhibit biofilm formation in clinical *E. coli* strains was investigated.

2. Materials and methods

2.1. Growth media and microbial strains

Following growth media were used in this study:

Brain heart infusion broth (BHI) (HiMedia Laboratories, Mumbai, India);

Luria Bertani medium (LB) (1 % Trypton (Biolife Italiana, Milan, Italy); 0.5 % yeast extract (Biolife Italiana); 1 % NaCl (HiMedia Laboratories);

LB-NaCl (1 % Trypton (Biolife Italiana); 0.5 % yeast extract (Biolife Italiana));

Modified artificial urine (AU) (Sarigul et al., 2019) (0.17 % Na_2SO_4 (Thermo Fisher Scientific Inc., Waltham, MA, USA); 0.072 % $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2 \text{H}_2\text{O}$ (CARLO ERBA Reagents srl, Cornaredo, MI, USA); 0.0881 % $\text{C}_4\text{H}_7\text{N}_3\text{O}$ (SIGMA-ALDRICH Co., St. Luis, MO, USA); 1.5 % H_2NCONH_2 (SIGMA-ALDRICH Co.); 0.2308 % KCl (SIGMA-ALDRICH Co.); 0.1756 % NaCl, 0.0185 % CaCl_2 (SIGMA-ALDRICH Co.); 0.1266 % NH_4Cl (Acros Organics, Geel, Belgium); 0.0035 % $\text{K}_2\text{C}_2\text{O}_4 \cdot \text{H}_2\text{O}$ (CARLO ERBA Reagents srl.); 0.1082 % $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$ (Acros Organics); 0.2912 % $\text{NaH}_2\text{PO}_4 \cdot 2 \text{H}_2\text{O}$ (SIGMA-ALDRICH Co.); 0.0831 % $\text{Na}_2\text{HPO}_4 \cdot 2 \text{H}_2\text{O}$

(SIGMA-ALDRICH Co.) dissolved in distilled water and mixed with LB-NaCl 1:1 before use);

M63 (1.36 % KH_2PO_4 (Acros Organics); 0.2 % $(\text{NH}_4)_2\text{SO}_4$ (SERVA Electrophoresis GmbH, Heidelberg, Germany); 0.02 % $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 0.000005 % $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (SIGMA-ALDRICH Co.); 0.2 % glucose (Biolife Italiana); 0.5 % casamino acids (SIGMA-ALDRICH Co.);

Mueller Hinton Broth (MH) (Torlak, Belgrade, Serbia)

Tryptic Soy Broth (TSB) (Biolife Italiana)

The 14 clinical *E. coli* strains (ECU11-ECU114) were isolated from urine samples of patients diagnosed with UTI and kindly provided by the medical laboratory “Zdravlje”, Sabac, Serbia. The strains were cultured in LB-NaCl at 37 °C and 180 rpm. *Pedobacter* sp. BG31 strain is part of laboratory proprietary bacterial collection isolated from *Abramis brama* (bream) gills (BG) (Atanaskovic et al., 2024) and it was cultured in BHI at 37 °C and 180 rpm.

2.2. 16S rDNA sequencing

Genomic DNA (gDNA) from *Pedobacter* sp. BG31 and clinical *E. coli* strains was extracted using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Amplification of the 16S rRNA gene was performed using the bacteria-specific primers 27 F and 1492 R (Invitrogen, Waltham, MA, USA) and FastGene Taq DNA Polymerase (Nippon Genetics, Dören, Germany). The reaction mixture (50 µL) contained 1 U Taq DNA polymerase, 1X dilution buffer B, 200 µM dNTP, 0.4 µM primer and 150–200 ng gDNA. Amplification was performed over 35 cycles with an annealing temperature of 50 °C and an elongation time of 1.4 min. The resulting PCR product was purified using the GeneJET PCR Purification Kit (Thermo Fisher Scientific Inc.), and sequencing was performed from both ends using the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems®, Waltham, MA, USA) on an Applied Biosystems® 3130 Genetic Analyzer (USA). Sequence data were analyzed and compiled using the SeqMan tool from DNASTAR Lasergene Software (DNASTAR, Madison, WI, USA). A BLASTn search was performed in the NCBI GenBank database (Altschul et al., 1990). The 16S rDNA sequence was deposited in GeneBank under accession number PQ359472.

2.3. Whole genome sequencing and analysis

Genomic DNA (gDNA) from *Pedobacter* sp. BG31 was extracted using the QIAamp DNA Mini Kit (Qiagen) and sequenced on the Illumina HiSeq 2500 platform at MicrobesNG (Birmingham Research Park, Birmingham, UK), which offers a sequencing depth of 30X. Genome assembly was performed using De Bruijn Graph methods, excluding contigs shorter than 200 base pairs (bp). The Burrows-Wheeler Aligner (BWA) was used for genome mapping, while genome annotation was performed using the Rapid Annotations using Subsystems Technology (RAST) server. Kraken 1 was used to identify the closest reference genome, and BWAMEM was applied to map reads to this reference and assess data quality. The draft genome sequence has been submitted to GenBank under accession number JBNHNZK000000000.

2.4. Bioinformatic analysis

The gene sequence of PG-BG31 was deposited in GeneBank under accession number PQ439955. The protein sequence was entered into the UniProt database (The UniProt Consortium et al., 2023) to determine the sequence homology and into the SWISS-MODEL (Waterhouse et al., 2018) to determine structural homology. Subcellular localization and the presence of a signal peptide were determined using SignalP version 5.0 (Almagro Armenteros et al., 2019). The InterProScan tool was used for functional annotation of the protein sequence (Zdobnov and Apweiler, 2001). Protein modeling was performed with AlphaFold2 (Jumper et al., 2021). Catalytic and substrate-binding motifs were visualized using Discovery Studio Visualizer v24.1.0.23298 (Copyright © 2023, Dassault Systèmes Biovia Corp) (Systèmes, 2020).

2.5. Enzyme expression and purification

The Takara pET6xHN expression vector set (In-Fusion Ready) (Takara Bio USA, Inc., San Jose, CA, US) was used for the recombinant expression of PG-BG31. The gene sequence was amplified with Phusion™ High-Fidelity DNA Polymerase (Thermo Fisher Scientific Inc.) and gene-specific primers with 15–18 nt overlap with the expression vector (F: 5' TAAGGCTCTGTCGACCAAGGGTACTATAACGTAATTGAA 3'; R: 5' CAGAATTCGCAAGCTTTTGTACTTTATCGCCCGAAA3'). The 20 µL reaction mixture contained 1 U Phusion DNA polymerase, 1X Phusion HF buffer dilution, 200 µM dNTP, 1 µM primers and 150–200 ng *Pedobacter* sp. BG31 gDNA. Amplification was performed over 35 cycles with an annealing temperature of 60 °C and an elongation time of 1 min. The resulting PCR product (1 µL) was added to the ligation mixture containing 5X In-Fusion HD Enzyme Premix (1 µL), pET6xHN-C In Fusion Ready Vector (1 µL) and H₂O (7 µL). The mixture was incubated at 50 °C for 15 min, and then used to transform the ultracompetent DH5α *E. coli* cells (ThermoFisher Scientific Inc.). Positive clones for PG-BG31 were identified by PCR with a T7 promoter forward primer and a gene-specific reverse primer. The plasmid DNA was then subsequently transformed into HI-Control BL21(DE3) *E. coli* cells (Lucigen, Middleton, WI, USA). For recombinant PG-BG31 expression, transformed HI-Control BL21(DE3) cells were cultured overnight in LB medium containing 100 µg/mL ampicillin (LB/Amp100) and 1 % glucose at 37 °C and 180 rpm. A 2 % inoculum was transferred to 200 mL LB/Amp100 medium and incubated at 37 °C and 180 rpm until the optical density (OD) at 600 nm reached 0.4–0.6. PG-BG31 expression was induced by adding 1 mM IPTG for 22 h at 25 °C and 180 rpm. The protein was purified using a HisTALON™ Gravity Column Purification Kit (Takara Bio USA) and eluted with an imidazole-containing buffer (150 mM). The buffer was then exchanged for 20 mM sodium phosphate with 100 mM NaCl at pH 7.0. For further purification, the protein was applied to the size exclusion chromatography column, HiLoad Superdex 200 prep grade 26/60 (Cytiva Sweden AB, Uppsala, Sweden), with a range of 10–600 kDa. The purification buffer contained 50 mM NaH₂PO₄ and 100 mM NaCl, pH 7.2. The proteins were visualized by SDS-PAGE electrophoresis (12.5 %) and subsequent staining with Coomassie Brilliant Blue R250. The Bradford method was used to determine the protein concentration (Bradford, 1976).

2.6. Assessment of enzymatic activity

The PG-BG31 activity was investigated with polygalacturonic acid (PGA) (SIGMA-ALDRICH Co.). The experiments were carried out at 37 °C and pH 6.0, with 0.5 % substrate and 100 µg/mL enzyme. The release of reducing sugars was monitored at exponential time points. Reactions were terminated by adding an equal volume of DNS reagent (1.6 % NaOH (SIGMA-ALDRICH Co.); 1 % 3, 5-dinitrosalicylic acid (SIGMA-ALDRICH Co.); 30 % KNa-tartrate (SIGMA-ALDRICH Co.)) and the vials were placed in a water bath set at 96 °C for 7 min (Contreras Esquivel and Voget, 2004). The absorbance was measured at 540 nm using the Tecan Infinite200.

2.7. Determination of the optimum temperature and pH and stability

The optimum temperature and pH for enzyme activity were determined using PGA (0.5 %). The temperature conditions varied between 7 and 90 °C at a pH of 6.0 and the pH conditions varied between 4.0 and 11.0 at 37 °C using different buffers including citrate-sodium buffer (100 mM; pH 4.0, 5.0), sodium phosphate buffer (20 mM; pH 6.0, 7.0) and Tris-HCl buffer (20 mM; pH 8.0, 9.0, 10.0, 11.0). The temperature and pH stability of the purified enzyme was determined by measuring the enzymatic activity after incubation for 2 h at temperatures between 7 and 90 °C and pH values ranging between 4.0 and 11.0, followed by enzymatic reactions carried out at 37 °C and pH 6.0. Reactions were terminated after 15 min with DNS reagent and after boiling for 7 min

absorbance was measured at 540 nm using the Tecan Infinite200 plate reader. The enzymatic activities were expressed in relation to the highest activities measured.

2.8. Screening for biofilm-forming *E. coli* isolates

The static biofilm test was performed in 96-well polystyrene microtiter plates (Biolite, Thermo Fisher Scientific Inc) to evaluate the potential of biofilm formation of 14 clinical *E. coli* isolates. Bacterial cultures grown overnight in LB-NaCl at 37 °C and 180 rpm were diluted to 5×10^7 CFU/mL in AU, M63, BHI, LB, LB-NaCl, double diluted LB-NaCl (LB-NaCl/2), MH or TSB. Aliquots of 100 µL were added to each well and incubated at 37 °C for 24 h. Afterward, the biofilms were washed and stained with 0.1 % crystal violet (CV) (SERVA Electrophoresis GmbH) (w/v). Biofilm biomass was quantified after dissolution of CV in 30 % acetic acid (Fisher Chemicals, Waltham, MA, US) (v/v) by measuring the absorbance at 570 nm using the Tecan Infinite200 (Merritt et al., 2005). Assays were performed in hexaplicate and repeated three times.

The isolates were categorized into four groups based on their biofilm production levels, determined using the three-standard-deviation method (Singh et al., 2017). The criteria for biofilm formation (OD_{cut}) was established using the following formula:

$$OD_{cut} = OD_{avg} \text{ of negative control} + 3 \times \text{standard deviation of ODs of negative control}$$

Sterile broth served as the negative control. Isolates were classified as non-biofilm-formers if their crystal violet measurements were below the OD_{cut}. Weak biofilm-formers had crystal violet measurements between the OD_{cut} and $2 \times OD_{cut}$, while moderate biofilm-formers had values between $2 \times OD_{cut}$ and $4 \times OD_{cut}$. Strong biofilm-formers were identified with crystal violet measurements exceeding $4 \times OD_{cut}$.

2.9. Effect of PG-BG31 on biofilm formation

2.9.1. Quantification of biofilm biomass

Quantification of biofilm biomass was performed as described above (Section 2.8). Biofilms of the strong biofilm-formers ECUI2, ECUI5, ECUI6 and ECUI13 were grown in AU with or without treatment. Concentrations 1.125, 2.5, 5, 10 and 100 µg/mL of each enzyme were used to determine the dose-dependent activity of PG-BG31, proteinase K (Thermo Fisher Scientific Inc.), DNase I (SIGMA-ALDRICH Co.) and cellulase from *Trichoderma reesei* ATCC 26921 (SIGMA-ALDRICH Co.). After 24 h, the biofilms were washed and stained with CV. The amount of biofilm was calculated in relation to the untreated control. Assays were performed in hexaplicate and repeated three times.

2.9.2. Static mature biofilm disruption assay

Overnight cultures of ECUI2, ECUI5, ECUI6 and ECUI13 were diluted to 5×10^7 CFU/mL in AU and 100 µL was added to the wells in 96-well microtiter plates and incubated for 24 h at 37 °C. After the removal of nonadherent cells and two washing steps with PBS, the biofilms were treated with PG-BG31 (0 µg/mL, 10 µg/mL and 100 µg/mL) for an additional 24 h. Afterward, the biofilms were washed and stained with 0.1 % CV. Biofilm biomass was quantified after the dissolution of CV in 30 % acetic acid (v/v) by measuring the absorbance at 570 nm using the Tecan Infinite200. Assays were performed in hexaplicate and repeated three times.

2.9.3. Visualization of PG-BG31 antibiofilm effect by fluorescent microscopy

To visualize biofilms under a fluorescent microscope, overnight bacterial cultures were diluted to 5×10^7 CFU/mL in AU medium and added to tilted 6-well plates (Sastedt AG & Co., Nümbrecht, Germany) containing plastic coverslips (1 mL per well), with or without PG-BG31

(10 µg/mL). Following a 24-h incubation at 37 °C, the biofilms were washed and stained using 2.5 µM Syto®9 green fluorescent dye and 2.5 µM propidium iodide (PI) red fluorescent dye from the LIVE/DEAD® BacLight™ Bacterial Viability Kit (Thermo Fisher Scientific Inc.). Biofilms were examined under a fluorescent microscope (Olympus BX51, Applied Imaging Corp., San Jose, CA, USA) at 10 × magnification. The anti-biofilm effect was evaluated by comparing biofilm formation in the presence and absence of the enzyme. Assays were performed in triplicates and repeated 3 times.

2.9.4. Inhibition of biofilm formation in the catheter model

For the visualization of biofilms on catheter surface overnight bacterial cultures of *E. coli* ECU12, ECU15, ECU16 and ECU13 were diluted to 5×10^7 CFU/mL in AU and 200 µL aliquots with or without enzyme were added to 2-mL Eppendorf tubes (SARSTEDT AG & Co. KG). The silicone catheter (Coloplast, Humlebaek, Denmark) was cut into 1 cm segments, which were partially immersed in the bacterial culture and incubated at 37 °C for 24 h. The biofilms were then washed and stained with 0.1 % CV. Assays were performed in triplicates and repeated 3 times.

The bacterial counting method was used to evaluate the effect of PG-BG31 on biofilm formation on 1 cm long catheter pieces. Overnight bacterial cultures of ECU12, ECU15, ECU16 and ECU13 were diluted to 5×10^7 CFU/mL in AU, and 200 µL aliquots were added to 2-mL Eppendorf tubes (SARSTEDT AG & Co. KG) with 10 µg/mL PG-BG31 or without treatment. The catheter segments were partially immersed in the liquid and incubated at 37 °C for 24 h. Afterward, pieces were washed in PBS and sonicated twice in a sonic water bath (DK Sonic, West Yorkshire United Kingdom) in 1 mL PBS at 2-minute intervals, with vortexing in between. Biofilm colonies were counted in sterile dilutions after growth at 37 °C on LB agar plates (Haney et al., 2018). The number of CFU was converted to the log reduction (log CFU/biofilm) and the percentage reduction compared to the untreated control. Assays were performed in triplicates and repeated 3 times.

2.9.5. Effect of PG-BG31 biofilm pretreatment on antibiotic susceptibility

To evaluate the antibiotic efficacy, biofilms were grown on catheter segments in the presence or absence of PG-BG31 (10 µg/mL) as previously described (Section 2.9.4). After 24 h catheters segments were washed two times in PBS and transferred to new Eppendorf tubes containing 700 µL of AU medium with the appropriate amount of ciprofloxacin (1 × MIC, 2 × MIC or 4 × MIC). Fully submerged catheter segments were incubated for an additional 24 h at 37 °C, after which the CFU counts were determined as described previously (Section 2.9.4). Assays were performed in triplicates and repeated 3 times.

2.10. The minimum inhibitory concentration

The minimum inhibitory concentration (MIC) of selected antibiotics (fosfomycin (Toronto Research Chemicals Inc.), trimethoprim (Sigma Aldrich Co.), ampicillin (Sigma Aldrich Co.) and ciprofloxacin (Sigma Aldrich Co.) was determined using standard broth microdilution assays according to European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2024). The inoculums were 5×10^4 colony-forming units (CFU)/mL. The MIC values corresponded to the lowest concentration that inhibited the visual growth after 20 h at 37 °C. Assays were performed in triplicates and repeated 3 times.

2.11. Cytotoxicity

Human MRC5 lung fibroblasts from American Type Culture Collection (ATCC) were cultured as monolayers in RPMI-1640 medium, HEPES (Thermo Fisher Scientific Inc.) supplemented with Antibiotic-Antimycotic solution (Thermo Fisher Scientific Inc.) and 10 % (v/v) fetal bovine serum (FBS) (Thermo Fisher Scientific Inc.). Cells were incubated at 37 °C in a humidified environment with 95 % air and 5 %

CO₂. Cytotoxicity was assessed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay after 48 h of exposure to PG-BG31 (10, 50, 100 and 200 µg/mL). After 30 min MTT was removed and 50 µL DMSO (Fisher Chemicals) was added to each well. To determine cell viability, the absorbance at 690 nm measured with the Tecan Infinite200 was subtracted from the absorbance at 540 nm and expressed as a percentage compared to the untreated cells (Aleksic et al., 2017). Assays were performed in tetraplicates and repeated 3 times.

2.12. *Caenorhabditis elegans* toxicity test

C. elegans strain AU37, obtained from the *Caenorhabditis* Genetics Center (CGC, University of Minnesota, Minneapolis, USA) was grown on nematode growth medium (NGM) (1.7 % agar, 0.29 % NaCl, 0.25 % peptone (Torlak), 0.01 % CaCl₂, 0.0005 % cholesterol (SIGMA-ALDRICH Co.), 0.34 % KH₂PO₄ and 0.012 % MgSO₄). The nematodes were fed with *E. coli* OP50 and kept at 15 °C. Hypochlorite bleach and NaOH were used to synchronize the population. The effect of PG-BG31 at concentrations of 10, 100 and 500 µg/mL on the survival of *C. elegans* were studied according to a previously established protocol (Stiernagle, 2006). The experiment was performed in 96-well microplates, with each well containing 25–40 L4-stage worms in 100 µL of M9 (6.4 % Na₂HPO₄·7H₂O, 1.5 % KH₂PO₄, 0.25 % NaCl, 0.5 % NH₄Cl) and LB medium (95:5 v/v) supplemented with 5 µg/mL cholesterol. Worms were counted at the beginning of the experiment and after 24 and 48 h using a stereomicroscope (SMZ143-N2GG, Motic, Germany). The relative toxicity of the enzyme was evaluated by comparing the survival rate with a control group without enzyme treatment under the same conditions. Assays were performed in tetraplicates and repeated 3 times.

2.13. Statistical analysis

The statistical analysis of enzyme activity was conducted using the Student's *t*-test in SPSS version 20 software (IBM, Armonk, NY, USA), with a significance level set at *P* < 0.05. Concentrations inhibiting 50 % of biofilms (BIC50 values) were determined using a non-linear regression curve in GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA).

3. Results and discussion

3.1. Identification and bioinformatics analysis of PG-BG31

E. coli bacteria are a major cause of urinary tract infections and often pose a major challenge for catheterized patients, where they lead to persistent infections (Tenke et al., 2017). The new enzymes capable of inhibiting *E. coli* adhesion and biofilm formation hold great potential for the development of functionalized catheters that could significantly reduce the incidence of these infections and prevent associated complications. In the search for new enzymes that could be used to prevent *E. coli* biofilms, we screened the genomes of environmental isolates from our bream gill (BG) bacterial collection for genes encoding potentially novel polygalacturonase. The gene encoding a 525 amino acid protein belonging to glycoside hydrolase family 28 was identified in the genome of the environmental strain *Pedobacter* sp. BG31 (named PG-BG31). SignalP, which is used to predict the presence and location of signal peptides, predicted a 22 amino acid long signal sequence, suggesting the extracellular location of the enzyme. According to the UniProt database, the central database for protein-related information, the closest homologous protein of PG-BG31 is polygalacturonase (A0A0Q4GWD2) from *Pedobacter* sp. Leaf216, which has a sequence identity of 85.1 %.

Polygalacturonases generally contain four conserved motifs, two catalytic regions (G/QDD and G/SHG) and two substrate-binding regions (NTD and RIK) (Palanivelu, 2005), all of which are found within the PG-BG31 sequence: GHG 135–137, NTD 231–233, GDD 253–255, GHG 287–289 and RIK 319–321. InterProScan, a bioinformatics tool

that scans protein sequences to identify functional domains, motifs, and other structural features, classified PG-BG31 as a pectin lyase-like superfamily (SSF51126) along with other galacturonases, pectin and pectate lyases, and tailspike proteins. Despite a lack of clear sequence similarity, structural parallels suggest that galacturonases, pectate lyases, tailspike proteins, and other right-handed parallel β -helix proteins likely evolved from a common ancestral protein (Jenkins et al., 1998). Tailspike phage proteins, which serve as the primary point of contact with the bacterial host, often possess enzymatic activity that enables them to bind to and degrade components such as LPS and CPS that form protective layers around bacterial cells (Sorensen et al., 2021).

