

Институт по инженерна химия – БАН

Институт по микробиология - БАН



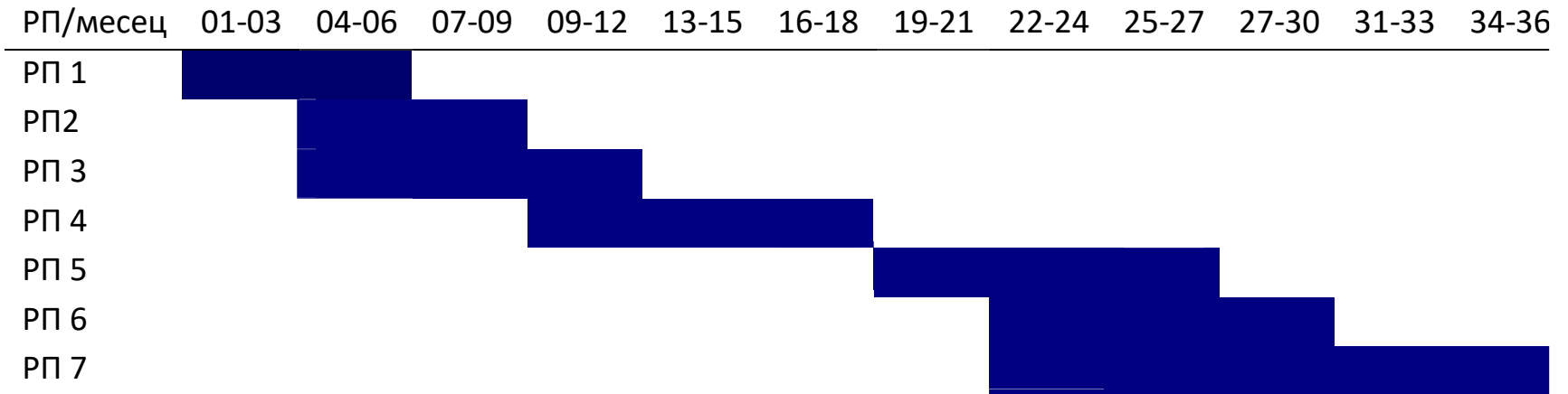
# Получаване на 2,3-бутандиол чрез ферментация на отпадна биомаса от новоизолирани и рекомбинантни щамове

Договор с ФНИ № ДН 17/1 от 2017 г.

Ръководител:

проф. дн Калоян Петров

# Работни пакети



## Работни пакети за I етап:

- РП<sub>1</sub> – Изолиране на нови продуценти на 2,3-БД
- РП<sub>2</sub> – Идентификация на непатогенни продуценти
- РП<sub>3</sub> – Подбор и анализ на химичния състав на отпадни продукти
- РП<sub>4</sub> – Анализ на ензимни активности на новоизолираните непатогенни щамове

# Цел и задачи при реализацията на РП1 – РП4

## ЦЕЛ:

Изолиране на продуцент на 2,3-БД, притежаващ следните характеристики:

- ✓ Непатогенен
- ✓ С подходящ субстратен спектър
- ✓ Свръхпродуцент на 2,3-БД

## Задачи:

- ✓ Изолиране на продуценти на 2,3-БД (РП1)
- ✓ Идентификация и подбор на непатогенните продуценти (РП2)
- ✓ Изследване на ензимната активност и субстратния спектър на непатогенните продуценти (РП3 и РП4)
- ✓ Изследване на продуктивните възможности на селектираните щамове (РП4)
- ✓ Избор на продуцент

## ❖ РП1 – Изолиране на нови продуценти на 2,3-БД

- Общ брой изследвани щамове – 140 (119 новоизолирани + 21 колекционни)

Изолиране на чисти  
култури от единична  
колония



Култивиране в течна среда  
с 20 г/л глюкоза и тест  
Voges-Proskauer



- ✓ Червеното оцветяване е индикация за синтез на ацетоин и 2,3-БД

Количествена оценка на  
синтезирания 2,3-БД чрез  
HPLC



- ✓ Изолирани продуценти на 2,3-БД – 77 щама (69 новоизолирани + 8 колекционни)

## ❖ РП<sub>2</sub> – Идентификация на новоизолираните продуценти

- Общ брой изследвани щамове – 77 (69 новоизолирани + 8 колекционни)

Морфологични критерии



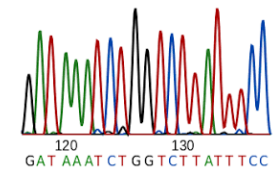
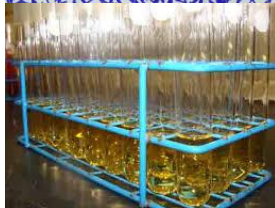
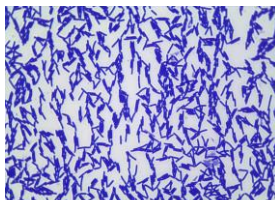
Физиологични критерии  
(Мезофилни аероби)