A limited number of experimentally validated homologs of PG-BG31 were found using the Swiss-Model database. Our analysis revealed that PG-BG31 has the highest structural similarity to exopolysaccharonases (PDB: 3jur.1.A) with 33.09 % sequence identity, suggesting a close functional and structural relationship with this enzyme family. To assess the reliability of the homology models, we used Global Model Quality Estimation (GMQE) scores, which provide an estimate of model accuracy based on sequence identity and alignment coverage. The homology model for this family yielded a GMQE score of 0.59, indicating relatively high reliability in predicting structural alignment. To a slightly lesser extent, the enzyme also shows similarity to endopolysaccharonases (PDB: 7b7a.1.A), with 28.36 % sequence identity and a corresponding GMQE score of 0.46, supporting a possible overlap in catalytic capabilities with this group. In addition, PG-BG31 shows some similarity to several phage tailspike proteins (e.g., PDB: 4urr.1.A; 7eea.1.A; 2vdm.1.C), with sequence identities around 16 % and GMQE scores between 0.31 and 0.34. This limited similarity suggests possible structural or evolutionary links, but may also indicate different functional roles compared to tailspike proteins. Overall, these results suggest that while the enzyme is most closely related to polygalacturonases, it also shares broader structural features with phage-derived proteins, as reflected in both sequence identity and model quality scores.

Using AlphaFold2, which predicts the three-dimensional conformation of the protein based on its amino acid sequence, we generated a structural model of the protein with high confidence (Fig. 1). For the

used model, generated by AlphaFold2, both predicted local distance difference test (pLDDT) and predicted aligned error (PAE) showed very high local and global confidence in modeled structure (Supplementary Fig. 1). Values dominantly > 90 % and low expected position error (near 0 Å), except for some lower results at the start and at the end of the sequence, suggest high accurate prediction and good relative placement of the domains in the predicted structure. Protein A0A0Q5U0G6 (A0A0Q5U0G6_9SPHI) from *Pedobacter* sp. *Leaf194*, which is available in the AlphaFold2 database, has a sequence identity of 78.86 % and a high GMQE score of 0.96, indicating a high-quality model. However, it is important to note that this model has not been experimentally validated. The model was visualized using Discovery Studio Visualizer so that we can see the position of the active catalytic and substrate binding motifs.

3.2. Heterologous expression and characterization of PG-BG31

His-tagged PG-BG31 was overexpressed in *E. coli* and purified by standard metal affinity chromatography, followed by size exclusion chromatography. This yielded 5 mg protein per 1 g cell pellet with a purity of approximately 98 %, as determined by Coomassie Brilliant Blue R-250 staining (Supplementary Fig. 2). The enzyme was eluted as a single peak from the size-exclusion chromatography column, resembling the size of a trimer (Supplementary Fig. 2A), which correlates with its bacteriophage tailspike protein homologs.

The catalytic activity of PG-BG31 was examined using polygalacturonic acid as a substrate, measuring the release of reducing sugars with DNS reagent after heat treatment (Supplementary Fig. 3). To determine the optimal conditions for PG-BG31 activity, tests were performed at different temperatures and pH values. The enzyme showed peak activity between 25 and 42 °C (Fig. 2A) and a pH of 5.0–6.0 (Fig. 2B), aligning with human body temperatures and the slightly elevated temperatures during infection. Its optimal activity under somewhat acidic conditions suggests a potential for the treatment of urinary tract infections, where many uropathogens maintain a urine pH of around pH 6 (Lai et al., 2021). In addition, PG-BG31 was stable for two hours at temperatures up to 65 °C (Fig. 2C) and over the entire range of pH values tested (pH 4–11) (Fig. 2D). This indicates that it is suitable for both storage and potential use in high pH immobilization protocols.

3.3. Evaluation of anti-biofilm activity on *E. coli*

E. coli biofilms exhibit considerable differences in their structure, composition, and behavior depending on environmental conditions, such as the type of growth medium and available substrates (Alqasim et al., 2014). To evaluate the anti-biofilm activity of PG-BG31 against *E. coli*, we first examined the biofilm-forming ability of 14 clinical *E. coli* isolates in different culture media to identify the strongest biofilm producers (Fig. 3). We used AU, M63, BHI, LB, LB-NaCl, LB-NaCl/2, MH and TSB to test for biofilm formation and the three-standard deviation method to categorize the strains into four groups: non-biofilm-formers ($A_{570} < 0.17$), weak biofilm-formers ($0.17 < A_{570} < 0.34$), moderate biofilm-formers ($0.34 < A_{570} < 0.68$) and strong biofilm-formers ($A_{570} > 0.68$) (Supplementary Table 1). As expected, the AU medium, which is similar in composition to urine, was found to be the most effective medium in promoting biofilm formation in urinary *E. coli* isolates. Of the media tested, it yielded the fewest non-biofilm-forming isolates and the highest number of highly biofilm-forming isolates. Therefore, this medium was selected for further biofilm experiments together with the highly biofilm-forming isolates ECU12, ECU15, ECU16 and ECU113.

Next, we investigated the biofilm-inhibitory effect of PG-BG31 using the four biofilm-forming strains. The effect of PG-BG31 on biofilm formation was evaluated by CV staining, fluorescence microscopy and CFU counting. It showed dose-dependent inhibition of biofilm formation (Fig. 4) with BIC50 values between 2 and 5 μ g/mL (Supplementary

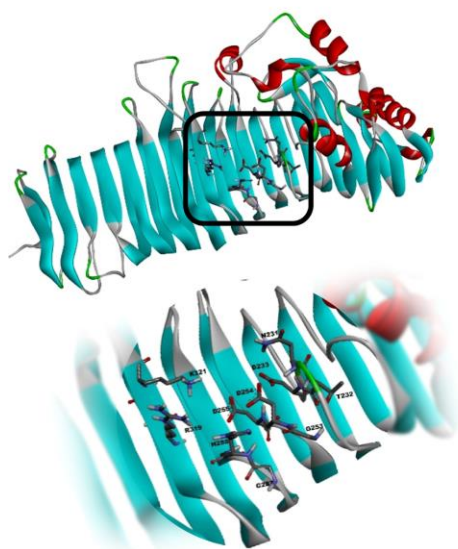


Fig. 1. Tertiary structure prediction model of PG-BG31 created with AlphaFold2 and visualized in the Discovery Studio Visualizer. Key residues in the catalytic and substrate-binding motifs are highlighted.

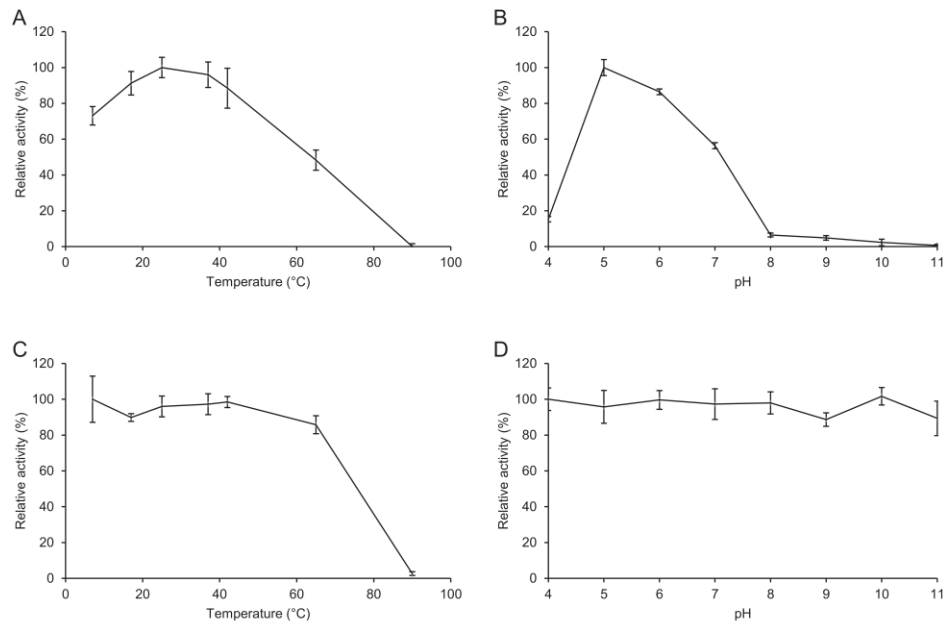


Fig. 2. Influence of temperature (A) and pH (B) on PG-BG31 activity. Enzyme stability after 2 h pre-incubation at different temperatures (C) and pH values (D). All reactions were performed with polygalacturonic acid as a substrate and the product was detected using a DNS reagent. Values are presented as mean $\pm$ SD.

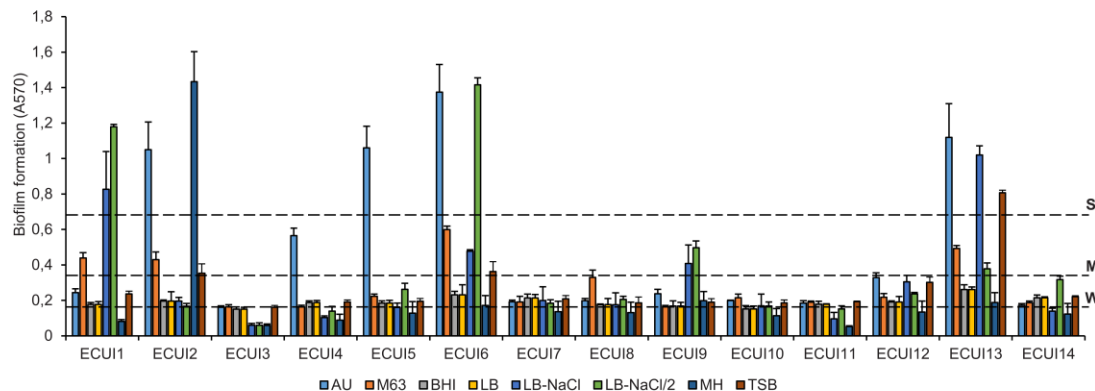


Fig. 3. Selection of biofilm-forming *E. coli* isolates. Biofilm formation in 96-well microtiter plates was quantified by CV staining. Dashed lines represent the margins between the absence of biofilm formation, weak (W), moderate (M) and strong (S) biofilm formation. Values are expressed as mean $\pm$ SD.

Table 2). The tested concentrations of PG-BG31 had no effects on bacterial growth (Supplementary Fig. 4), suggesting that the significant reduction in biofilms was due to the reduced number of bacteria adhering to the surface. Without bactericidal or bacteriostatic pressure, PG-BG31 is less likely to drive the development of antimicrobial resistance, making it a promising alternative for biofilm control without selecting for resistant strains.

PG-BG31 was not very effective in eliminating pre-formed biofilms, since only biofilms of ECUI2 were reduced by 30 % at an enzyme concentration of 10 $\mu\text{g/mL}$ (Supplementary Fig. 5), suggesting that the enzyme could be used for biofilm prevention rather than treatment of pre-formed biofilms.

Since cellulose and curly fibers are described in the literature as

major constituents of EPS in *E. coli* biofilms (Hu et al., 2020; Lim et al., 2019; Samaniego et al., 2024), and extracellular DNA is an important structural component of biofilms in various pathogenic bacteria (Okshevsy and Meyer, 2015), we next investigated the efficacy of proteinase K, DNase and cellulase to inhibit ECUI2, ECUI5, ECUI6 and ECUI13 biofilms. In most cases, these enzymes had no significant effect on biofilm formation and in some cases they even showed a stimulatory effect, with a few exceptions (Fig. 4). Proteinase K inhibited biofilm formation of ECUI5 in a dose-dependent manner (BIC50 3.149 $\mu\text{g/mL}$), while DNase reduced biofilm formation in ECUI5 and ECUI6 (BIC50 13.66 and 6.956 $\mu\text{g/mL}$, respectively). Additionally, cellulase inhibited biofilm formation in ECUI5 (BIC50 4.195 $\mu\text{g/mL}$). However, none of these enzymes were effective against all four strains, and their BIC50

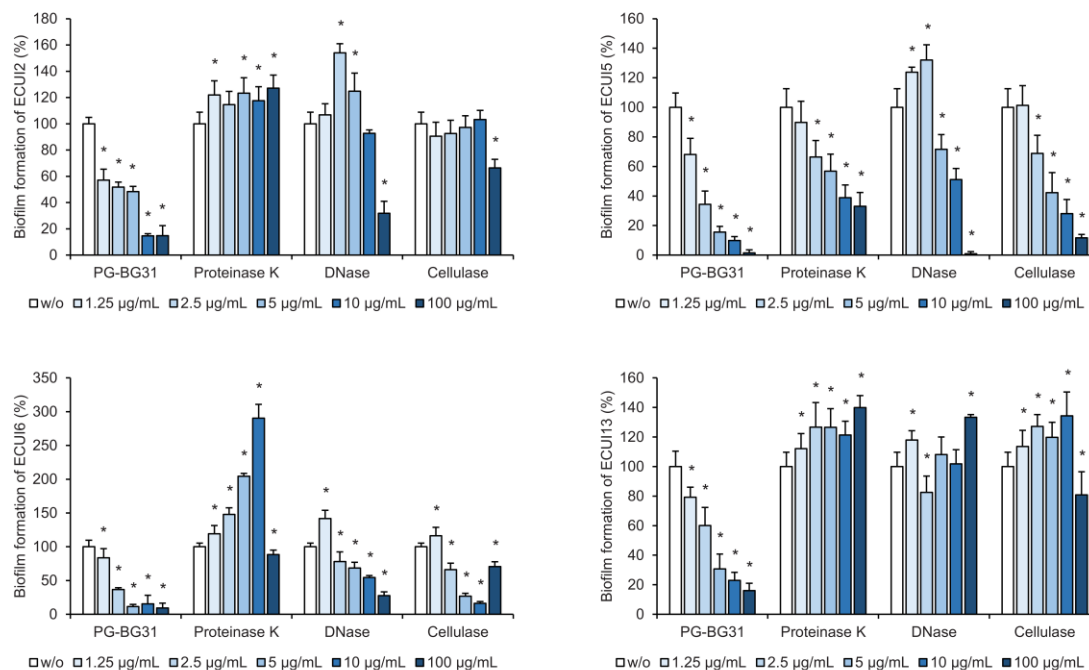


Fig. 4. Effect of PG-BG31, Proteinase K, DNase and Cellulase on *E. coli* biofilm formation. Dose-dependent inhibition of biofilm formation quantified with CV relative to control without treatment (w/o). Values are expressed as mean $\pm$ SD. * $p < 0.05$.

values were higher than those observed for PG-BG31.

The efficacy of PG-BG31 was validated using fluorescence microscopy in combination with the Live/Dead® BacLight Kit, which contains Syto®9 and propidium iodide (PI) dyes that bind to nucleic acids. Syto®9, a green fluorescent dye, penetrates the membranes of both viable and non-viable cells, while the red fluorescent PI selectively penetrates cells with damaged membranes, indicating non-viable cells. Incubation with PG-BG31 resulted in a significant reduction in biofilm formation in all strains tested (Fig. 5). The consistent and significant reduction in biofilm formation in all strains underscores the broad-spectrum efficacy of PG-BG31 as an anti-biofilm agent.

Various enzymes have been investigated for their ability to inhibit biofilm formation on catheters by specifically targeting the biofilm matrix or quorum-sensing molecules (Grover et al., 2016; Ivanova et al., 2021; Ivanova et al., 2015; Vogel et al., 2020). This approach disrupts established biofilms and prevents bacterial adhesion more effectively than passive coatings while offering a safer alternative to broad-spectrum antibiotics. However, few studies have looked specifically at *E. coli*, particularly with different clinical isolates (Ivanova et al., 2021; Ivanova et al., 2015). Therefore, further research is needed to identify the most effective enzymes for combating a wide range of *E. coli* variants. Therefore, the efficacy of PG-BG31 was also tested for biofilm formation on the catheter surface. Biofilms were visualized by CV staining (Supplementary Fig. 6) and quantified by CFU counting. Biofilms that grew in the presence of the enzyme showed a reduction in bacterial CFU counts of more than 90 % for all strains compared to untreated controls (Fig. 6). The treated biofilms of ECUI2, ECUI5, ECUI6 and ECUI13 showed a reduction of 1.87, 2.53, 1.88 and 1.06 $\log_{10}$ CFU/biofilm, respectively.

3.4. Potentiation of antibiotics effect by enzyme pretreatment

In clinical practice, antibiotics are still the most effective treatment for UTIs. Although the European Association of Urology and the American Urological Association may recommend the use of supplements such as cranberry products, xyloglucan, hibiscus, propolis, centaury, lovage, or rosemary to help alleviate symptoms, antibiotics remain indispensable for the treatment of symptomatic UTIs (EAU, 2025; AUA, 2022). The most commonly used antibiotics for uncomplicated UTIs are trimethoprim-sulfamethoxazole, trimethoprim, β -lactams, fluoroquinolones, nitrofurantoin, and fosfomycin-tromethamine (Jancel and Dudas, 2002). We investigated whether PG-BG31 pretreatment can improve antibiotic therapy in catheter infections associated with biofilms. First, we determined the minimum inhibitory concentrations (MICs) of fosfomycin, trimethoprim, ampicillin and ciprofloxacin for ECUI2, ECUI5, ECUI6 and ECUI13 (Supplementary Table 3). Since ciprofloxacin and trimethoprim had the lowest MIC values among the antibiotics tested and affected most of the strains, they were selected as the antimicrobial agents for use in the catheter biofilm model. To evaluate their efficacy, they were applied at concentrations of MIC, 2 $\times$ MIC and 4 $\times$ MIC to pre-formed biofilms grown both in the absence and presence of PG-BG31 (Fig. 6). In biofilms formed without PG-BG31 treatment, none of these concentrations achieved a 4-log reduction (equivalent to a 99.99 % reduction in viable bacteria), except for ECUI2 which had a 4-log reduction when treated with 2 $\times$ MIC concentration of antibiotics. In comparison, biofilms grown in the presence of PG-BG31 showed a significantly increased sensitivity to both ciprofloxacin and trimethoprim. A 4-log reduction was achieved at the MIC concentration of ciprofloxacin for ECUI2, at 2 $\times$ MIC for ECUI5 and ECUI13, and at 4 $\times$ MIC for ECUI6 (Fig. 6A). In combination with trimethoprim 4-log reduction was achieved at the MIC concentration for ECUI2, at 2 $\times$ MIC for ECUI6, and at 4 $\times$ MIC for ECUI13 (Fig. 6B). This observation

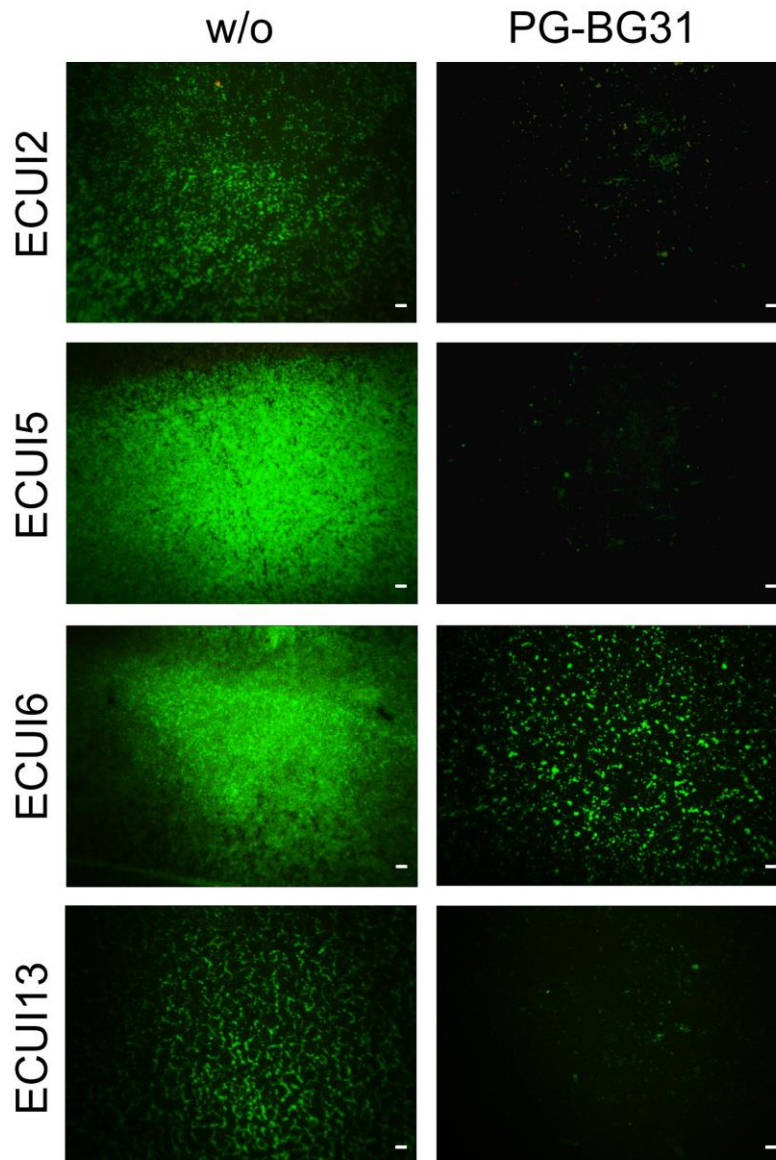


Fig. 5. Visualization of PG-BG31 anti-biofilm effect by fluorescent microscopy. Biofilms were formed for 24 h on plastic coverslips with or without treatment (10 $\mu\text{g}/\text{mL}$ PG-BG31) and analyzed by fluorescent microscopy using a Live/Dead staining kit. Live cells were stained with Syto®9 (green) and dead cells were stained with PI (red). Scale bars represent 10 μm .

highlights the synergistic effect of PG-BG31 in enhancing the antimicrobial efficacy of ciprofloxacin and trimethoprim, possibly by reducing the biofilm biomass and weakening the biofilm structure, thereby increasing bacterial exposure to the antibiotic. These results highlight the potential of incorporating enzymatic treatments into prophylactic strategies for catheterized patients, alongside conventional antibiotics, to effectively combat biofilms, particularly in the prevention and treatment of catheter-associated infections.

3.5. Assessment of enzyme toxicity

To determine the safety doses of PG-BG31, its toxicity was evaluated using *in vitro* and *in vivo* models.

The effect of PG-BG31 on cell viability was investigated using human lung fibroblasts (MRC5) as a model of healthy cells. MRC5 cells were treated with increasing concentrations of the enzyme for 24 h and then analyzed for metabolic activity. As shown in Fig. 7A, treatment with PG-BG31 had no effect on cell viability at any of the concentrations tested.

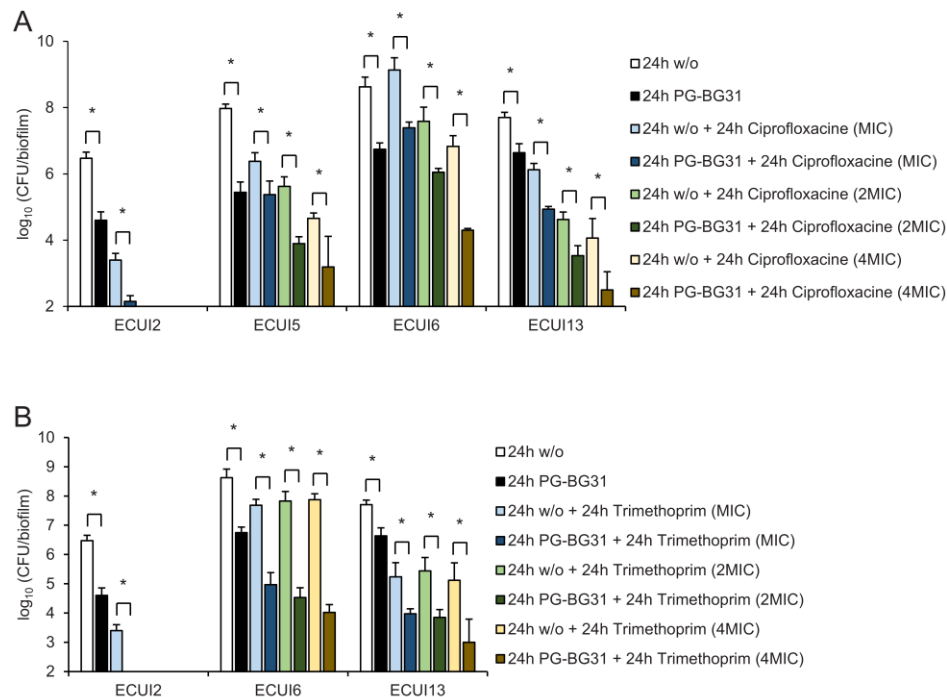


Fig. 6. Prevention of biofilm formation on the silicone catheter and potentiation of antibiotic therapy. Biofilms were grown in the absence (w/o) or presence of PG-BG31 (10 µg/mL), after which the biofilm eradication was performed with different doses (1 × MIC, 2 × MIC or 4 × MIC) of ciprofloxacin (A) or trimethoprim (B). The remaining biofilm was quantified by the number of CFUs. Logarithmic values are presented as mean ± SD. * $p < 0,05$.

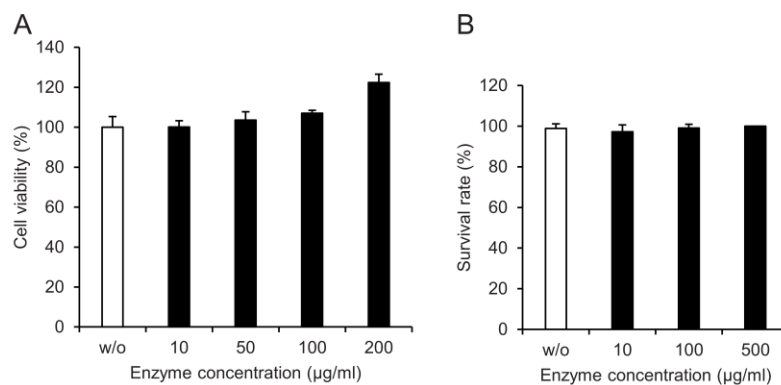


Fig. 7. Evaluation of the cytotoxicity of PG-BG31 on MRC5 cells (A) and *in vivo* toxicity using the *Caenorhabditis elegans* model (B). The effect of the enzyme was estimated in comparison to the control without treatment (w/o). Values are presented as means ± SD.

The toxicity of PG-BG31 *in vivo* was investigated using a liquid culture model of *C. elegans* AU37. Nematodes were exposed to increasing enzyme concentrations for 48 h, and survival rates were subsequently assessed. Treatment with PG-BG31 did not result in toxicity to *C. elegans* under the conditions tested, even at doses of 500 µg/mL (Fig. 7B).

The fact that PG-BG31 had no effect on *C. elegans* survival and exhibited no cytotoxicity indicates a favorable biocompatibility profile, suggesting its suitability for functionalization of materials for long-term medical use.

4. Conclusion

In this study, we describe a novel bacterial polygalacturonase with inhibitory activity against *E. coli* biofilms. We show that PG-BG31 effectively inhibits the biofilm formation of all tested *E. coli* strains, which was confirmed by three independent methods. PG-BG31 is active over a wide temperature range and at a slightly acidic pH and remains stable under both acidic and basic conditions. Biofilms pretreated with PG-BG31 showed increased susceptibility to subsequent antibiotic

treatment, highlighting the potential of this enzyme to improve current therapeutic approaches. Importantly, PG-BG31 showed no toxicity to human cells and the *C. elegans* model, supporting its safety profile for potential therapeutic applications. Therefore, we propose PG-BG31 as a candidate for the functionalization of catheters to reduce biofilm formation, representing a novel agent for preventing UTIs associated with *E. coli* biofilms in the clinical setting. Future studies should investigate the feasibility of this approach *in vivo* and its potential applicability to other biofilm-forming pathogens.

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CRedit authorship contribution statement

Lidija Senerovic: Writing – review & editing, Visualization, Supervision, Methodology, Conceptualization. **Mario Zlatović:** Visualization, Investigation. **Andjela Dilparic:** Investigation. **Marija Atanaskovic:** Writing – original draft, Methodology, Investigation, Data curation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jbiotec.2025.07.011.

Data availability

Data will be made available on request.

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3.3 A novel β -1,2-mannosidase GH130-BG31 from glycoside hydrolase family 130 targets healthcare-associated bacterial biofilms and potentiates antibiotic activity



A novel β -1,2-mannosidase GH130-BG31 from glycoside hydrolase family 130 targets healthcare-associated bacterial biofilms and potentiates antibiotic activity

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Exopolysaccharides

ABSTRACT

Hospital-acquired infections (HAIs) pose a major global health challenge, largely due to persistent biofilms formed by multidrug-resistant pathogens. In this study, we investigate GH130-BG31, a β -1,2-mannosidase from the GH130 glycoside hydrolase family, and demonstrate for the first time that it exhibits strong activity against mannose-rich biofilms associated with HAIs. By targeting β -1,2-mannosidic linkages in exopolysaccharides, GH130-BG31 inhibits 80% of biofilm formation in *Escherichia coli* and *Staphylococcus epidermidis* at 10 μ g/mL, and in *Klebsiella pneumoniae* at 100 μ g/mL. At 100 μ g/mL, it also reduces early adhesion of *Pseudomonas aeruginosa* by 30% and *Acinetobacter baumannii* by 50%. The enzyme partially degrades preformed *K. pneumoniae* and *S. epidermidis* biofilms and significantly enhances the efficacy of various antibiotics. In the catheter-associated *K. pneumoniae* model, the combination of GH130-BG31 (100 μ g/mL) with colistin (4 μ g/mL) reduces biofilm formation by up to 8 logs, and with 8 μ g/mL colistin, it disrupts mature biofilms by 4 logs. The enzyme functions optimally at 37 °C and neutral to slightly acidic pH, and remains stable at temperatures up to 37 °C and within a pH range of 4–7. It also shows no cytotoxicity in human cell lines or in *Caenorhabditis elegans*. Overall, GH130-BG31 emerges as a promising enzyme for preventing and controlling clinically relevant HAI-associated biofilms and as a potential adjuvant to antibiotic therapy.

1. Introduction

Hospital-acquired infections (HAIs) represent a major global public health challenge. According to recent estimates, approximately 136 million cases of HAIs resistant to antibiotics occur worldwide annually, with the highest burden observed in middle-income countries [1]. Patients with HAIs experience prolonged hospitalization by 2–20 days, exhibit a mortality rate ranging from 7% to 64.6%, and represent a substantial economic burden due to extended hospital stays, additional diagnostic testing, and the need for invasive procedures [2].

Biofilms play a crucial role in the persistence and severity of HAIs. These structured microbial communities adhere to the surfaces of indwelling medical devices such as catheters, ventilators, and implants, and are encased within a protective extracellular matrix that shields

them from host immune responses and antibiotic treatments [3]. The ability of bacterial pathogens such as *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Staphylococcus epidermidis*, and *Escherichia coli* to form biofilms significantly contributes to their resistance to antimicrobial therapy and facilitates chronic, recurrent infections [4]. The biofilm matrix also promotes the exchange of resistance genes among bacteria, exacerbating the challenge of multidrug resistance in healthcare settings [5]. Consequently, biofilms represent a formidable barrier to effective infection control, highlighting the urgent need for innovative treatments and preventive strategies targeting biofilm-associated infections in hospitals.

The biofilm matrix is a complex mixture of extracellular polymeric substances (EPS), including polysaccharides, proteins, lipids, and extracellular DNA (eDNA), that collectively provide structural integrity,

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mediate cell-to-cell interactions, and protect the biofilm from environmental stressors [6]. Among these components, polysaccharides play a central role in maintaining the architecture and function of the biofilm. They facilitate surface adhesion, provide mechanical stability, act as a barrier against environmental factors, and contribute to nutrient retention and intercellular communication [7]. They provide the main structural framework of the biofilm matrix, which allows the formation of a hydrated polymer network that gives the biofilm its mechanical stability, supports the development of complex three-dimensional structures, and enables the stratification of bacterial communities within the biofilm. Glycosyl composition analyses have demonstrated a high prevalence of mannose in the polysaccharide fraction of multiple biofilm-forming bacteria, including *P. aeruginosa*, *A. baumannii*, *K. pneumoniae*, *S. epidermidis*, and *E. coli* [8]. The best described example of mannose playing a critical role in bacterial biofilm formation is the Psl polysaccharide of *P. aeruginosa*. Psl is composed of repeating penta-saccharide units of mannose, glucose, and rhamnose in a 3:1:1 ratio [9]. Mannose, being the major sugar component of the Psl exopolysaccharide, is essential for the structural integrity, cell adhesion, and biofilm stability of *P. aeruginosa*. Similarly, in fungal pathogens such as *Candida albicans*, mannose-rich mannan-glucan complexes are integral to biofilm architecture and development [10].

While biofilms formed by single species of these pathogens already pose a significant clinical challenge due to their heightened antibiotic resistance and immune evasion, the problem is further exacerbated in mixed-species biofilms. In such polymicrobial communities, different microorganisms can cooperate synergistically, often leading to enhanced biofilm biomass, increased structural complexity, and greater resistance to antimicrobial agents. Mixed biofilms are frequently observed in chronic wounds, respiratory tract infections (e.g., in cystic fibrosis patients), and on indwelling medical devices, where their presence is associated with worse clinical outcomes and treatment failure [11]. Given the widespread occurrence of mannose-containing polysaccharides in both bacterial and fungal biofilms, mannose represents an attractive and broadly relevant target for matrix-disrupting therapeutics. A strategy capable of degrading mannose-rich components could provide a unified approach to weaken biofilms formed by multiple pathogens simultaneously, including those in mixed-species communities. Such matrix-focused intervention would bypass species-specific barriers and enhance antibiotic efficacy, immune clearance, and treatment outcomes across a range of clinically significant infections.

Therapeutic strategies that target biofilm exopolysaccharides are being actively explored and show promise for both bacterial and fungal biofilms. One of the most effective approaches involves the use of matrix-degrading enzymes, which break down the structural exopolysaccharide components of the biofilm matrix. This enzymatic degradation disrupts the integrity of the biofilm, increases antibiotic penetration, and enhances the effectiveness of antimicrobial treatments [12]. Glycoside hydrolases, such as Dispersin B, α -amylase, β -glucosidase, cellulase, and polygalacturonase, have been shown to be capable of dispersing previously formed biofilms or preventing biofilm formation [13–16]. A notable example of a mannose-targeting enzyme is PslG, a glycoside hydrolase encoded within the *Psl* biosynthetic operon of *P. aeruginosa*. Although PslG shares the closest homology with glycoside hydrolase family 39, which typically lacks specificity for mannose, its biofilm-disruptive function involves binding to mannose residues within the Psl polymer, thereby facilitating biofilm disintegration [17]. However, there are no reports of its antibiofilm activity against other bacterial pathogens. Other enzymes capable of targeting mannose-containing polysaccharides, such as α -mannosidase and β -mannosidase, have been reported to exert significant antibiofilm activity. However, their therapeutic utility is limited due to cytotoxic effects observed at concentrations required for biofilm disruption [18].

Given the high abundance of mannose residues in biofilms formed by clinically relevant pathogens, there is a pressing need to identify novel,

non-toxic glycoside hydrolases capable of targeting mannose-rich exopolysaccharides. In this context, we investigated an enzyme from the glycoside hydrolase family 130 (GH130), a group primarily associated with mannose metabolism, including the phosphorylytic and hydrolytic cleavage of β -linked manno-oligosaccharides in bacterial carbohydrate-processing pathways [19]. Although GH130 enzymes have been studied in the context of glycan degradation, their potential for antibiofilm applications has not yet been explored. The aim of this study is, therefore, to explore and characterize a new mannose-specific glycoside hydrolase with potent antibiofilm activity, which could serve as an effective agent in the development of biofilm-targeted antimicrobial strategies.

2. Materials and methods

2.1. Microbial strains and growth media

The following microbial strains were used in this study: *P. aeruginosa* PAO1 ATCC 15692; *A. baumannii* ATCC 19606; *K. pneumoniae* ATCC BAA 2146; *E. coli* ATCC 25922; *S. epidermidis* ATCC 13076; *C. albicans* SC 5314.

The following growth media were used in this study: Luria Bertani medium (LB) (1% tryptone (Biolife Italiana, Milan, Italy); 0.5% yeast extract (Biolife Italiana); 1% NaCl (HiMedia Laboratories); LB-NaCl (1% tripton (Biolife Italiana); 0.5% yeast extract (Biolife Italiana)); Brain heart infusion broth (BHI) (HiMedia Laboratories, Mumbai, India); TYS10 (1% tripton (Biolife Italiana); 0.5% yeast extract (Biolife Italiana); 1.5% agar technical (Biolife Italiana); 10% (W/V) filtered sucrose (Fisher Scientific, Geel, Belgium) added after autoclaving); YPD (1% yeast extract; 2% peptone (Torlak, Belgrade, Serbia); 2% glucose (CARLO ERBA Reagents srl, Cornaredo, MI, USA); MSA (1% pepton (Torlak); 0.1% beef extract (OXOID, Hampshire, England); 7.5% NaCl; 1% manitol (Biolife Italiana); 1.2% agar technical; 0.0025% phenol red (SIGMA-ALDRICH Co.); Modified artificial urine (AU) (0.17% Na₂SO₄ (Thermo Fisher Scientific Inc., Waltham, MA, USA); 0.072% Na₃C₆H₅O₇·2H₂O (CARLO ERBA Reagents srl); 0.0881% C₄H₇N₃O (SIGMA-ALDRICH Co., St. Luis, MO, USA); 1.5% H₂NCONH₂ (SIGMA-ALDRICH Co.); 0.2308% KCl (SIGMA-ALDRICH Co.); 0.1756% NaCl, 0.0185% CaCl₂ (SIGMA-ALDRICH Co.); 0.1266% NH₄Cl (Acros Organics, Geel, Belgium); 0.0035% K₂C₂O₄·H₂O (CARLO ERBA Reagents srl); 0.1082% MgSO₄·7H₂O (Acros Organics); 0.2912% NaH₂PO₄·2H₂O (SIGMA-ALDRICH Co.); 0.0831% Na₂HPO₄·2H₂O (SIGMA-ALDRICH Co.) dissolved in distilled water and mixed with LB-NaCl 1:1 before use); Nematode Growth Medium (NGM) (1.7% agar technical; 0.29% NaCl; 0.25% peptone (Torlak); 0.01% CaCl₂; 0.0005% cholesterol (SIGMA-ALDRICH Co.); 0.34% KH₂PO₄ (Fisher Chemicals, Waltham, MA, US) and 0.012% MgSO₄); M9 (6.4% Na₂HPO₄·7H₂O (Acros Organics); 1.5% KH₂PO₄, 0.25% NaCl, 0.5% NH₄Cl).

2.2. Bioinformatic analysis

The GH130-BG31 gene sequence was submitted to the GenBank under accession number PX515007. To assess sequence and structural homology, the corresponding protein sequence was analyzed using the UniProt database [20] and SWISS-MODEL [21], respectively. SignalP version 5.0 [22] was employed to predict subcellular localization and the presence of a signal peptide. Protein structure modeling was conducted with AlphaFold2 [23], and the resulting model was visualized in Discovery Studio Visualizer v24.1.0.23298 (Copyright © 2023, Dassault Systèmes Biovia Corp) [24]. Enzyme sequences from the GH130 family were retrieved from the CAZy database [25] and aligned with GH130-BG31 using Clustal Omega [26]. The alignment was subsequently visualized with Jalview [27].

2.3. Enzyme expression and purification

Recombinant expression of GH130-BG31 was performed using the Takara pET6xHN expression vector set (In-Fusion Ready) (Takara Bio USA, Inc., San Jose, CA, USA). The gene was amplified from *Pedobacter* sp. BG31 genomic DNA using Phusion™ High-Fidelity DNA Polymerase (New England Biolabs) and gene-specific primers designed with 15–18 nucleotide overlaps complementary to the vector sequence (Forward: 5'-TAAGGCCTCTGTCGACCAGGATAAAGTCCAGATTG-3'; Reverse: 5'-CAGAATTCGCAAGCTTTTGGAGAAGCAGGATTATAAA-3'). The PCR reaction (20 µL) included 1 U of Phusion polymerase, 1× Phusion HF buffer, 200 µM dNTPs, 1 µM of each primer, and 150–200 ng of genomic DNA. Amplification was carried out over 35 cycles, using an annealing temperature of 60 °C and a 1-minute extension time. The resulting PCR product (1 µL) was recombined into the pET6xHN-C In-Fusion Ready vector by mixing it with 1 µL of 5× In-Fusion HD Enzyme Premix, 1 µL of vector, and 7 µL of nuclease-free water. The reaction mixture was incubated at 50 °C for 15 min and used to transform ultracompetent *E. coli* DH5α cells (Thermo Fisher Scientific Inc.). Positive clones carrying the GH130-BG31 insert were verified by colony PCR using a T7 promoter forward primer and a gene-specific reverse primer. Plasmid DNA from confirmed clones was isolated and transformed into HI-Control *E. coli* BL21(DE3) cells (Lucigen, Middleton, WI, USA) for protein expression. The transformed cells were initially grown overnight in LB medium supplemented with 100 µg/mL ampicillin (LB/Amp100) at 37 °C, shaking at 180 rpm. The overnight culture was diluted 50× and grown under the same conditions until reaching an OD₆₀₀ of 0.4–0.6. Protein expression was induced by the addition of 1 mM IPTG, and cells were incubated for an additional 22 h at 25 °C, 180 rpm. GH130-BG31 was purified using a HisTALON™ Gravity Column Purification Kit (Takara Bio USA), and the bound protein was eluted using an imidazole-containing buffer (150 mM imidazole). The elution buffer was subsequently exchanged for 50 mM sodium phosphate buffer with 50 mM NaCl at pH 7.2. Protein fractions were analyzed by SDS-PAGE (12.5%), and visualized using Coomassie Brilliant Blue R-250 staining. The concentration of purified GH130-BG31 was determined using the Bradford protein assay [28].

2.4. Assessment of enzymatic activity

2.4.1. Preparation of crude extract of *C. albicans* mannan

Mannan crude extract was prepared according to modified protocol of Cuskin et al. [19]. *C. albicans* was grown in YPD medium at 30 °C overnight from 2% inoculum in 200 mL medium. Cell pellets were resuspended in distilled water and autoclaved two times at 121 °C, 15 psi, 15 min. Mannan was precipitated with 4 volumes of ice-cold ethanol overnight. Precipitate was pelleted by centrifugation at 5.000 g for 10 min, resuspended in water, dialyzed overnight against water and freeze-dried in a lyophilizer (Alpha 1-2 Ldplus, Martin Christ Gefrier-trocknungsanlagen GmbH, Osterode am Harz, Germany). Lyophilized material was stored at –20 °C.

2.4.2. Time-dependent activity of the enzyme

The enzyme activity assay was carried out at 37 °C and pH 7.0, with 20 mg/mL mannan crude extract or mannan from *Saccharomyces cerevisiae* (SIGMA-ALDRICH Co.) and 100 µg/mL enzyme. The release of reducing sugars was monitored every 15 min. Reactions were terminated by adding an equal volume of DNS reagent (1.6% NaOH (SIGMA-ALDRICH Co.); 1% 3, 5-dinitrosalicylic acid (SIGMA-ALDRICH Co.); 30% KNa-tartrate (SIGMA-ALDRICH Co.)) and the vials were placed in a water bath set at 96 °C for 7 min. The absorbance was measured at 540 nm using the Tecan Infinite200.

2.4.3. Temperature and pH-dependent activity of the enzyme

The optimum temperature and pH for enzyme activity were determined using mannan crude extract (20 mg/mL). The temperature

conditions varied between 6 and 80 °C at neutral pH and the pH conditions varied between 4.0 and 11.0 at 37 °C using different buffers, including citrate-sodium buffer (100 mM; pH 4.0, 5.0), sodium phosphate buffer (20 mM; pH 6.0, 7.0) and Tris-HCl buffer (20 mM; pH 8.0, 9.0, 10.0, 11.0). The temperature and pH stability of the purified enzyme was determined by measuring the enzymatic activity after incubation for 2 h at temperatures between 6 and 80 °C and pH values ranging between 4.0 and 11.0, followed by enzymatic reactions carried out at 37 °C and pH 7.0. Reactions were terminated after 30 min with the DNS reagent and, after boiling for 7 min, absorbance was measured at 540 nm using the Tecan Infinite200 plate reader. The enzymatic activities were expressed in relation to the highest activities measured.

2.4.4. High-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD)

The enzymatic reaction was carried out as a function of time and substrate concentration. The GH130-BG31 (10 µg/mL) was incubated with crude mannan extract (20 mg/mL) at 37 °C, to assess the time dependence of enzymatic activity. The reaction was terminated at intervals of 15 min, by boiling for 15 min. To assess the dependence on substrate concentration GH130-BG31 (10 µg/mL) was incubated at 37 °C for 15 min with serially diluted substrate (20 mg/mL, 10 mg/mL, 5 mg/mL, 2.5 mg/mL, 1.25 mg/mL, 0.6 mg/mL, 0.3 mg/mL, 0.15 mg/mL). The experiments were performed in triplicate. Mannose release was analyzed using HPAEC-PAD. Chromatographic measurements were performed on a Dionex ICS-3000 DP system (Dionex, Sunnyvale, CA, USA) equipped with a quaternary gradient pump, an electrochemical detector (Au working electrode and Ag/AgCl reference electrode), an autosampler (AS-DV), and Chromeleon 6.7 Chromatography Management Software. Separations were carried out on a CarboPac PA10 column (4 × 250 mm) with a corresponding guard column (4 × 50 mm) (Dionex), maintained at 30 °C. The mobile phase consisted of (A) ultrapure water (conductivity 0.055 µS/cm) and (B) 300 mmol/L NaOH. The gradient program was applied at a constant flow rate of 1.0 mL/min as follows: 0.0–8.0 min, 10–15% B; 8.0–12.0 min, 15% B; 12.0–20.0 min, 15–20% B. After each run, the system was returned to initial conditions and equilibrated prior to the next injection. External calibration was performed using mannose standards prepared by serial dilution of a 1 mg/mL stock solution in ultrapure water (conductivity 0.055 µS/cm). Mannose (≥99.9% purity) (SIGMA-ALDRICH Co.). All calibration standards were injected in duplicate. The coefficient of determination (R²) for the calibration curve was 0.9987.

2.5. Enzyme effect on biofilm formation

2.5.1. Quantification of biofilm biomass

Inhibition of biofilm formation: Bacterial cultures grown overnight in LB-NaCl (*P. aeruginosa*, PAO1 ΔpelA, *E. coli*, *S. epidermidis* and *K. pneumoniae*) or BHI (*A. baumannii*) at 37 °C and 180 rpm were diluted to 5 × 10⁷ CFU/mL in the corresponding medium. Aliquots of 100 µL with or without treatment were added to wells of a 96-well microtiter plate and incubated at 37 °C for 24 h. Treatment involved GH130-BG31 at concentrations of 1.25, 2.5, 5, 10, 50 and 100 µg/mL. As a negative control to confirm that the observed effect was due to the enzyme's catalytic activity, the enzyme was heat-inactivated by boiling at 100 °C for 10 min, and the sample was visually inspected for precipitation. Afterward, the biofilms were washed and stained with 0.1% crystal violet (CV) (SERVA Electrophoresis GmbH) (w/v). Biofilm biomass was quantified after dissolution of CV in 30% acetic acid (Fisher Chemicals) (v/v) and measuring the absorbance at 570 nm using the Tecan Infinite200 [29]. The amount of biofilm was calculated in relation to the untreated control. Assays were performed in hexaplicate and repeated three times.

Decomposition of preformed biofilms: Biofilms were first formed as described above, without treatment, by incubation at 37 °C for 24 h. Following removal of planktonic cells and two washes with phosphate-

buffered saline (PBS) (0.8% NaCl; 0.02% KCl; 0.024% KH_2PO_4 ; 0.287% $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$), the biofilms were exposed to GH130-BG31 at concentrations of 10, 50, 100, and 200 $\mu\text{g/mL}$ for an additional 24 h. After treatment, biofilms were washed, stained with 0.1% (w/v) crystal violet, and the dye was dissolved in 30% (v/v) acetic acid. Biofilm biomass was determined by measuring absorbance at 570 nm using the Tecan Infinite200 reader and expressed as a percentage relative to untreated controls. Each assay was conducted in hexaplicate and independently repeated three times.

2.5.2. Determination of biofilm metabolic activity

Inhibition of bacterial adhesion: To measure the metabolic activity of adhered cells, *P. aeruginosa*, PAO1 ΔpelA and *A. baumannii* were incubated for 3 h in 96-well plates. Bacterial cultures grown overnight in LB-NaCl (*P. aeruginosa*, PAO1 ΔpelA) or BHI (*A. baumannii*) at 37 °C and 180 rpm were diluted to 5×10^7 CFU/mL in the corresponding medium. Aliquots of 100 μL with GH130-BG31 (10 and 100 $\mu\text{g/mL}$) or without treatment were added to wells of a 96-well microtiter plate and incubated at 37 °C for 3 h. Non-adherent cells were then removed and adherent cells were washed two times with PBS. Then the medium containing resazurin (0.16 mM) (SIGMA-ALDRICH Co.), was added to the wells. Fluorescence (λ_{ex} : 570 nm and λ_{em} : 615 nm) was measured after 1 h of incubation for *A. baumannii* and 4 h of incubation for *P. aeruginosa* and PAO1 ΔpelA (37 °C, dark) using the Tecan Infinite200. Negative control with spontaneous transformation of resazurin to resorufin was considered as blank and was subtracted from the rest of the measurements. The relative effect on bacterial adhesion was determined in comparison to the control without treatment. Assays were performed in hexaplicate and repeated three times.

Decomposition of preformed biofilms: Biofilms of *E. coli*, *K. pneumoniae* and *S. epidermidis* were established as described above (Section 2.5.1), without treatment, by incubation at 37 °C for 24 h. Following removal of planktonic cells and two washes with PBS, the biofilms were exposed to GH130-BG31 at concentrations of 10, 50, 100, and 200 $\mu\text{g/mL}$ for an additional 24 h. After treatment, biofilms were washed and the medium containing resazurin (0.16 mM) (SIGMA-ALDRICH Co.) was added to the wells. Fluorescence (λ_{ex} : 570 nm and λ_{em} : 615 nm) was measured after 30 min (37 °C, dark) using the Tecan Infinite200. Negative control with spontaneous transformation of resazurin to resorufin was considered as blank and was subtracted from the rest of the measurements. The relative effect on biofilms was determined in comparison to the control without treatment. Assays were performed in hexaplicate and repeated three times.

2.5.3. Bacterial adhesion analysis by CFU counts

P. aeruginosa PAO1 ΔpelA and *A. baumannii* were grown overnight in LB-NaCl (*P. aeruginosa*, PAO1 ΔpelA) or BHI (*A. baumannii*) at 37 °C and 180 rpm, then diluted to 5×10^7 CFU/mL in the corresponding medium. Aliquots of 100 μL with GH130-BG31 (10 or 100 $\mu\text{g/mL}$) or without treatment were added to wells of a 96-well microtiter plate and incubated at 37 °C for 3 h. Non-adherent cells were then removed and adherent cells were washed twice with PBS and sonicated in a sonic water bath (DK Sonic, West Yorkshire, United Kingdom) in 200 μL PBS with 10 s sonication and 10 s shaking intervals, repeated 5 times. Biofilm colonies were counted in serial dilutions after growth at 37 °C on LB agar plates. The number of CFU was converted to log reduction (log CFU/mL). Assays were performed in triplicate and repeated three times.

2.5.4. Visualization of PG-BG31 antibiofilm effect by fluorescent microscopy

Inhibition of biofilm formation: To observe biofilms under a fluorescence microscope, overnight cultures of *E. coli*, *K. pneumoniae* and *S. epidermidis* were adjusted to 5×10^7 CFU/mL in LB-NaCl and dispensed into tilted 6-well plates (Sartstedt AG & Co., Nümbrecht, Germany) at 1 mL per well, with or without GH130-BG31 (100 $\mu\text{g/mL}$). Biofilms were cultivated either as single-species or mixed-species

communities, in ratios of 1:1:1 for three-species combinations or 1:1 for two-species mixtures. After 24 h of incubation at 37 °C, biofilms were gently washed and stained with 2.5 μM Syto®9 (green fluorescent dye) and 2.5 μM propidium iodide (PI, red fluorescent dye) from the LIVE/DEAD® BacLight™ Bacterial Viability Kit (Thermo Fisher Scientific Inc.). Syto®9 penetrates the membranes of both viable and non-viable cells, while PI selectively penetrates cells with damaged membranes, indicating non-viable cells [30]. Visualization was performed using a fluorescence microscope (Olympus BX51, Applied Imaging Corp., San Jose, CA, USA) at 10 $\times$ magnification. All assays were carried out in triplicate and repeated three times.

Decomposition of preformed biofilms: Biofilms were first allowed to form under the same conditions described above, without treatment, by incubating at 37 °C for 24 h. After removing planktonic cells and washing twice with PBS, the established biofilms were exposed to GH130-BG31 (100 $\mu\text{g/mL}$) for another 24 h. Subsequently, the biofilms were washed and stained as previously described. Imaging was carried out using an Olympus BX51 fluorescence microscope (Applied Imaging Corp., San Jose, CA, USA) at 10 $\times$ magnification. Each assay was conducted in triplicate and repeated three times.

2.5.5. Catheter infection model

Inhibition of biofilm formation: For the evaluation of biofilms on the catheter surface, an overnight bacterial culture of *K. pneumoniae* was diluted to 5×10^7 CFU/mL in AU and 200 μL aliquots with or without enzyme (100 $\mu\text{g/mL}$) and with or without antibiotic were added to 2-mL Eppendorf tubes (SARSTEDT AG & Co. KG). The silicone catheter (Coloplast, Humlebaek, Denmark) was cut into 1 cm segments, which were partially immersed in the bacterial culture and incubated at 37 °C for 24 h. Afterward, the bacterial counting method was used to evaluate the effect of GH130-BG31 on biofilm formation. Catheter pieces were washed in PBS and sonicated twice in a sonic water bath in 1 mL PBS at 2-minute intervals, with vortexing in between. Biofilm colonies were counted in serial dilutions after growth at 37 °C on LB agar plates [31]. The number of CFU was converted to the log reduction (log CFU/biofilm) and the percentage reduction compared to the untreated control. Assays were performed in triplicate and repeated 3 times.

Decomposition of preformed biofilms: Biofilms were first formed on catheter segments as described above, by incubation without treatment at 37 °C for 24 h. Following the removal of nonadherent cells and two PBS washes, preformed biofilms were treated with GH130-BG31 (100 $\mu\text{g/mL}$) in combination with an antibiotic for an additional 24 h. The viable cell count method was then used to determine the effect of GH130-BG31 on biofilm degradation. Colony counts were expressed as log CFU per biofilm, and the reduction in viable biofilm cells was calculated as both log and percentage decreases relative to the untreated control. All assays were conducted in triplicate and repeated three times.

2.6. Toxicity evaluation

2.6.1. Cytotoxicity

Human MRC5 lung fibroblasts obtained from the American Type Culture Collection (ATCC) were cultured as monolayers in RPMI-1640 medium supplemented with HEPES, Antibiotic-Antimycotic solution, and 10% (v/v) fetal bovine serum (FBS) (all from Thermo Fisher Scientific Inc.). The cells were maintained at 37 °C in a humidified incubator containing 5% CO_2 and 95% air. Cytotoxicity of GH130-BG31 at concentrations of 10, 50, 100, and 200 $\mu\text{g/mL}$ was evaluated using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay after 48 h of exposure. Following incubation, the MTT reagent was removed, and 50 μL of DMSO (Fisher Chemicals) was added to each well. Cell viability was determined by measuring absorbance at 540 nm and correcting for background absorbance at 690 nm using a Tecan Infinite200 reader. Results were expressed as a percentage relative to untreated control cells [32]. All experiments were conducted in quadruplicate and repeated three times.

2.6.2. Hemolysis

The hemolytic activity of GH130-BG31 was evaluated using red blood cells (RBCs) to assess its biocompatibility. Fresh defibrinated sheep blood (Torlak, Belgrade, Serbia) was centrifuged at 800 ×g for 5 min to separate erythrocytes. The RBC pellet was washed three times with sterile PBS and resuspended to obtain a 1% erythrocyte suspension. Aliquots of 100 µL were incubated with or without treatment at 37 °C for 24 h. GH130-BG31 was tested at concentrations of 10, 50, 100 and 200 µg/mL, while 0.1% Triton X-100 was used as a positive control (100% hemolysis). After incubation, samples were centrifuged at 1000 ×g for 5 min, and the supernatant was collected for analysis. Hemoglobin release was quantified by measuring absorbance at 540 nm using a microplate reader. The experiment was performed in quadruplicate and repeated three times.

2.6.3. *Caenorhabditis elegans* toxicity test

The *C. elegans* AU37 strain, obtained from the Caenorhabditis Genetics Center (CGC, University of Minnesota, Minneapolis, USA), was maintained on NGM agar plates and fed with *E. coli* OP50 at 15 °C. Population synchronization was achieved using hypochlorite bleach and NaOH. The impact of GH130-BG31 at concentrations of 10, 100, and 500 µg/mL on *C. elegans* survival was assessed following a previously described protocol [33]. Experiments were conducted in 96-well microtiter plates, each containing 25–40 L4-stage worms in 100 µL of M9/LB medium (95:5 v/v) supplemented with 5 µg/mL cholesterol. Worm viability was recorded at the start of the experiment and after 24 and 48 h using a stereomicroscope (SMZ143-N2GG, Motic, Germany). Enzyme toxicity was determined by comparing worm survival rates to a control group not exposed to the enzyme under identical conditions. All assays were carried out in quadruplicate and repeated three times.

2.7. Statistical analysis

The statistical analysis of enzyme activity was conducted using the Student's *t*-test and ANOVA in SPSS version 20 software (IBM, Armonk, NY, USA), with a significance level set at $P < 0.05$. Apparent Michaelis–Menten parameters, K_m ,app and V_{max} ,app were estimated by fitting the data to the Michaelis–Menten equation: $v = (V_{max} \times [S]) / (K_m + [S])$. Nonlinear regression analysis was performed in R (v.4.3.2) using the nls() function. Concentrations inhibiting 50% of biofilms (BIC50 values) were calculated by fitting a four-parameter log-logistic model using the drc package in R (v.4.3.2).

3. Results and discussion

3.1. Identification and bioinformatic analysis of GH130-BG31

In the search for novel enzymes capable of targeting mannose-containing polysaccharides, we conducted genome mining of the *Pedobacter* sp. BG31 strain, part of the bacterial collection isolated from bream gills [13]. This analysis led to the identification of a gene encoding a 375-amino-acid protein classified within glycoside hydrolase family 130 (GH130). The analysis indicated that GH130-BG31 is not located within a predicted operon and does not appear to be part of a recognizable polysaccharide utilization locus (Supplementary Fig. 1). GH130 includes enzymes primarily involved in the hydrolysis or phosphorylation of β -1,2- and β -1,4-mannosidic linkages, commonly found in short-chain mannanoligosaccharides. Members of this family function as β -mannoside phosphorylases, β -1,2-mannosidases, or glycosyl-transferases, and play an important role in mannan degradation and oligosaccharide metabolism. GH130 enzymes are predominantly found in bacteria, especially those adapted to degrade complex carbohydrates from fungal or plant sources [19].

Signal peptide prediction using SignalP indicated the presence of a 23-amino-acid N-terminal signal sequence, suggesting the enzyme is secreted extracellularly. According to the UniProt database, the closest

homolog is a GH130 protein from *Haoranjania flava* (UniProt ID: A0AAE3ILLO), sharing 75.6% sequence identity. Structural modeling using Swiss-Model showed the highest similarity to BT3780 (PDB ID: 5A7V), with a sequence identity of 62.39%. The reliability of the generated homology model was supported by a Global Model Quality Estimation (GMQE) score of 0.84, indicating a high level of confidence in the structural prediction. Protein BT3780 is a β -1,2-mannosidase enzyme encoded by *Bacteroides thetaiotaomicron* [19]. BT3780 cleaves β -1,2-mannose units, releasing mannose rather than mannose-1-phosphate, distinguishing it from glycoside phosphorylases. The enzyme plays a role in the depolymerization of yeast mannan polysaccharides by targeting the β -1,2-mannose caps on the side chains, particularly evident in *Candida albicans* α -mannan, but not in *Saccharomyces cerevisiae*, which lacks these β -1,2 linkages.

To gain insight into the putative catalytic mechanism of GH130-BG31, we performed a multiple sequence alignment with representative members of the GH130 family. The enzyme showed a higher degree of similarity to sequences annotated with glycoside hydrolase activity (EC 3.2.1.197) than to those with phosphorylase activity (EC 2.4.1.281, EC 2.4.1.319, EC 2.4.1.320, EC 2.4.1.339, and EC 2.4.1.340). Furthermore, GH130-BG31 contains two motifs (Trp²²², Gly²²³, Glu²²⁴ and Thr²⁶⁴, Glu²⁶⁵, Cys²⁶⁶) proposed to be a catalytic base of β -mannosidases and Lys¹⁹⁶ as a proposed catalytic residue (highlighted in purple and red, respectively, in Fig. 1A) [19]. GH130-BG31 lacks four out of six residues necessary for Pi interaction (highlighted in blue in Fig. 1A), supporting its classification as a glycoside hydrolase rather than a phosphorylase, within the GH130 family [34].

Using AlphaFold2, which predicts three-dimensional protein structures from amino acid sequences, we obtained a high-confidence structural model of the protein (Fig. 1B). The reliability of this model, as indicated by both the predicted local distance difference test (pLDDT) and the predicted aligned error (PAE) metrics, demonstrated strong local and global confidence in the predicted conformation (Supplementary Fig. 2). We further visualized the enzyme's active site using Discovery Studio Visualizer (Fig. 1B), marking the residues red and purple, corresponding to Fig. 1A.

3.2. Heterologous expression and characterization of GH130-BG31

His-tagged GH130-BG31 was overexpressed in *E. coli* and purified using standard metal affinity chromatography. This yielded 2 mg of protein per 1 g cell pellet, with a purity of approximately 98%, as determined by Coomassie Brilliant Blue R-250 staining (Supplementary Fig. 3). The catalytic activity of GH130-BG31 was examined using crude extract of *C. albicans* mannan as a substrate, measuring the release of reducing sugars with DNS reagent following heat treatment (Supplementary Fig. 4). The release of mannose was verified by thin-layer chromatography (TLC), both compared to other monosaccharides (Supplementary Fig. 5A), and time dependently (Supplementary Fig. 5B), which is consistent with the expected glycoside hydrolase activity. The product release was also analyzed by HPAEC-PAD (Fig. 2A). The enzyme showed time-dependent mannose release, reaching a plateau after approximately 60 min (Fig. 2B). Initial reaction rates were determined from the linear phase of product formation (15 min). Substrate-dependent activity followed a Michaelis–Menten-like trend, and apparent kinetic parameters were calculated as K_m ,app = 8.885 mg/mL and V_{max} ,app = 0.007 mg/mL/min (Fig. 2C). Due to the heterogeneous nature of the polysaccharide substrate, the values obtained represent apparent kinetic parameters (K_m ,app and V_{max} ,app) rather than true kinetic constants. These results provide insight into the enzyme's affinity and activity toward biologically relevant polysaccharide substrates. *C. albicans* mannan contains an acid-labile region rich in β -1,2 oligomannosides, which differentiates it from the mannan found in *S. cerevisiae* [35,36]. Therefore, we used *S. cerevisiae* mannan as a substrate to evaluate the enzyme's specificity. The enzyme showed no activity toward mannan derived from *S. cerevisiae*, indicating its specificity

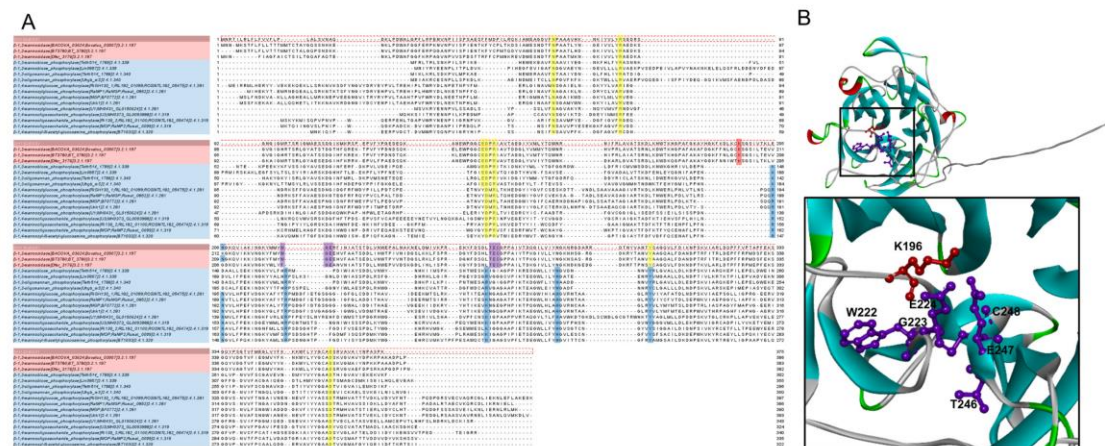


Fig 1. Structural analysis of GH130-BG31. Characterized members of the GH130 family are presented in the alignment (A). Enzymes classified as glycoside hydrolases are indicated in red, while phosphorylases are shown in blue. The sequence of GH130-BG31 is outlined with a red dashed line. Conserved residues involved in substrate binding are highlighted in yellow. Motifs containing residues predicted to form the catalytic base of β -mannosidases are marked in purple, and the lysine residue proposed as the catalytic residue for β -1,2-mannosidases is shown in red. Residues interacting with phosphate are depicted in blue. The catalytic site is visualized using Discovery Studio Visualizer in the model of GH130-BG31 created with AlphaFold2 (B). Highlighted residues correspond to the purple and red motifs from the alignment (A).

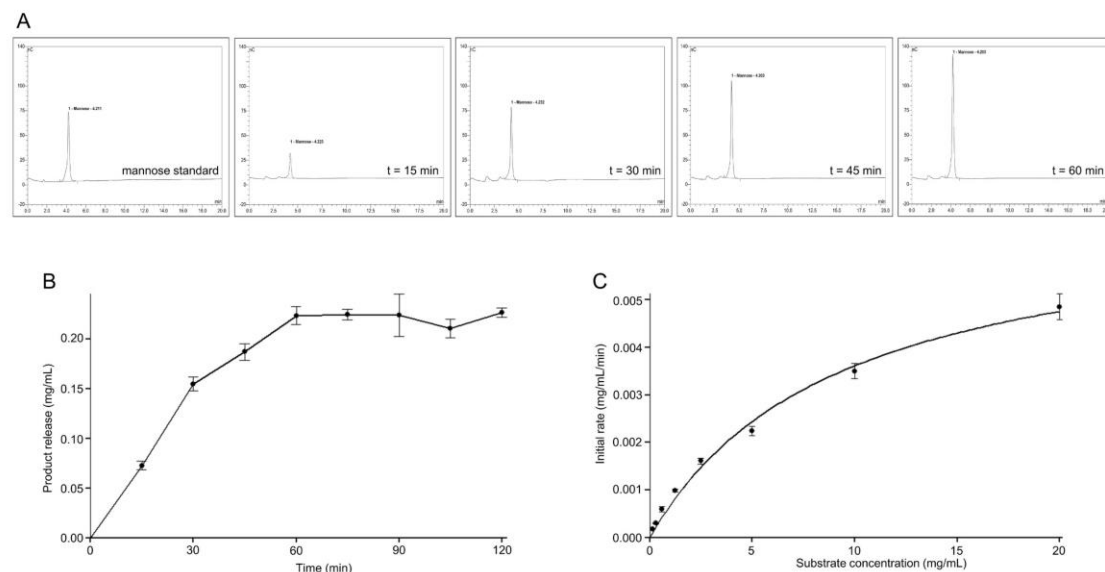


Fig. 2. HPAEC-PAD analysis of reactions catalyzed by GH130-BG31. The enzyme GH130-BG31 was incubated with a crude extract of *C. albicans* mannan, and the released products were analyzed at 15-minute intervals (A, B). Product formation was further evaluated across varying substrate concentrations (C). Values are presented as mean $\pm$ SD of triplicates ($n = 3$).

for β -1,2 mannosidic linkages (Supplementary Fig. 6).

The biochemical characterization of GH130-BG31 revealed that the enzyme exhibits optimal activity at moderate temperatures (25–50 °C) (Fig. 3A) and within near-neutral pH (5.0–7.0) ranges (Fig. 3B), consistent with conditions commonly encountered in biological and environmental systems. The enzyme retained full activity after 2 h of incubation at temperatures up to 37 °C (Fig. 3C), indicating satisfactory thermal stability for potential applications in physiological or mild

processing conditions. Moreover, its stability across a broad pH range (4–11) (Fig. 3D) suggests structural robustness and adaptability to diverse environmental conditions. Together, these properties indicate that GH130-BG31 is a stable and catalytically efficient enzyme under conditions relevant for both laboratory and applied biotechnological settings.

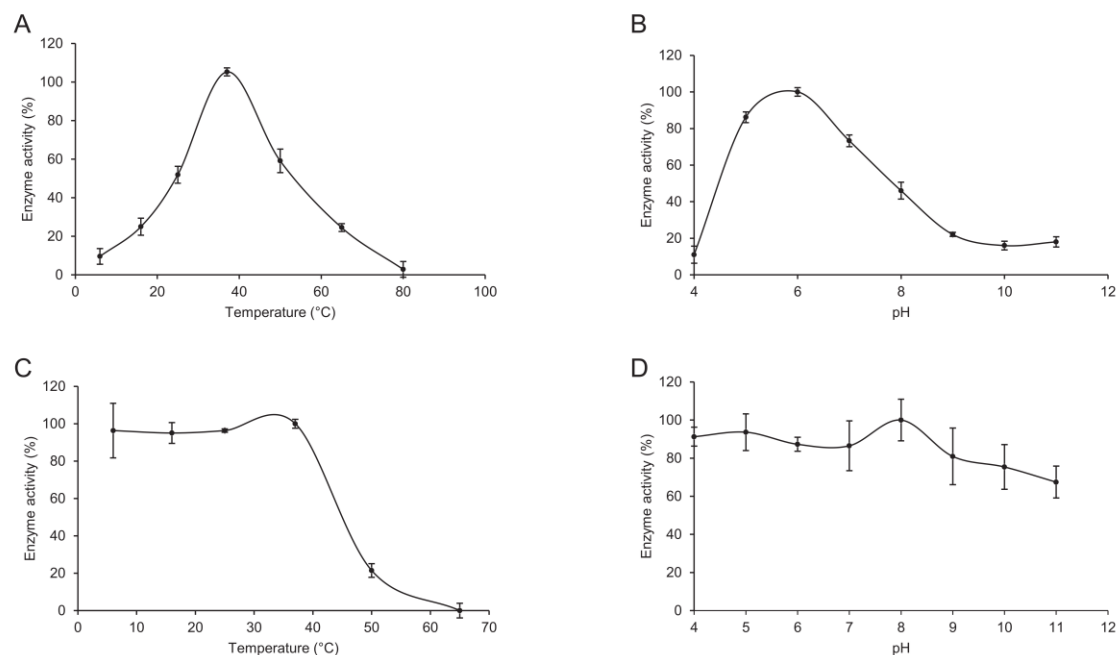


Fig. 3. Influence of temperature (A) and pH (B) on GH130-BG31 activity. Enzyme stability after 2 hours of pre-incubation at different temperatures (C) and pH (D). All reactions were performed with crude mannan extract from *C. albicans* as substrate, and the product was detected using a DNS reagent. Values are presented as mean $\pm$ SD of triplicates ($n = 3$).

3.3. Evaluation of antibiofilm activity

Based on the findings that the biofilm matrix of *P. aeruginosa*, *A. baumannii*, *K. pneumoniae*, *S. epidermidis* and *E. coli* has a high total mannose content, we selected these bacterial strains to evaluate the GH130-BG31 antibiofilm effect [8]. Glycosyl linkage analysis in the same study revealed a high presence of 2-linked mannopyranosyl residues, which can possibly be targeted by GH130-BG31. The crystal violet staining revealed that GH130-BG31 dose dependently prevents biofilm formation in *K. pneumoniae*, *E. coli* and *S. epidermidis* with $\sim 80\%$ inhibition at a concentration of $100 \mu\text{g/mL}$ for *K. pneumoniae* and $10 \mu\text{g/mL}$ for *E. coli* and *S. epidermidis* biofilms (Fig. 4). Calculated BIC_{50} values were 2.827 , 2.909 and $8720 \mu\text{g/mL}$ for *E. coli*, *S. epidermidis* and *K. pneumoniae*, respectively. We proved that this antibiofilm effect cannot be attributed to the enzyme's effect on bacterial growth (Supplementary Fig. 7), indicating a biofilm-specific mechanism. A heat-inactivated enzyme (HIE) control was employed to confirm that the observed antibiofilm effect resulted from the enzyme's catalytic activity rather than its mere presence. As anticipated, the HIE showed no impact on biofilm formation in any of the tested strains. We confirmed the inhibitory effect on *K. pneumoniae*, *E. coli* and *S. epidermidis* by CFU counts, both in single species and mixed species biofilms. The CFU counts revealed an approximately 2-log reduction in each case, which corresponds to 99% of the bacteria (Supplementary Fig. 8).

Numerous studies have confirmed the presence of mannose as a major constituent in the EPS fractions of *E. coli*, *S. epidermidis*, and *K. pneumoniae*, however, the specific polysaccharides containing these mannose residues and their precise glycosidic linkages remain unidentified [8,37–39]. The exopolysaccharide (EPS) composition can also vary considerably between bacterial strains, even within the same species, leading to structural and functional diversity in biofilm matrices. As a result, the exact mechanism through which GH130-BG31 exerts its

antibiofilm activity cannot yet be fully defined. Nonetheless, the enzyme's ability to inhibit biofilm formation in these species implies that their EPS may contain β -1,2-mannosidic linkages susceptible to cleavage. Importantly, enzymes with characterized substrate specificities, like GH130-BG31, can also serve as valuable tools for identifying glycosidic linkages in uncharacterized polysaccharide samples, thereby contributing both to functional biofilm disruption and structural elucidation of bacterial EPS [40].

The enzyme showed no effect on *P. aeruginosa* and *A. baumannii* biofilm formation (Fig. 4). We hypothesized that the absence of activity against *P. aeruginosa* biofilm was most likely a consequence of the complex structure of EPS within the *P. aeruginosa* biofilm matrix. To test this hypothesis, we constructed a PAO1 ΔpelA mutant, which lacks expression of the Pel polysaccharide (Supplementary Fig. 9). We expected that the absence of Pel and the dominant presence of Psl would increase the enzyme's antibiofilm efficacy compared to the treatment of the wild-type strain. Even though *P. aeruginosa* is one of the best-characterized bacterial pathogens with respect to exopolysaccharide composition, the reported linkage patterns do not fully match the expected proportions based on the currently proposed structure [9]. Specifically, the analysis reported by Bales and colleagues suggests that additional or less-characterized linkages, potentially including other 1,2-mannosidic linkages, may be present within the polymer [8]. However, no improved effect of GH130-BG31 against the PAO1 ΔpelA was observed (Fig. 4). Since it is evident that *P. aeruginosa* and *A. baumannii* contain mannose in their biofilms, we further evaluated whether the enzyme affects bacterial adhesion in the initial 3 h of incubation. Using resazurin staining, the enzyme reduced bacterial adhesion in both strains, by approximately 30% in *P. aeruginosa* strains and approximately 50% in *A. baumannii* at $100 \mu\text{g/mL}$ (Fig. 5). Consistent with these findings, quantification of adhered cells by colony counting also demonstrated a statistically significant reduction in

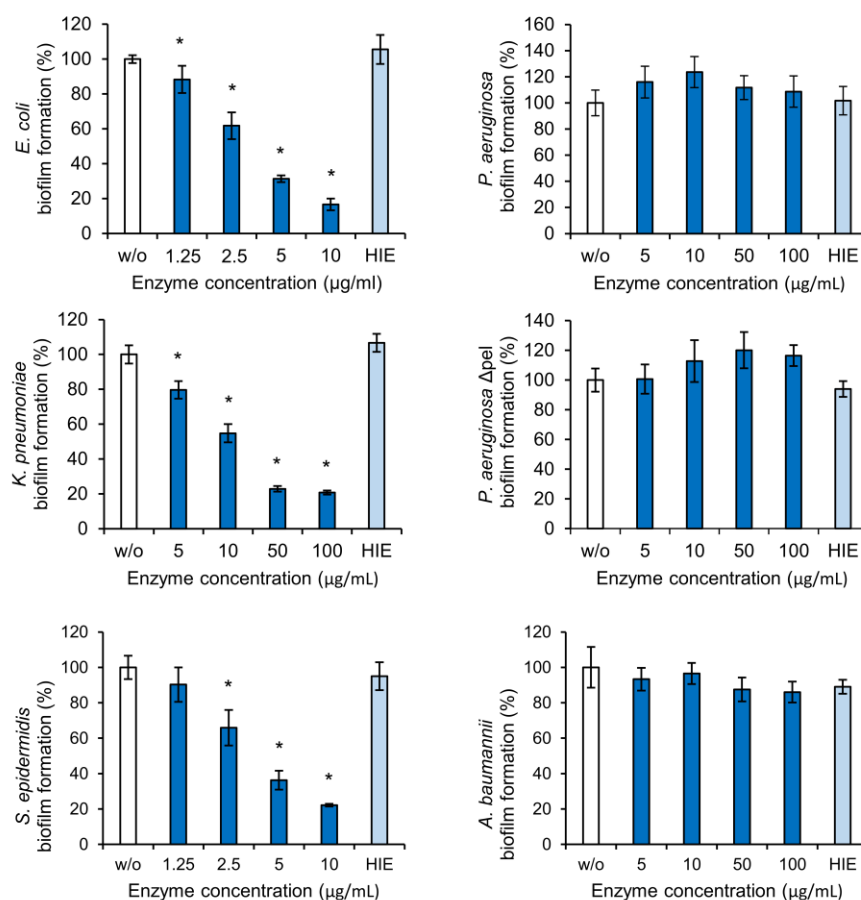


Fig. 4. Effect of GH130-BG31 on *E. coli*, *K. pneumoniae*, *S. epidermidis*, *P. aeruginosa*, PAO1 $\Delta pelA$ and *A. baumannii* biofilm formation. Dose-dependent inhibition of biofilm formation was quantified with crystal violet relative to the untreated control (w/o). The effect of heat-inactivated enzyme (HIE) was included as an additional negative control. Values are expressed as mean $\pm$ SD of hexaplicates ($n = 6$). * $p < 0.05$.

adhesion upon treatment, however, the decrease was less than 1 log in both species (Fig. 5). This suggests that mannose-containing exopolysaccharides play an important role during the early phase of biofilm formation, while other components of the biofilm matrix become more dominant during biofilm growth and maturation.

The lack of significant inhibition of *P. aeruginosa* biofilm formation by GH130-BG31 can likely be attributed to the structural characteristics of the exopolysaccharide Psl. The mannose residues in Psl are predominantly linked through β -1,3 glycosidic bonds within the linear backbone, while the side branches are connected by α -1,2 linkages [41]. Since GH130-BG31 functions as a β -1,2-mannosidase, its substrate specificity limits its ability to efficiently cleave β -1,3 linkages present in Psl. Consequently, the enzyme may only partially interfere with early adhesion processes, where minor structural accessibility or alternative glycosidic linkages could be transiently exposed, explaining the modest reduction in adhesion observed within the first 3 h. Once the biofilm matrix becomes more established and densely packed, the enzyme's activity is likely hindered due to both steric constraints and the lack of appropriate glycosidic targets, resulting in minimal impact on mature biofilm formation.

The limited inhibitory effect of GH130-BG31 on *A. baumannii* biofilm

formation, despite a moderate ($\sim 50\%$) reduction in initial adhesion, can be explained by the distinct and poorly characterized composition of *A. baumannii* exopolysaccharides (EPS). Previous studies have associated *A. baumannii* biofilm development with a cell-associated poly- β -1,6-linked GlcNAc polymer [42]. However, this observation does not correlate with the compositional analysis by Bales et al. [8] showing only $\sim 1\%$ GlcNAc and 80–90% mannose in both tested strains. This discrepancy suggests the presence of a previously uncharacterized exopolysaccharide, and the limited activity of GH130-BG31 may therefore be due to the absence of suitable β -1,2-mannosidic linkages or strain-specific variations in the structural organization of the EPS.

To evaluate the effect of GH130-BG31 on preformed biofilms, we used *K. pneumoniae*, *E. coli* and *S. epidermidis*, monitoring the reduction both in biomass by crystal violet and metabolic activity by resazurin. The enzyme caused approximately 40% reduction in biomass and about 70% reduction in metabolic activity in *K. pneumoniae* at a concentration of 50 $\mu\text{g/mL}$, and a similar effect in *S. epidermidis* at 100 $\mu\text{g/mL}$ (Fig. 6). Only minor was observed on the preformed biofilm of *E. coli*, which is consistent with its comparatively low mannose content ($\sim 10\%$) relative to *K. pneumoniae* and *S. epidermidis* (40–50%) [8].

The efficacy of GH130-BG31 was visualized using fluorescence

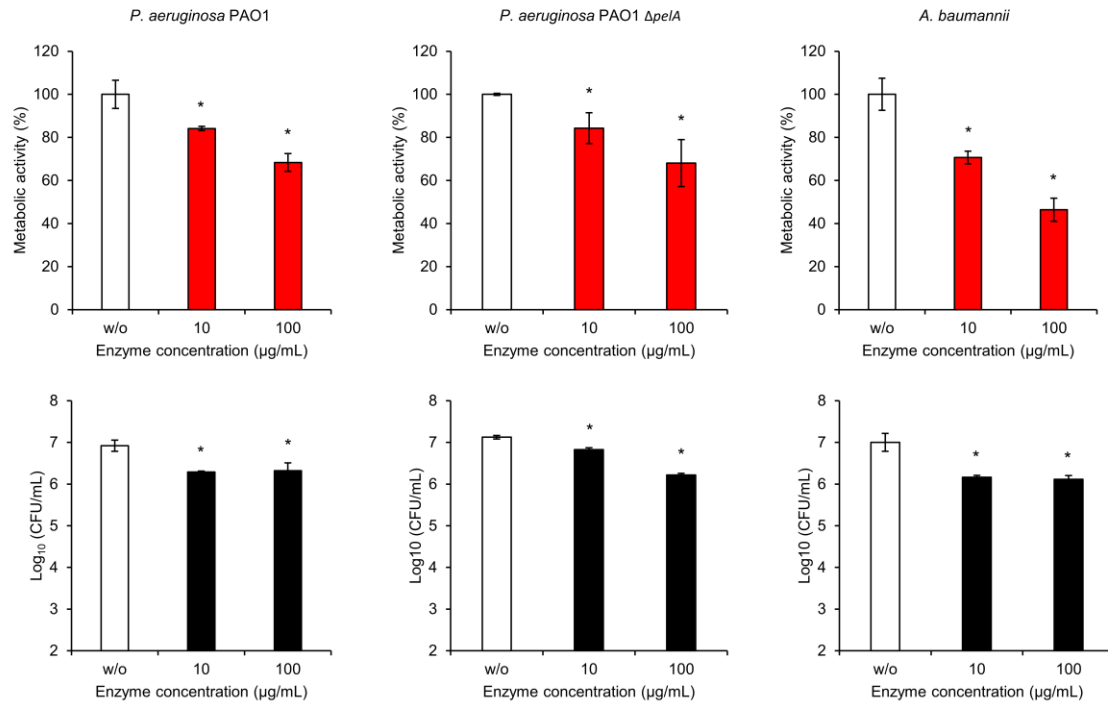


Fig. 5. Effect of GH130-BG31 (100 µg/mL) during the adhesion phase (3 h). Metabolic activity (red) of adhered cells was quantified by resazurin staining, relative to the untreated control (w/o). Values are expressed as mean $\pm$ SD of hexaplicates (n = 6). Direct viable cell counts (black) were determined by plating serial dilutions after sonication. Logarithmic values of CFU counts are presented as mean $\pm$ SD of quadruplicates (n = 4). * p < 0.05.

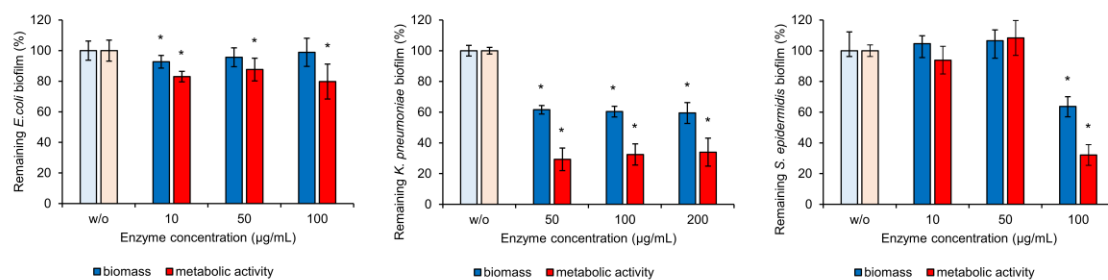


Fig. 6. Effect of GH130-BG31 on 24 hour-old biofilms. Biofilm biomass and metabolic activity were quantified after 24 hours of treatment of preformed biofilms, using CV and resazurin staining, respectively. Values are presented relative to the untreated control (w/o) as mean $\pm$ SD of six replicates (n = 6). * p < 0.05.

microscopy in combination with the Live/Dead® BacLight Kit. Incubation with GH130-BG31 resulted in a significant reduction in biofilm formation in both single-species and mixed-species biofilms (Fig. 7A). As for biofilm decomposition, an inhibitory effect was visible in *K. pneumoniae* and *S. epidermidis*, while there was no effect in *E. coli*, which was also reflected in mixed-species biofilms that contained *E. coli*. Quantitative analysis of fluorescence micrographs using ImageJ showed a marked reduction in biofilm biomass after enzyme treatment under all tested conditions (Supplementary Table 3). In mono-species biofilms, treatment led to substantial decrease in mean fluorescence intensity, integrated density, and biofilm surface coverage, with reductions of approximately 84% to 92%. Among the strains tested, *E. coli* was the most susceptible, while *K. pneumoniae* exhibited slightly lower, though still significant, reductions. In mixed-species biofilms, the inhibitory

effect remained significant but was consistently lower than in mono-species conditions, with reductions of approximately 70% to 82% (Fig. 7B). The triple-species biofilm demonstrated the lowest level of inhibition across all measured parameters. All three quantitative metrics displayed consistent trends, indicating that the observed effect represents a true reduction in biofilm biomass and coverage rather than changes in fluorescence intensity alone. These findings suggest that increased biofilm complexity and interspecies interactions may partially mitigate the enzyme's antibiofilm activity. Quantitative analysis of mature biofilms degradation revealed reduced enzyme efficacy compared to its effect on biofilm formation (Supplementary Table 4). No reduction was observed in *E. coli* biofilms. Moderate biofilm degradation was observed in *K. pneumoniae* and *S. epidermidis*, with reductions of approximately 26–30%. In mixed-species biofilms, the effect was further

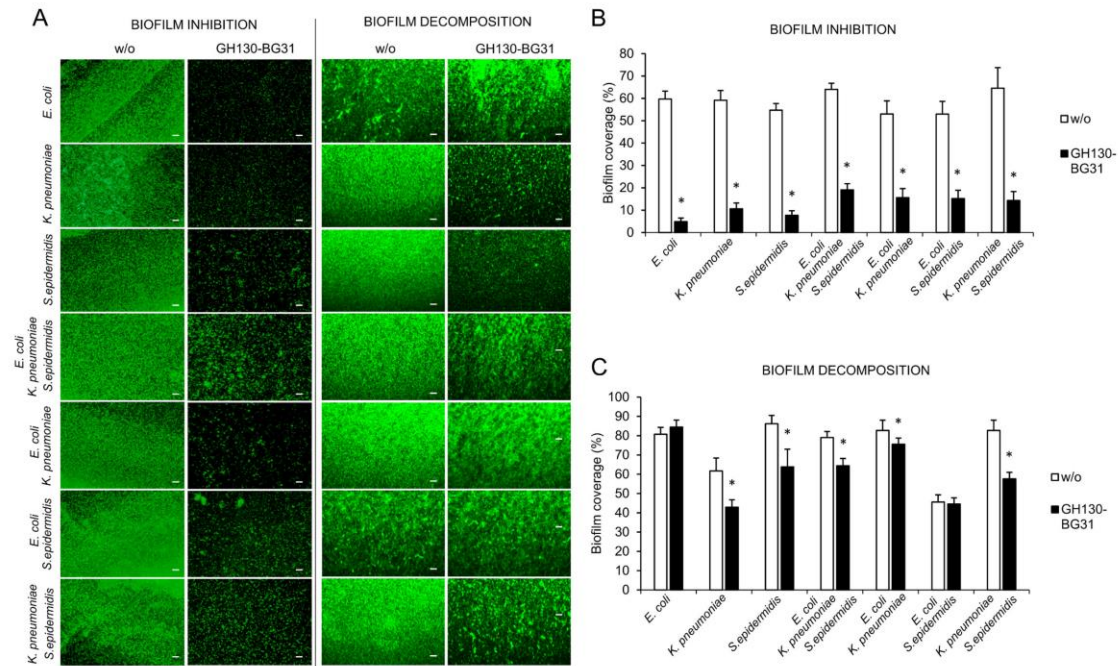


Fig. 7. Visualization of the anti-biofilm effect of GH130-BG31 by fluorescence microscopy. Biofilms were formed for 24 h on plastic coverslips with (100 μ g/mL GH130-BG31) or without treatment (w/o) to observe biofilm inhibition, or the treatment was introduced after 24 h of biofilm formation to observe biofilm decomposition. The effect was analyzed by fluorescence microscopy using a Live/Dead staining kit (A). Live cells were stained with Syto®9 (green) and dead cells with PI (red). Scale bars represent 10 μ m. Biofilm coverage (B, C) was calculated using ImageJ software. Values are expressed as mean $\pm$ SD of quintuplicates (n = 5). * $p < 0.05$.

diminished, ranging from negligible (~2%) to moderate (~30%) reductions, depending on species composition (Fig. 7C). Overall, these results indicate that the enzyme has limited activity against established biofilms, with efficacy influenced by both species and community complexity. The effects observed by fluorescence microscopy were consistent with the results obtained from crystal violet and resazurin assays. The biofilms predominantly appeared green, confirming that the enzyme does not exhibit a bactericidal effect at the tested concentrations.

3.4. Combined effect of GH-BG31 and antibiotics

Next, we evaluated the combined effect between the enzyme and selected antibiotics. To do this, we first selected a panel of ten antibiotics representing different classes and mechanisms of action to determine the minimum inhibitory concentrations (MICs) for the bacterial strains. According to EUCAST [43] guidelines, *E. coli* was sensitive to ciprofloxacin, tetracycline, and ampicillin, while *S. epidermidis* was sensitive to rifampicin, gentamicin, and ciprofloxacin. In contrast, *K. pneumoniae* was resistant to all antibiotics in the initial panel. Therefore, an additional set of ten antibiotics was tested, among which only colistin

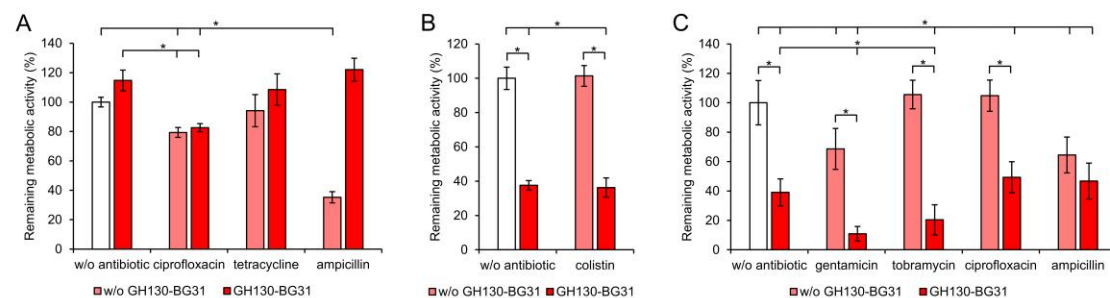


Fig. 8. Combined effect of GH130-BG31 and antibiotics on preformed biofilms of *E. coli* (A), *K. pneumoniae* (B), and *S. epidermidis* (C). The 24 hour-old biofilms were treated with the MIC of the selected antibiotics with or without GH130-BG31 (100 μ g/mL) for a further 24 h. Remaining metabolic activity was quantified by resazurin staining relative to the untreated control (w/o). Values are expressed as mean $\pm$ SD of six replicates (n = 6). * $p < 0.05$.

showed a relevant MIC value (0.25 µg/mL) according to EUCAST breakpoints (Supplementary Table 5). These antibiotics, which effectively killed bacteria, were subsequently used to assess the combined effect with the enzyme on preformed biofilms, as evaluated by resazurin staining (Fig. 8). Using a Bliss independence model, we determined a synergistic effect between the enzyme and either gentamicin or tobramycin in *S. epidermidis*, and between the enzyme and ciprofloxacin in *E. coli* biofilm decomposition (Supplementary Fig. 10). An antagonistic effect was observed for ciprofloxacin and for ampicillin in *S. epidermidis* and ampicillin in *E. coli* biofilm decomposition. An additive effect was observed for tetracycline in *E. coli* and colistin in *K. pneumoniae* biofilm decomposition.

E. coli and *K. pneumoniae* are the two most prevalent pathogens found in catheter-associated urinary tract infections (CAUTIs) [44,45]. *K. pneumoniae* infections are frequently observed in patients with other comorbidities, such as malignancies, chronic organ diseases, or immunodeficiency. Consequently, mortality rates are typically higher compared to *E. coli* infections, which occur more commonly in patients with fewer underlying conditions and are generally associated with lower fatality [46]. The clinical impact of *K. pneumoniae* is further amplified by its frequent multidrug resistance, particularly among extended-spectrum β -lactamase (ESBL)-producing and carbapenem-resistant isolates, which complicates treatment and contributes to increased morbidity and mortality [45]. The presence of catheters facilitates *K. pneumoniae* colonization and biofilm formation, further enhancing its persistence and resistance to antibiotics [47]. These factors underscore the importance of recognizing *K. pneumoniae* as a severe pathogen in catheter-associated and invasive urinary tract infections, particularly in populations with compromised host defenses or in healthcare environments with elevated resistance rates.

Therefore, we further evaluated the efficacy of GH-BG31 in a catheter model of *K. pneumoniae* infection. The efficacy of the enzyme was tested in combination with colistin to prevent biofilm formation and to disrupt preformed biofilms. The biofilm inhibition assays demonstrated that the enzyme alone (100 µg/mL) reduced *K. pneumoniae* biofilm formation from 8.42 log CFU (untreated control) to 5.37 log CFU, corresponding to an approximately 3-log (~99.6%) reduction (Fig. 9A). This inhibitory effect was also observed with scanning electron microscopy (SEM) (Supplementary Fig. 11). The enzyme enhanced the anti-biofilm effect of colistin at all tested concentrations (Fig. 9A).

In the biofilm decomposition assay, preformed *K. pneumoniae* biofilms were partially reduced by the enzyme alone, decreasing from 8.51 log CFU in the untreated control to 7.24 log CFU, demonstrating a modest biofilm-disrupting effect with approximately 1.3-log (~94.7%)

reduction (Fig. 9B and Supplementary Fig. 11). The combination of the enzyme with colistin consistently improved biofilm clearance, particularly at higher antibiotic concentrations (Fig. 9B). Although disruption of mature biofilms was less pronounced than the prevention of their formation, the overall results highlight the enzyme's ability to enhance colistin activity and reduce *K. pneumoniae* biofilm resilience, supporting its potential use as an adjuvant in biofilm-targeted therapy.

Only a few studies to date have attempted to separate and characterize the exopolysaccharides associated with *K. pneumoniae* biofilms. However, those that have done so consistently report a high mannose content, suggesting that mannose-rich polysaccharides are a key structural component of the *K. pneumoniae* biofilm matrix [8,38,39]. A more detailed and systematic characterization of exopolysaccharide composition would be of great significance, as our findings demonstrate that glycoside hydrolases, such as GH130-BG31, can potentiate antibiotic activity by disrupting the biofilm matrix and improving drug penetration. This is particularly relevant for *K. pneumoniae*, which is a major cause of HAI, including pneumonia, bloodstream infections, and device-associated infections. The species is frequently multidrug-resistant, often producing extended-spectrum β -lactamases (ESBLs) or carbapenemases, which severely limit treatment options [48]. Understanding the precise biochemical structure of its biofilm EPS could enable the development of targeted enzymatic strategies to weaken biofilm defenses and restore antibiotic susceptibility, offering a promising complementary approach to combat multidrug-resistant *K. pneumoniae* infections.

3.5. Assessment of enzyme toxicity

To assess the safety profile of GH130-BG31, its toxicity was evaluated using both in vitro and in vivo models. Cytotoxicity was measured using the MTT assay on human MRC5 fibroblasts, treated with increasing concentrations of GH130-BG31 for 24 h. No reduction in fibroblast metabolic activity was observed at any tested concentration (Fig. 10A), indicating that GH130-BG31 does not impair cell viability. Hemolytic activity was evaluated to determine its hemocompatibility. No hemolysis was detected at any tested concentration after 24 h of incubation, with absorbance values comparable to the negative control (Fig. 10B). In vivo toxicity was examined using the *C. elegans* AU37 liquid culture model. Nematodes were exposed to varying concentrations of GH130-BG31 for 48 h, and survival rates were recorded. GH130-BG31 did not affect nematode viability, even at concentrations as high as 500 µg/mL (Fig. 10C). The absence of cytotoxic effects in human cells and lack of toxicity in *C. elegans* support the biocompatibility of GH130-

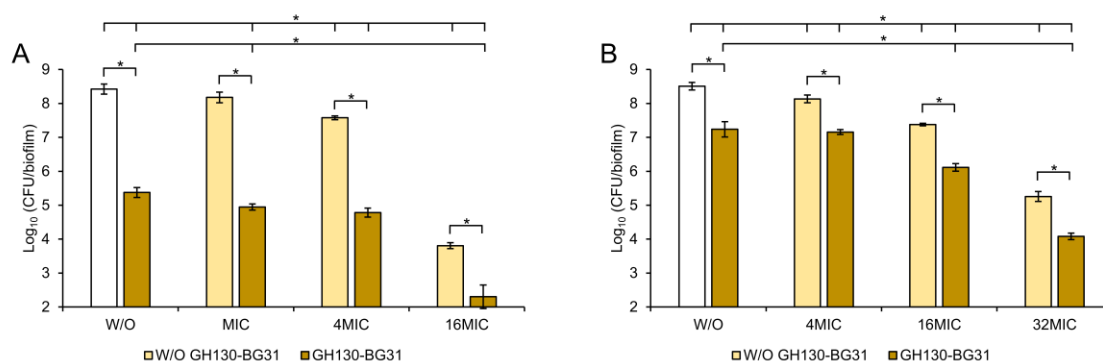


Fig. 9. Evaluation of anti-biofilm activity using a catheter biofilm model. In the biofilm inhibition experiment (A), biofilms were developed on silicone catheters either without treatment (w/o) or in the presence of GH130-BG31 (100 µg/mL) and varying concentrations of colistin (1 × MIC, 4 × MIC, or 16 × MIC). In the biofilm degradation experiment (B), preformed overnight biofilms were subsequently treated with GH130-BG31 (100 µg/mL) and colistin (4 × MIC, 16 × MIC, 32 × MIC). Biofilm biomass was determined by counting CFUs, and logarithmic values are shown as mean ± SD of triplicates (n = 3). * $p < 0.05$.

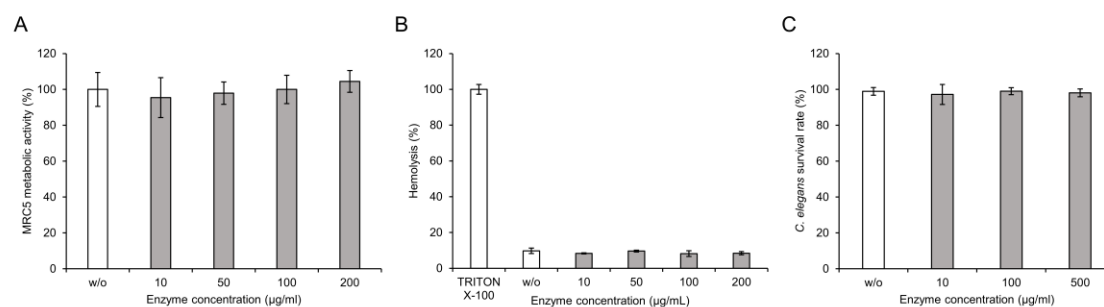


Fig. 10. Evaluation of the cytotoxicity of GH130-BG31 on MRC5 cells (A), hemolytic activity (B) and *in vivo* toxicity using the *Caenorhabditis elegans* model (C). The effect of the enzyme was determined in comparison with the untreated control (w/o). Values are presented as means $\pm$ SD of quadruplicates (n = 4).

BG31, highlighting its potential for safe application in the functionalization of materials intended for long-term medical use.

4. Conclusion

In this study, we demonstrated for the first time that a glycoside hydrolase belonging to the GH130 family exhibits pronounced anti-biofilm activity, both by preventing biofilm formation and by degrading established biofilms. The characterized enzyme, GH130-BG31, functions as a highly efficient β -1,2-mannosidase affecting bacterial adhesion of *P. aeruginosa* and *A. baumannii*, as well as preventing biofilm formation by *E. coli*, *K. pneumoniae*, and *S. epidermidis*. Moreover, GH130-BG31 effectively reduces the development of mixed-species biofilms composed of *E. coli*, *K. pneumoniae*, and *S. epidermidis*.

In addition to its effect on biofilm formation, GH130-BG31 demonstrates the ability to partially decompose preformed biofilms of *K. pneumoniae* and *S. epidermidis*, and to potentiate antibiotic efficacy. This synergistic effect was confirmed in a catheter infection model under urine-like conditions, where GH130-BG31 enhanced the activity of colistin against *K. pneumoniae* biofilms. The enzyme exhibited optimal activity at physiological temperature and neutral pH, with notable stability up to 37 °C and across a broad pH range. Importantly, GH130-BG31 showed no toxicity toward human cells or the *C. elegans* model, supporting its favorable safety profile and potential for therapeutic application.

Collectively, these findings identify GH130-BG31 as a promising candidate for the enzymatic control of biofilm-associated infections, particularly in combination with antibiotics, and highlight the therapeutic potential of GH130 family glycoside hydrolases in combating biofilm-related microbial persistence.

CCRediT authorship contribution statement

Marija Atanaskovic: Writing – original draft, Visualization, Methodology, Investigation, Data curation. **Mina Mandic:** Investigation. **Mihajlo V. Jakanovski:** Investigation, Methodology. **Linda De Vooght:** Methodology. **Paul Cos:** Supervision. **Lidija Senerovic:** Writing – review & editing, Visualization, Supervision, Methodology, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijbiomac.2026.152628>.

Data availability

Data will be made available on request.

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4. DISKUSIJA

4.1 Bakterijska kolekcija izolovana iz škrga ribe kao izvor novih glikozid-hidrolaza

Laboratorijska kolekcija, koju čini 75 bakterijskih izolata izolovanih sa škrga deverike (*Abramis brama*) (BG kolekcija), je testirana na glikozid-hidrolaznu aktivnost. Korišćene su minimalne hranljive podloge koje kao glavni izvor ugljenika imaju celulozu, ksilozu, škrob ili alginat i na osnovu veličine hidrolitičkih zona i fenotipske i filogenetske raznovrsnosti bakterijskih izolata izabrana su četiri kandidata čiji su genomi zatim sekvencirani. Ovi bakterijski sojevi su identifikovani kao *Aeromonas* sp. BG2, *Rhodococcus* sp. BG20, *Microbacterium* sp. BG28 (odeljak 3.1) i *Pedobacter* sp. BG31 (odeljak 3.2; odeljak 3.3).

Gram-negativne bakterije iz roda *Aeromonas* naseljavaju prirodne vodene sredine, uključujući reke i jezera, ali se često izoluju i iz sistema za distribuciju pijaće vode. Neke vrste su identifikovane kao oportunistički patogeni koji izazivaju infekcije kod ljudi, riba i drugih životinja, prvenstveno putem faktora virulencije kao što su aerolizinski toksini, lipopolisaharidi, ekstracelularni enzimi i sistemi sekrecije tipa III (Janda & Abbott, 2010). Vrste ovog roda pokazale su hidrolitičku aktivnost na različitim polisaharidima, uključujući alginat, celulozu, hitin, pektin i škrob (Hsu et al., 1985).

Rod *Rhodococcus* obuhvata Gram-pozitivne bakterije koje se odlikuju značajnom efikasnošću u procesima bioremedijacije i biokonverzije teško razgradivih polutanata. Ovaj potencijal primarno proističe iz njihove sposobnosti adaptacije na nepovoljne ekološke uslove i visoke tolerancije na toksična jedinjenja (Nazari et al., 2022). Vrste ovog roda pokazuju i izraženu QQ aktivnost pa se mogu koristiti i u biokontroli (Bourigault et al., 2021). Neke vrste mogu biti i oportunistički patogeni (Weinstock & Brown, 2002). Genomska analiza vrsta iz roda *Rhodococcus* je pokazala da imaju potencijal za razgradnju najzastupljenijih polisaharida kao što su celuloza, pektin, škrob i glikogen (Ghimire et al., 2021).

Vrste roda *Microbacterium* nalaze se u raznovrsnim okruženjima, uključujući biljke, tlo, hranu, morske i slatkovodne ekosisteme. To su Gram-pozitivne bakterije i neke vrste su ekstremofili otporni na sušu i zračenje. Uglavnom se smatraju nepatogenim za ljude, ali mogu izazvati infekcije kod imunokompromitovanih osoba, često sa visokom stopom smrtnosti (Hill et al., 2025). U genomima vrsta roda *Microbacterium* pronađeni su geni za celulaze, hemicelulaze, amilaze, pektinaze, hitinaze i druge endoglikozidaze i egzoglikozidaze (S. Gupta et al., 2022).

Vrste roda *Pedobacter* su Gram-negativne i pretežno su izolovane iz zemljišta, ali su takođe identifikovane i u vodi (uključujući i pijaću vodu), rashlađenim prehrambenim proizvodima, ribama, kompostu, mulju, glečerima, kao i u drugim ekstremnim sredinama. Patogeni sojevi u okviru ovog roda do sada nisu identifikovani (Viana et al., 2018). Vrste ovog roda poslužile su kao izvor brojnih glikozid-hidrolaza, uključujući galaktozidaze, ksilanaze, hitozanaze, karagenaze i celulaze (Miyazaki et al., 2015; Ravindran & Yang, 2016; Sharma et al., 2018; Song et al., 2019; B. Zhu et al., 2018).

U cilju identifikacije enzima sa potencijalom za razgradnju bakterijskih egzopolisaharida prisutnih u medicinski relevantnim biofilmovima (alginat, Pel, Psl, celuloza, PNAG i kolanska kiselina), sprovedena je detaljna bioinformatička analiza 173 gena koji kodiraju glikozid-hidrolaze u anotiranim genomima ispitivanih bakterijskih izolata. Selekcija kandidata za heterolognu ekspresiju zasnivala se na nekoliko kriterijuma, uključujući predviđenu supstratnu specifičnost enzima, prisustvo katalitičkih domena karakterističnih za razgradnju polisaharida sličnih ciljnim egzopolisaharidima, kao i prisustvo signalnih peptida i modularnih domena koji ukazuju na potencijalnu ekstracelularnu aktivnost. Na osnovu ovih kriterijuma, odabrano je 17 enzima koji pripadaju različitim funkcionalnim klasama: celulaze i β -glukozidaze, koje ciljaju glukozne ostatke prisutne u celulozi, Psl-u i kolanskoj kiselini; hitinaze, β -N-acetilheksozaminidaze i endo- α -1,4-poligalaktozaminidaze, koje ciljaju amino-šećere prisutne u Pel-u i PNAG-u; β -manozidaze, koje

ciljaju manozne ostatke u Psl-u; ramnogalakturonil hidrolaze i poligalakturonaze, odabrane zbog sposobnosti da ciljaju polisaharide bogate uronskim kiselinama, poput alginata i kolanske kiseline; te α -amilaze i β -ksilanaze, koje su uključene na osnovu dokumentovane uspešnosti ovih klasa u tretmanima biofilma, iako nemaju očigledno specifičnu metu u okviru odabranih egzopolisaharida (Craig et al., 2011; J.-H. Lee et al., 2018; Mustafa et al., 2025; Redman et al., 2021).

Od 17 kloniranih gena samo četiri je eksprimirano u *E. coli* sistemu za heterolognu ekspresiju u solubilnoj formi. Sve četiri glikozid-hidrolaze su uspešno prečišćene, a tri od njih su pokazale antibiofilm aktivnost. Ova tri enzima su opisana u tri naučna rada iz ove disertacije i to su β -glukozidaza BglB-BG28 (odjeljak 3.1), poligalakturonaza PG-BG31 (odjeljak 3.2) i β -1,2-manozidaza GH130-BG31 (odjeljak 3.3).

Bakterijski ekspresioni sistemi se često koriste u istraživanjima zbog sposobnosti brzog rasta bakterija na jeftinim supstratima, dobro okarakterisanih genoma i dostupnosti velikog broja klonirajućih vektora i mutantnih sojeva. Zbog detaljno proučenog genoma i širokog spektra razvijenih alata za genetičku manipulaciju *E. coli* se i dalje najčešće koristi za proizvodnju rekombinantnih proteina. Međutim, ekspresija u ovom organizmu nije podjednako efikasna za sve gene, pri čemu je proizvodnja intracelularnih enzima znatno uspešnija u poređenju sa sekretovanim (L. Liu et al., 2013). Ovo se može uočiti i u našim rezultatima gde je prinos intracelularne BglB-BG28 (odjeljak 3.1 str. 20) bio 20 puta veći od prinosa PG-BG31 (odjeljak 3.2 str. 33) i 50 puta veći od prinosa GH130-BG31 (odjeljak 3.3 str. 45) koje su ekstracelularni enzimi. Ekspresioni sistemi zasnovani na rodu *Bacillus* mogu se razmatrati u budućim eksperimentima kao dodatni pristup za ekspresiju onih enzima kod kojih u *E. coli* nije postignut optimalan prinos jer *Bacillus* ima visok sekretorni kapacitet i može eksportovati eksprimirane proteine direktno u medijum (L. Liu et al., 2013).

Osim podataka iz anotacije genoma, korišćen je i skup komplementarnih bioinformatičkih alata za funkcionalnu karakterizaciju i predikciju specifične aktivnosti glikozid-hidrolaza, uključujući analizu sekvence (NCBI BLAST, Clustal Omega, UniProt, InterProScan), predviđanje subcelularne lokalizacije i signalnih peptida (SignalP), strukturno modelovanje (SWISS-MODEL, AlphaFold2), kao i vizuelizaciju sekvenci i struktura (Jalview, Discovery Studio Visualizer). Alati zasnovani na poravnanju sekvenci, poput BLAST-a i anotacija u UniProt bazi, omogućavaju brzo i relativno pouzdano predviđanje funkcije enzima kada postoji visok stepen sličnosti sa eksperimentalno okarakterisanim proteinima (Altschul et al., 1990; The UniProt Consortium et al., 2023). Međutim, pouzdanost ovih predikcija značajno opada kod udaljenih homologa, naročito u slučaju enzima kao što su glikozid-hidrolaze, kod kojih male promene u aminokiselinskoj sekvenci ili aktivnom mestu mogu dovesti do značajnih razlika u supstratnoj specifičnosti i katalitičkoj efikasnosti. InterProScan alat dodatno unapređuje funkcionalnu interpretaciju identifikacijom konzerviranih domena i motiva, ali i dalje ne može sa sigurnošću predvideti enzimsku aktivnost bez eksperimentalne potvrde (Zdobnov & Apweiler, 2001). Strukturni pristupi, uključujući homologno modelovanje (SWISS-MODEL) i predikcije bazirane na mašinskom učenju (AlphaFold2), pružaju dublji uvid u prostornu organizaciju enzima, potencijalna aktivna mesta i interakcije sa supstratima, što je posebno korisno u hipotezama o mehanizmu delovanja (Jumper et al., 2021; Waterhouse et al., 2018). Baze podataka i anotacije zasnovane samo na homologiji sekvenci nose rizik od propagacije pogrešnih ili previše opštih funkcionalnih opisa, dok su eksperimentalno potvrđeni podaci znatno pouzdaniji, ali često ograničeni u obimu. U ovom istraživanju su bioinformatički alati korišćeni kao snažan polazni okvir za selekciju enzima i racionalan dizajn eksperimenata (odjeljak 3.1 str. 20; odjeljak 3.2 str. 32; odjeljak 3.3 str. 45). S obzirom na veliku raznovrsnost glikozid-hidrolaza i često nizak procenat identičnosti odnosno sličnosti sa eksperimentalno okarakterisanim enzimima, za identifikaciju novih glikozid-hidrolaza su kombinovane baze podataka zasnovane na analizi sličnosti sekvenci i baze eksperimentalno potvrđenih podataka, nakon čega je aktivnost glikozid-hidrolaza potvrđena eksperimentalnim analizama.

4.2 Biohemijska karakterizacija enzima BglB-BG28, PG-BG31 i GH130-BG31

Biohemijska karakterizacija prečišćenih rekombinantnih enzima urađena je primenom specifičnih supstrata i metoda detekcije, odabranih u skladu sa njihovim pretpostavljenim mehanizmom delovanja i predviđenom specifičnošću. Aktivnost β -glukozidaze BglB-BG28 ispitivana je korišćenjem hromogenog supstrata (odeljak 3.1 str. 20), što omogućava visoku osetljivost, dobru reproducibilnost i kvantitativnu analizu reakcije u realnom vremenu, čime je omogućeno pouzdano određivanje kinetičkih parametara enzima (odeljak 3.1 str. 20). Za praćenje razgradnje prirodnih supstrata detektovano je oslobađanje redukujućih šećera pomoću DNS reagensa (odeljak 3.1 str. 20, odeljak 3.2 str. 33, odeljak 3.3 str. 45). U poređenju sa p-nitrofenilnim supstratima, DNS metoda je manje precizna i podložnija interferencijama jer meri ukupnu količinu redukujućih šećera, bez razlikovanja pojedinačnih produkata hidrolize (Sengupta et al., 2000). Zbog toga je u ovom istraživanju korišćena prvenstveno za određivanje relativne enzimske aktivnosti. Ipak, njena značajna prednost leži u širokoj primenljivosti, jer omogućava detekciju različitih mono- i oligosaharida nezavisno od njihove strukture, što je čini posebno pogodnom za analizu aktivnosti enzima sa različitim i kompleksnim polisaharidnim supstratima, kao i za inicijalnu evaluaciju aktivnosti. Iz tog razloga, kao i zbog karakteristika polisaharidnih supstrata koji su heterogeni - promenljive dužine lanaca i stepena polimerizacije, za PG-BG31 i GH130-BG31 nisu određene kinetičke konstante. S obzirom da je kao supstrat za GH130-BG31 korišćen mananski ekstrakt iz *Candida albicans* koji nije komercijalni supstrat, prisustvo manoze kao produkta reakcije je potvrđena prvo pomoću tankoslojne hromatografije (TLC) (odeljak 3.3 str. 45), a potom i senzitivnijom metodom, anjonsko-izmenjivačkom hromatografijom visokih performansi sa pulsnom amperometrijskom detekcijom (HPAEC-PAD) (odeljak 3.3 str. 45). Izolacija egzopolisaharida direktno iz bakterijskih biofilmova predstavljala bi biološki relevantniji model ispitivanja aktivnosti antibiofilm enzima, ali je tehnički izuzetno zahtevna zbog niskih prinosa, heterogenosti sastava, prisustva proteina, eDNK i drugih komponenti matriksa, kao i velike varijabilnosti između uzoraka (Bales et al., 2013). Zbog toga je karakterizacija enzima sprovedena na komercijalno dostupnim ili standardizovanim supstratima, što omogućava bolju uporedivost rezultata i veću reproducibilnost. Poseban izazov predstavljala je karakterizacija β -1,2-manozidazne aktivnosti, budući da su komercijalni supstrati za ovaj tip enzima veoma ograničeni. Iz tog razloga, manan je izolovan iz *C. albicans*, čiji ćelijski zid sadrži visoke količine relativno homogenih mananskih polisaharida sa dobro definisanim vezama (Shibata et al., 2007) (odeljak 3.3 str. 45). Izolacija polisaharida iz kvasaca je znatno jednostavnija u poređenju sa bakterijskim biofilmovima, jer ćelijski zid *C. albicans* sadrži dominantne polisaharidne komponente, a sam proces ne zahteva razbijanje kompleksnog matriksa biofilma (Cuskin et al., 2015).

Glikozid-hidrolaze mogu delovati kao endo-enzimi, raskidanjem veza unutar polisaharidnog lanca ili kao egzo-enzimi postepenim oslobađanjem monosaharida ili kratkih oligosaharida sa krajeva polisaharidnih lanaca (Morais et al., 2023). U kontekstu razgradnje egzopolisaharida bakterijskih biofilmova, glikozid-hidrolaze endo- i egzo- tipa imaju komplementarne, ali funkcionalno različite uloge (Samaniego et al., 2024). Glikozid-hidrolaze endo- tipa se generalno smatraju efikasnijim za inicijalnu destabilizaciju matriksa biofilma, jer brzo smanjuju molekulsku masu i viskoznost egzopolisaharida, dovodeći do gubitka strukturnog integriteta biofilma. Nasuprot tome, glikozid-hidrolaze egzo- tipa deluju postepeno, te same po sebi obično imaju ograničen kapacitet da u kratkom vremenu dovedu do potpune razgradnje strukture biofilma. Ipak, egzo-enzimi imaju značajnu ulogu u potpunijoj depolimerizaciji proizvoda razgradnje, naročito kada deluju sinergistički sa endo-hidrolazama (Samaniego et al., 2024).

Sva tri enzima ispitana u ovoj disertaciji pripadaju grupi glikozid-hidrolaza egzo- tipa, što ukazuje da njihova primena u razgradnji egzopolisaharida nije optimalna kao samostalna strategija za uklanjanje biofilma, ali bi mogla imati značajnu ulogu u kasnijim fazama razgradnje, odnosno u uklanjanju krajnjih produkata i sprečavanju ponovne polimerizacije ili reorganizacije matriksa. U praktičnoj primeni, najefikasniji pristup razgradnji ekstracelularnih polimernih supstanci

podrazumeva kombinaciju endo- i egzo-enzima, gde endo-hidrolaze omogućavaju brzo narušavanje strukture matriksa, dok egzo-hidrolaze doprinose potpunijoj razgradnji oslobođenih fragmenata. Ova činjenica ujedno pruža i objašnjenje za to što sva tri enzima iz ove disertacije primarno deluju na inhibiciju formiranja biofilma, dok na već formiran biofilm imaju slabiji efekat ili uopšte ne utiču na njegovu strukturu (odjeljak 3.1 str. 21; odeljak 3.2 str. 33; odeljak 3.3 str. 47). Kao glikozid-hidrolaze egzo- tipa ovi enzimi nisu u stanju da efikasno i brzo naruše već uspostavljenu strukturu egzopolisaharidnog matriksa, već deluju prvenstveno u ranim fazama razvoja biofilma. Glikozid-hidrolaze egzo- tipa imaju visoku specifičnost prema monosaharidima i njihovim anomernim konfiguracijama, što rezultuje manje kompleksnom strukturom njihovog aktivnog mesta. Nasuprot tome, glikozid-hidrolaze endo- tipa prepoznaju veće regione unutar polisaharidnog lanca, pa njihova aktivna mesta imaju veće džepove, kao i multimodalne domene za vezivanje za polisaharid (Kobata, 2013). Zbog kompleksne strukture moguće su poteškoće u heterolognoj ekspresiji glikozid-hidrolaza endo- tipa u standardnim bakterijskim ekspresionim sistemima, poput *E. coli* (Watts et al., 2021). U cilju optimizacije ekspresije glikozid-hidrolaza endo- tipa u budućim eksperimentima može se razmotriti primena sistema ekspresije poput *Bacillus* spp., koji imaju visoku sposobnost sekrecije rekombinovanih proteina, ili eukariotskih sistema poput *Pichia pastoris* i *Komagataella phaffii*, koji omogućavaju pravilnije savijanje i obradu složenijih enzima (Lambertz et al., 2014). Uz opisane glikozid-hidrolaze egzo- tipa, uspešna heterologna ekspresija glikozid-hidrolaza endo- tipa bi omogućila razvoj efikasnijih enzimskih koktela za ciljanu razgradnju egzopolisaharida iz biofilma.

Uticaj temperature i pH na aktivnost i stabilnost ispitivanih enzima ukazuje da oni mogu efikasno delovati u uslovima predviđenim za njihovu primenu. BglB-BG28 pokazuje maksimalnu katalitičku aktivnost pri 42 °C i neutralnom pH, uz širok operativni opseg aktivnosti u temperaturnom intervalu od 8 do 55 °C i pH vrednostima od 3.0 do 11.0, što predstavlja značajnu prednost za primenu u prehrambenoj industriji (odjeljak 3.1 str. 20). Ovakva fleksibilnost omogućava efikasno delovanje enzima u uslovima koji mogu varirati duž proizvodnih i hladnih lanaca, gde temperaturna odstupanja od preporučenih vrednosti nisu neuobičajena. Posebno je važno što BglB-BG28 zadržava antibiofilm aktivnost i pri niskim temperaturama (8 °C), gde tokom produžene inkubacije značajno smanjuje formiranje biofilma *S. Enteritidis*, kao i pri 25 °C, gde gotovo u potpunosti inhibira biofilm (odjeljak 3.1 str. 22). S obzirom na sposobnost *Salmonella* spp. da formiraju biofilme na abiotičkim površinama, ova svojstva enzima BglB-BG28 ukazuju na njegov potencijal za primenu u čišćenju i dezinfekciji rashladne opreme i prehrambenih postrojenja. Nasuprot tome, enzimi PG-BG31 i GH130-BG31 pokazuju optimalnu aktivnost u temperaturnim i pH opsezima koji odgovaraju fiziološkim uslovima ljudskog organizma, što ih čini pogodnim kandidatima za medicinsku primenu u prevenciji urinarnih infekcija asociраних са катетером. Optimalna aktivnost enzima PG-BG31 pri 25–42 °C i blago kiselom pH (5.0–6.0) posebno je relevantna u kontekstu urinarnog trakta, gde mnogi uropatogeni održavaju pH vrednosti oko 6.0 (Lai et al., 2021), dok njegova visoka termička i pH stabilnost dodatno proširuje mogućnosti skladištenja i imobilizacije (odjeljak 3.2 str. 33). Slično tome, enzim GH130-BG31 pokazuje stabilnost i punu aktivnost pri telesnoj temperaturi i u blago kiselim do neutralnim pH uslovima, uz izuzetnu pH stabilnost u širokom rasponu (odjeljak 3.3 str. 46), što ukazuje na njegovu strukturnu robusnost. Zajedno, ovi rezultati potvrđuju da su biohemijska svojstva sva tri enzima usklađena sa zahtevima njihovih predviđenih primena.

4.3 Antibiofilm aktivnost opisanih glikozid-hidrolaza

Opisane glikozid-hidrolaze deluju na različite bakterijske patogene ciljajući specifične polisaharidne komponente matriksa biofilma. Enzim BglB-BG28 pokazao je antibiofilm aktivnost protiv *E. coli* i *S. enterica* subsp. *enterica* serovara Enteritidis, Typhimurium i Infantis, patogena čiji biofilmovi u značajnoj meri sadrže celulozu kao strukturni element, naročito kada su formirani na abiotičkim površinama (odjeljak 3.1 str. 20). S obzirom na to da β-glukozidaze predstavljaju sastavni deo celulaznog kompleksa, degradacija β-1,4-glikozidnih veza u celulozi dovodi do

destabilizacije celokupne arhitekture biofilma (A. Singh et al., 2021). Iako polisaharidi biofilma *P. aeruginosa* nisu primarni supstrati za β -glukozidaze, prethodna istraživanja su pokazala da β -glukozidaza iz badema može značajno inhibirati formiranje biofilma ovog patogena, što ukazuje na širi, indirektan potencijal ove klase enzima (Banar et al., 2019).

Enzim PG-BG31 je pokazao inhibiciju formiranja biofilma kod različitih kliničkih izolata *E. coli*, što se može dovesti u vezu sa njegovom sposobnošću da razgrađuje polisaharide koji sadrže galakturonsku i glukuronsku kiselinu (Tan et al., 2020) (odjeljak 3.2 str. 30). Matriks biofilma *E. coli* uključuje i komponente kao što su lipopolisaharidi, kolanska kiselina i drugi kapsularni polisaharidi bogati glukuronskom kiselinom, koji predstavljaju potencijalne mete poligalakturonaze (Klein et al., 2013; Sande & Whitfield, 2021). Poligalakturonaze pripadaju grupi pektinolitičkih enzima koji imaju široku primenu u različitim biotehnološkim industrijama (Jayani et al., 2005). Antibiofilm aktivnost pektinolitičkih enzima već je prethodno opisana, budući da je komercijalna pektinaza iz *Aspergillus niger* uspešno sprečila formiranje biofilma laboratorijskog soja *E. coli* K-12, čime se potvrđuje relevantnost ove enzimske klase za kontrolu biofilmova (Villa et al., 2015).

Glikozid-hidrolaza GH130-BG31 je pokazala antibiofilm aktivnost protiv *E. coli*, *S. epidermidis* i *K. pneumoniae*, što se može pripisati visokom sadržaju manoze u egzopolisaharidima ovih patogena (odjeljak 3.3 str. 47). Sličan mehanizam opisan je za PslG, glikozid-hidrolazu *P. aeruginosa*, koja destabilizuje biofilm vezivanjem za manozne ostatke unutar Psl polisaharida, iako je njena antibiofilm aktivnost do sada potvrđena isključivo na ovom patogenu (Baker et al., 2015). Iako su neke α - i β -manozidaze takođe pokazale izraženu antibiofilm aktivnost protiv manozom bogatih biofilmova, njihova primena je ograničena zbog citotoksičnosti pri efektivnim koncentracijama (Banar et al., 2016). U tom kontekstu, karakterizacija GH130-BG31 predstavlja poseban doprinos, jer enzim pokazuje antibiofilm efikasnost protiv različitih klinički relevantnih patogena a ne ispoljava citotoksičnost. Pored toga, ovo je prva glikozid-hidrolaza iz familije GH130 sa opisanom antibiofilm aktivnošću.

U dosadašnjim istraživanjima delovanja glikozid-hidrolaza protiv biofilmova, ispitivanja su najčešće rađena na *P. aeruginosa*, koji predstavlja najčešće korišćen i najbolje okarakterisan model infekcija asociranih sa biofilmom (Baker et al., 2016; Fleming et al., 2016; Ostapska et al., 2022). Međutim, takav pristup može dovesti do previđanja enzima sa potencijalno visokom efikasnošću protiv drugih klinički značajnih patogena. U tom kontekstu, dostupni literaturni podaci ukazuju da je relativno mali broj glikozid-hidrolaza ispitan na patogenima kao što su *S. enterica*, *E. coli*, *K. pneumoniae* i *S. epidermidis*. Celulaze su među najčešće ispitivanim glikozid-hidrolazama i pokazale su sposobnost razgradnje biofilmova *Salmonella* spp. i *E. coli*, međutim, efekti su postignuti tek pri vrlo visokim koncentracijama (20 mg/ml), koje se mogu smatrati nepraktičnim za širu primenu (Lim et al., 2019; H. Wang et al., 2016). Enzim uključen u degradaciju kolanske kiseline, ispitivan na *S. Typhimurium* i *E. coli*, doveo je do umerene inhibicije formiranja (≈ 35 – 37%) i razgradnje biofilma (≈ 27 – 34%) pri znatno nižim koncentracijama (100 $\mu\text{g/ml}$) (Mayton et al., 2021). Disperzin B i srodne N-acetil- β -D-heksozaminidaze (npr. DisH) predstavljaju neke od potentnijih antibiofilm enzima, pri čemu Dispersin B pri koncentraciji od 50 $\mu\text{g/ml}$ dovodi do gotovo potpune inhibicije formiranja biofilma *E. coli* i *S. epidermidis*, dok DisH pri istoj koncentraciji smanjuje formiranje biofilma *E. coli* za oko 35% i razgrađuju prethodno formirani biofilm za oko 42% (Itoh et al., 2005; L. Zhu et al., 2018). U slučaju *K. pneumoniae*, α -amilaza pri koncentracijama od 32–64 $\mu\text{g/ml}$ inhibira oko 80% formiranja biofilma (Mustafa et al., 2025), a često se opisuju i polisaharid-depolimeraze poreklom iz bakteriofaga koje pokazuju efikasno razlaganje matriksa biofilma ovog patogena (Li et al., 2021; X. Sun et al., 2023; Y. Wu et al., 2019). U poređenju sa navedenim podacima iz literature, enzimi ispitivani u okviru ovog istraživanja pokazali su višu ili uporedivu antibiofilm efikasnost. Enzim BglB-BG28 je inhibirao formiranje oko 50% biofilma *S. Enteritidis*, *S. Typhimurium* i *S. Infantis*, kao i približno 80% biofilma *E. coli* pri koncentraciji od 200 $\mu\text{g/ml}$ (odjeljak 3.1 str. 21). Efekat na prethodno formirani biofilm je bio ograničen. Enzim je pokazao sposobnost da razgradi do 40% biofilma *S. Enteritidis*

starog 9 sati, dok se efekat na starije biofilme gubi (odjeljak 3.1 str. 23). Glikozid-hidrolaza PG-BG31 je ispoljila izrazito snažno delovanje na *E. coli*, sa inhibicijom formiranja biofilma od oko 90% pri koncentraciji od 5-100 µg/ml zavisno od izolata (odjeljak 3.2 str. 33). Enzim je pokazao slabu sposobnost razgradnje ($\approx$ 20–30%), 24 h starih biofilma za samo dva od četiri ispitivana soja *E. coli* (odjeljak 3.2 prilog). Najveću efikasnost pokazao je enzim GH130-BG31, inhibirajući formiranje oko 80% biofilma *E. coli* i *S. epidermidis* već pri koncentraciji od 10 µg/ml, dok je za *K. pneumoniae* sličan efekat postignut pri 100 µg/ml (odjeljak 3.3 str. 47). Pri koncentraciji od 100 µg/ml enzim je pokazao sposobnost razgradnje 24 h starih biofilma *S. epidermidis* i *K. pneumoniae* za oko 40% (odjeljak 3.3 str. 50). Ovi rezultati jasno ukazuju da enzimi identifikovani i okarakterisani u ovom istraživanju predstavljaju potentnije antibiofilm agense u poređenju sa većinom prethodno opisanih glikozid-hidrolaza, dodatno potvrđujući značaj proširenja istraživanja na druge medicinski relevantne patogene, koji se ređe koriste kao modeli biofilma.

4.4 Sposobnost izolata patogenih bakterija da formiraju biofilm u laboratorijskim uslovima

Antibiofilm efekat glikozid-hidrolaza ispitivan je na patogenima različitog porekla, uključujući referentne sojeve iz ATCC kolekcije, kliničke izolate i njihove delecione mutante za određene egzopolisaharide. Ono što je zajedničko za ove sojeve je heterogenost u sposobnosti formiranja biofilma koja zavisi od soja i uslova gajenja, a koja u velikoj meri određuje dizajn eksperimenata i interpretaciju dobijenih rezultata.

U slučaju *S. Enteritidis*, sistematsko ispitivanje panela kliničkih izolata i referentnog soja *S. Enteritidis* ATCC 13076 pokazalo je da sposobnost formiranja biofilma nije univerzalna osobina čak ni unutar istog serovara. Od 55 ispitivanih izolata, samo 4 su pokazala značajnu sposobnost formiranja biofilma, definisanu na osnovu intenziteta boje biomase biofilma nakon bojenja kristal violetom (optička gustina rastvorene boje > 1) (odjeljak 3.1 str. 20). Odabrani sojevi sa visokim kapacitetom formiranja biofilma ispoljili su RDAR morfoliju (eng. *red, dry and rough*), koji ukazuje na produkciju celuloze i kurli vlakana. Nasuprot tome, BDAR morfoliju (eng. *brown, dry and rough*) referentnog soja, koji ne sadrži celulozu, ukazuje na to da i suptilne razlike u sastavu matriksa mogu značajno promeniti arhitekturu biofilma i, posledično, njegovu osetljivost na agense koji ciljaju matriks (odjeljak 3.1 str. 20). Uzimajući u obzir ekologiju *S. Enteritidis* i pojačanu ekspresiju celuloze na sobnoj temperaturi i ovaj faktor je uključen u testiranje aktivnosti BglB-BG28. Na sobnoj temperaturi *S. Enteritidis* jeste formirala bogatiji biofilm, ali je i inhibitoran efekat enzima bio izraženiji nego u tretmanu na 37 °C (odjeljak 3.1 str. 22). Drugi značajan faktor u prehrambenoj industriji su i površine na kojima se biofilm formira. Za simuliranje realnih uslova u prehrambenoj industriji, pored mikrotitracionih ploča formirani su modeli biofilma i na plastičnim i staklenim pokrovnim stakalcima i na aluminijumskoj foliji. Iako je stepen formiranja biofilma varirao u zavisnosti od vrste površine, nijedna od ispitivanih površina nije umanjila inhibitorni efekat enzima na formiranje biofilma (odjeljak 3.1 str. 22).

Slično tome, sposobnost formiranja biofilma *E. coli* značajno varira između kliničkih izolata i uslova gajenja, što odražava dobro poznatu plastičnost biofilma *E. coli* kao odgovor na faktore sredine, poput sastava nutrijenata i osmolarnosti (Alqasim et al., 2014). Upotreba različitih hranljivih podloga, uključujući medijum koji imitira urin (AU medijum), omogućila je identifikaciju izolata sa najizraženijim fenotipom formiranja biofilma u uslovima relevantnim za infekcije urinarnog trakta (odjeljak 3.2 str. 35). Urinarna infekcija asocirana sa upotrebom urinarnog katetera simulirana je modelom biofilma na segmentima silikonskog katetera uronjenog u medijum koji simulira urin (odjeljak 3.2 str. 35).

Iako tačan sastav polisaharida bogatih manozom i veze koje cilja GH130-BG31 u biofilmovima *E. coli*, *S. epidermidis* i *K. pneumoniae* još uvek nisu u potpunosti okarakterisani, dosledna sposobnost ovog enzima da inhibira formiranje biofilma kod ovih vrsta ukazuje na postojanje strukturno očuvanih β -1,2-manozidnih motiva u njihovim egzopolisaharidima. U ovom slučaju za formiranje modela biofilma su korišćeni sojevi iz referentne ATCC kolekcije. *S*

obzirom na visok sadržaj manoze (80%) u egzopolisaharidima *P. aeruginosa*, izostanak efekta enzima na biofilm *P. aeruginosa* je bio neočekivan. Sa pretpostavkom da je manaza dominantno prisutna u Psl polisaharidu, i da je slabija efikasnost enzima posledica kompleksne strukture matriksa ove bakterije i značajne uloge drugih polisaharida u formiranju i održavanju stabilnosti biofilma generisan je $\Delta pelA$ mutant, s pretpostavkom da će to smanjiti kompleksnost matriksa i povećati dostupnost manoznih struktura enzimu. Međutim, efekat enzima je izostao i kod mutanta, što ukazuje na prisustvo manozidnih veza koje ne predstavljaju ciljnu strukturu za GH130-BG31. Isti rezultat dobijen je i za biofilm *A. baumannii* koji takođe poseduje visok sadržaj manoze (80%) u svojim egzopolisaharidima.

4.5 Potencijalna primena opisanih glikozid-hidrolaza

Glikozid-hidrolaze opisane u ovom istraživanju ispoljavaju značajan potencijal za razvoj specifičnih strategija usmerenih ka ciljanjoj degradaciji i kontroli biofilмова.

S obzirom na pokazanu aktivnost, BglB-BG28 može naći primenu kao enzimski pretretman u protokolima dezinfekcije površina u prehrambenoj industriji, gde bi razgradnjom strukturnih polisaharida biofilma, pre svega celuloznih vlakana *Salmonella* spp. i *E. coli*, omogućila efikasnije delovanje konvencionalnih dezinfekcionih sredstava. Njena kompatibilnost sa dezinfekcionim sredstvom Oxid S i sinergistički efekat u sekvencijalnom tretmanu potvrđuju da enzimski pretretman može značajno povećati uspešnost dezinfekcije i istovremeno smanjiti potrebu za agresivnijim hemijskim uslovima. Pokazano je da je za uklanjanje 80% biofilma *S. Enteritidis* potrebna dvostruko niža koncentracija Oxid S ukoliko je biofilm formiran u prisustvu 200 µg/ml BglB-BG28, u poređenju sa tretmanom samim dezinfekcionim sredstvom (odjeljak 3.1 str. 23). Iako postoji potencijal za kombinovanje sa deterdžentima, važno je naglasiti da jonski deterdženti dovode do denaturacije enzima, te je primena BglB-BG28 moguća isključivo u kombinaciji sa nejonskim deterdžentima (odjeljak 3.1 str. 24).

Sličan koncept enzimske destabilizacije matriksa biofilma opisan je za celulazu, koja na *E. coli* pokazuje veoma snažno antibiofilm delovanje u mikrotitracionoj ploči, gde pri koncentraciji od 20 mg/ml inhibira formiranje do 98,5% biofilma i uklanja oko 30% već formiranog biofilma (Lim et al., 2019). Međutim, potreba za tako visokom koncentracijom enzima može predstavljati značajno ograničenje za praktičnu primenu. U modelu biofilma formiranog na metalnim kuponima pokazano je da sekvencijalni tretman celulazom praćen natrijum-hipohloritom (NaClO) dovodi do redukcije preformiranog biofilma od 1,74 log, dok je još izraženiji efekat postignut primenom proteinaze K, zatim celulaze i NaClO, sa redukcijom od 2,84 log (Lim et al., 2019). Istovremeno, promena redosleda tretmana (celulaza–proteinaza K–NaClO) rezultovala je slabijim efektom (redukcija od 1,58 log), što ukazuje na značaj pravilnog sekvencijalnog razlaganja proteinskih i polisaharidnih komponenti matriksa biofilma. Pored enzima koji ciljaju celulozne strukture, u literaturi je opisan i enzim predviđen za degradaciju kolanske kiseline, važnog egzopolisaharida u biofilmovima enterobakterija. Ovaj enzim pri koncentraciji od 100 µg/ml inhibira formiranje biofilma *E. coli* za oko 40% i *S. Typhimurium* za oko 35%, dok u poređenju sa 10 mM KCl, koji simulira uslove ispiranja česmenskom vodom, uklanja 35% više biofilma *E. coli* i 27% više biofilma *S. Typhimurium*, sa pretpostavkom da će oslobođene planktonske bakterije biti osetljivije na dezinfekciona sredstva (Mayton et al., 2021).

Glikozid-hidrolaze PG-BG31 i GH130-BG31 mogu naći primenu u razvoju funkcionalizovanih urinarnih katetera. PG-BG31 je pokazala značajan efekat u inhibiciji formiranja biofilma na urinarnim kateterima, pri čemu je enzimski tretman omogućio znatno bolju eradikaciju biofilma naknadnom antibiotskom terapijom, potvrđujući ulogu glikozid-hidrolaza kao senzitizera biofilma (odjeljak 3.2 str. 35). Dodatno, enzim GH130-BG31 je ispoljio izražen aditivni efekat sa antibioticima, kako u prevenciji formiranja biofilma, tako i u razgradnji već formiranog biofilma na površini katetera (odjeljak 3.3 str. 50). Ovakvi rezultati su u saglasnosti sa prethodnim studijama koje pokazuju da imobilizacija glikozid-hidrolaza na medicinskim materijalima može značajno

produžiti funkcionalni vek implantata, smanjiti učestalost infekcija povezanih sa kateterima i doprineti racionalnijoj upotrebi antibiotika. Imobilizacija enzima na silikonske katetere predstavlja izazov zbog hemijske inertnosti i hidrofobnosti silikona, ali dosadašnja istraživanja pokazuju da ovakav pristup može značajno unaprediti prevenciju biofilмова. Pokazano je da imobilizacija PslG na površinu venskih katetera dovodi do smanjenja adhezije i formiranja biofilma *P. aeruginosa* za približno 99,9% u odnosu na netretirane površine (Asker et al., 2021). Slično tome, enzimski premazi koji sadrže amilazu, samostalno ili u kombinaciji sa drugim agensima, pokazali su značajno smanjenje formiranja biofilma *E. coli*, *S. aureus* i *P. aeruginosa*, kao i odlaganje razvoja infekcija povezanih sa kateterima u *in vitro* i *in vivo* modelima (A. Ivanova et al., 2021; K. Ivanova et al., 2015). Opisani rezultati ukazuju da imobilizacija enzima predstavlja perspektivnu strategiju za razvoj funkcionalizovanih medicinskih materijala sa antibiofilm svojstvima. U tom kontekstu, dalja istraživanja će obuhvatiti procenu mogućnosti imobilizacije PG-BG31 i GH130-BG31, uz fokus na očuvanje njihove aktivnosti i stabilnosti premaza na površinama katetera.

4.6 Biološka bezbednost i biokompatibilnost glikozid-hidrolaza

Jedan od ključnih preduslova za razvoj enzimskih strategija za borbu protiv biofilмова jeste njihova bezbednost po bakterijsku ekologiju, model organizme i ćelije domaćina, naročito u kontekstu dugotrajne ili ponovljene primene. Opšta pretpostavka je da antibiofilm enzimi čiji je mehanizam dejstva razgradnja komponenti matriksa biofilma ne ubijaju bakterijske ćelije i ne inhibiraju bakterijski rast (Kaplan, 2009). To je dokazano i na najčešće korišćenim glikozid-hidrolazama kao što su celulaza, α -amilaza, disperzin B, PelA i PslG (Baker et al., 2016; Fleming et al., 2016; Kaplan et al., 2024). Međutim, u literaturi se mogu pronaći i slučajevi gde glikozid-hidrolaze sa antibiofilm aktivnošću, kao što su β -glukozidaza i α -manozidaza, ujedno ispoljavaju i baktericidno dejstvo (Banar et al., 2016, 2019). U ovom radu pokazano je da nijedna od tri ispitivane glikozid-hidrolaze ne ispoljava direktno baktericidno ili bakteriostatsko delovanje, budući da nisu negativno uticale na rast planktonskih bakterijskih kultura (odjeljak 3.1 str. 21; odeljak 3.2 str. 34; odeljak 3.3 str. 47). Ovaj nalaz je od posebnog značaja, jer potvrđuje da enzimi deluju primarno na strukturne komponente matriksa biofilma, a ne na esencijalne metaboličke procese bakterijskih ćelija, čime se značajno smanjuje verovatnoća razvoja rezistencije.

Citotoksični potencijal enzima u ovom radu procenjen je na humanim ćelijskim linijama, pri čemu nije zabeležen nikakav negativan efekat na vijabilnost ćelija (odjeljak 3.2 str. 36; odeljak 3.3 str. 51). Odsustvo citotoksičnosti je od posebnog značaja u kontekstu potencijalne primene ovih enzima na medicinskim uređajima, kao što su urinarni kateteri, ali i u okruženjima gde može doći do indirektnog kontakta sa ljudskim tkivom, uključujući i prehrambenu industriju. Značajnost ovih rezultata ističu i podaci iz literature koji pokazuju da je od 16 testiranih komercijalnih glikozid-hidrolaza sa antibiofilm dejstvom, svih 16 imalo citotoksičan efekat na humanim fibroblastima nakon tročasovnog tretmana u dozama koje su pokazale antibiofilm dejstvo (Redman et al., 2021). Većina ovih glikozid-hidrolaza je poreklom iz gljiva, dok su dve izolovane iz bakterija, a po jedna iz algi, biljaka i životinja. Za razliku od njih većina opisanih antibiofilm glikozid-hidrolaza, poreklom iz bakterijskih operona za sintezu egzopolisaharida, ne ispoljavaju citotoksičnost prema humanim ćelijama u kulturi (Donelli et al., 2007; Snarr et al., 2017; S. Yu et al., 2015).

Ispitivanja na model organizmu *Caenorhabditis elegans* pokazala su da nijedna od glikozid-hidrolaza ne utiče negativno na preživljavanje nematoda, što ukazuje na dobar bezbednosni profil u *in vivo* uslovima (odjeljak 3.1 str. 25; odeljak 3.2 str. 37; odeljak 3.3 str. 51). *C. elegans* se često koristi kao pouzdan preliminarni model za procenu sistemske toksičnosti i biokompatibilnosti (Hunt, 2017), te ovi rezultati dodatno potvrđuju da opisani enzimi ne izazivaju štetne biološke efekte van ciljanog antibiofilm delovanja.

Sveukupno, dobijeni rezultati ukazuju da opisane glikozid-hidrolaze poseduju povoljan bezbednosni i toksikološki profil, koji ih jasno razlikuje od agresivnih hemijskih dezinfekcionih sredstava i nekih enzima sa širim, nespecifičnim delovanjem. Ovakva kombinacija odsustva

baktericidnosti, netoksičnosti prema eukariotskim modelima i visoke antibiofilm efikasnosti dodatno podržava njihovu primenu kao ciljanih, biokompatibilnih alata u savremenim antibiofilm strategijama.

5. ZAKLJUČCI

1. U mikrobioti škrga deverike (*A. brama*) identifikovani su bakterijski izolati sa značajnom glikozid-hidrolaznom aktivnošću, *Aeromonas* sp. BG2, *Rhodococcus* sp. BG20, *Microbacterium* sp. BG28 i *Pedobacter* sp. BG31, čiji anotirani genomi sadrže ukupno 173 gena koji kodiraju glikozid-hidrolaze. Uz tri funkcionalno okarakterisane glikozid-hidrolaze sa antibiofilm aktivnošću, ovi izolati predstavljaju značajan i nedovoljno istražen rezervoar enzima sa potencijalnom primenom u kontroli medicinski relevantnih biofilmova.
2. β -glukozidaza BglB-BG28 iz soja *Microbacterium* sp. BG28 inhibira formiranje biofilma bakterija *S. enterica* i *E. coli* i omogućava eradikaciju biofilma sekvencijalnim tretmanom dezinficijensom Oksicid S pri čemu se postiže isti efekat uz dvostruko nižu koncentraciju dezinfijensa u odnosu na tretman bez enzima.
3. Poligalakturonaza PG-BG31 iz soja *Pedobacter* sp. BG31 efikasno inhibira formiranje biofilma strukturno heterogenih kliničkih izolata *E. coli*.
4. Enzim PG-BG31 omogućava primenu nižih doza antibiotika u sekvencijalnom tretmanu za eradikaciju biofilma na modelu inficiranog urinarnog katetera.
5. β -1,2-manozidaza GH130-BG31 iz soja *Pedobacter* sp. BG31 inhibira formiranje biofilmova bogatih manozom bakterija *K. pneumoniae*, *E. coli* i *S. epidermidis*
6. Enzim GH130-BG31 pospešuje efekat kolistina u tretmanu silikonskih katetera inficiranih bakterijom *K. pneumoniae* pri formiranju biofilma i pri razgradnji prethodno formiranog biofilma.
7. β -glukozidaza BglB-BG28, poligalakturonaza PG-BG31 i β -1,2-manozidaza GH130-BG31 su efikasne u inhibiciji formiranja biofilmova, ali je efekat na prethodno formirane biofilmova značajno slabiji, što se može pripisati njihovom mehanizmu delovanja kao glikozid-hidrolazama egzo- tipa koje nisu u stanju da efikasno i brzo naruše već uspostavljenu strukturu egzopolisaharidnog matriksa, već deluju prvenstveno u ranim fazama sinteze.
8. β -glukozidaza BglB-BG28, poligalakturonaza PG-BG31 i β -1,2-manozidaza GH130-BG31 pokazuju optimalnu aktivnost i stabilnost u temperaturnim i pH uslovima relevantnim za njihovu potencijalnu primenu u prehrambenoj industriji i medicini.
9. β -glukozidaza BglB-BG28, poligalakturonaza PG-BG31 i β -1,2-manozidaza GH130-BG31 ne ispoljavaju direktno baktericidno ili bakteriostatsko delovanje, što smanjuje verovatnoću za razvoj rezistencije na tretmane ovim enzimima.
10. β -glukozidaza BglB-BG28, poligalakturonaza PG-BG31 i β -1,2-manozidaza GH130-BG31 nisu citotoksične prema humanim ćelijama u kulturi i ne utiču na preživljavanje nematode *C. elegans*, što ukazuje na njihov povoljan bezbednosni profil i potencijal za bezbednu primenu u antibiofilm strategijama

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BIOGRAFIJA

Marija Atanasković je rođena 29. januara 1996. godine u Šapcu. Osnovnu i srednju medicinsku školu je završila u Šapcu, nakon čega je 2015. godine upisala Biološki fakultet Univerziteta u Beogradu. Osnovne akademske studije je završila 2019. godine na modulu Molekularna biologija i fiziologija, sa prosečnom ocenom 9,55.

Iste godine je upisala master akademske studije, smer Profesor biologije na Biološkom fakultetu Univerziteta u Beogradu. Master rad pod nazivom „Primena digitalnih igara u nastavi biologije“ odbranila je 2020. godine sa ocenom 10.

Doktorske akademske studije je upisala 2021. godine na istom fakultetu, na podmodulu Molekularna mikrobiologija i biotehnologija. Od 2022. godine je zaposlena na Institutu za molekularnu genetiku i genetičko inženjerstvo, prvo kao istraživač-pripravnik, a zatim od 2024. godine kao istraživač-saradnik.

Kao stipendista Erasmus+ programa i Federacije evropskih mikrobioloških društava (FEMS), deo svog istraživanja realizovala je u Laboratoriji za mikrobiologiju, parazitologiju i higijenu, Univerziteta u Antverpen u Belgiji 2023. godine i u Laboratoriji za bakterijske biofilme, Minjo Uiverziteta u Portugaliji 2025. godine.

Iz njenog dosadašnjeg naučnoistraživačkog rada proizašlo je pet publikacija u međunarodnim časopisima. Na domaćim i međunarodnim naučnim skupovima je učestvovala sa ukupno sedam saopštenja.

Прилог 1.

Изјава о ауторству

Име и презиме аутора: Марија С. Атанасковић

Број индекса: М3006/2021

Изјављујем

да је докторска дисертација под насловом

Деполимеризација матрикса медицински значајних биофилмова гликозид-хидролазама пореклом из микробиоте шкрга деверике (*Abramis brama*)

- резултат сопственог истраживачког рада;
- да дисертација у целини ни у деловима није била предложена за стицање друге дипломе према студијским програмима других високошколских установа;
- да су резултати коректно наведени и
- да нисам кршио/ла ауторска права и користио/ла интелектуалну својину других лица.

Потпис аутора

У Београду, _____

Прилог 2.

Изјава о истоветности штампане и електронске верзије докторског рада

Име и презиме аутора: Марија С. Атанасковић

Број индекса: М3006/2021

Студијски програм: Молекуларна биологија

Наслов рада: Деполимеризација матрикса медицински значајних биофилмова гликозид-хидролазама пореклом из микробиоте шкрга деверике (*Abramis brama*)

Ментор: др Лидија Шенеровић, др Горан Вукотић

Изјављујем да је штампана верзија мог докторског рада истоветна електронској верзији коју сам предао/ла ради похрањивања у **Дигиталном репозиторијуму Универзитета у Београду**.

Дозвољавам да се објаве моји лични подаци везани за добијање академског назива доктора наука, као што су име и презиме, година и место рођења и датум одбране рада.

Ови лични подаци могу се објавити на мрежним страницама дигиталне библиотеке, у електронском каталогу и у публикацијама Универзитета у Београду.

Потпис аутора

У Београду, _____

Прилог 3.

Изјава о коришћењу

Овлашћујем Универзитетску библиотеку „Светозар Марковић“ да у Дигитални репозиторијум Универзитета у Београду унесе моју докторску дисертацију под насловом:

Деполимеризација матрикса медицински значајних биофилмова гликозид-хидролазама пореклом из микробиоте шкрга деверике (*Abramis brama*)

која је моје ауторско дело.

Дисертацију са свим прилозима предао/ла сам у електронском формату погодном за трајно архивирање.

Моју докторску дисертацију похрањену у Дигиталном репозиторијуму Универзитета у Београду и доступну у отвореном приступу могу да користе сви који поштују одредбе садржане у одабраном типу лиценце Креативне заједнице (Creative Commons) за коју сам се одлучио/ла.

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Кратак опис лиценци је саставни део ове изјаве).

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