Генетични критерии  
(секвенция на 16S rDNA)



Биохимични критерии  
(API тест)



Непатогенни:

- *Bacillus subtilis* – 33 щама
- *Bacillus licheniformis* – 9
- *Bacillus thuringiensis* – 8
- *Bacillus pumilus* – 3
- *Bacillus cereus* – 3
- *Bacillus toyonensis* – 2
- *Bacillus velezensis* – 2
- *Bacillus stratosphericus* – 1
- *Bacillus safensis* – 1
- *Bacillus tequilensis* – 1
- *Bacillus sonorensis* – 1
- *Paenibacillus polymixa* – 1

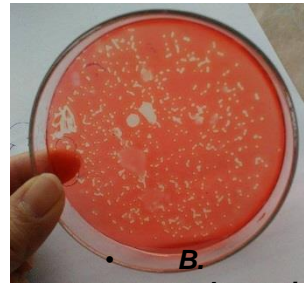
- ✓ Идентифицирани като непатогенни продуценти на 2,3-БД – 65 щама (57 новоизолирани + 8 колекционни)

- ✓ Идентифицирани като патогенни продуценти на 2,3-БД – 12 щама

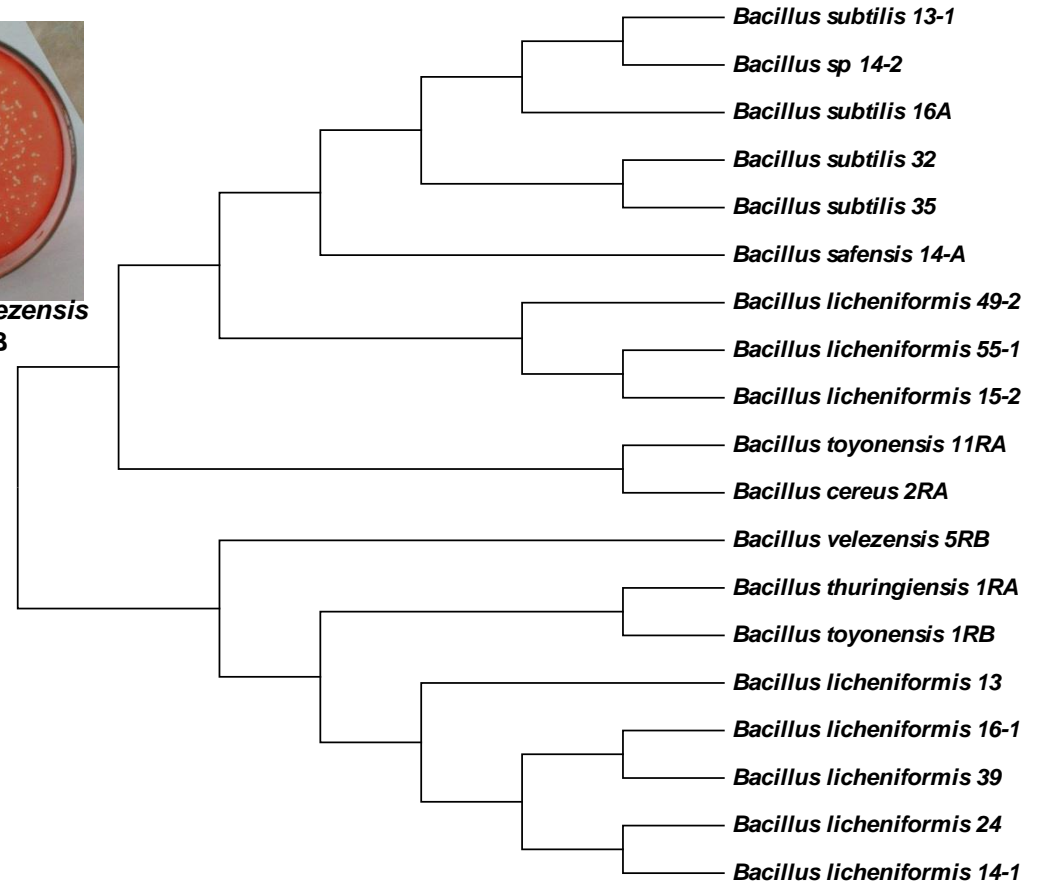
# ❖ РП4 – Изследване на новоизолираните продуценти за целулазна активност

- Общ брой изследвани щамове – 65 (57 новоизолирани + 8 колекционни)

Изследване на целулазна активност чрез обезцветяване на среда с 0.01 % карбоксиметил целулоза и Конго-червено

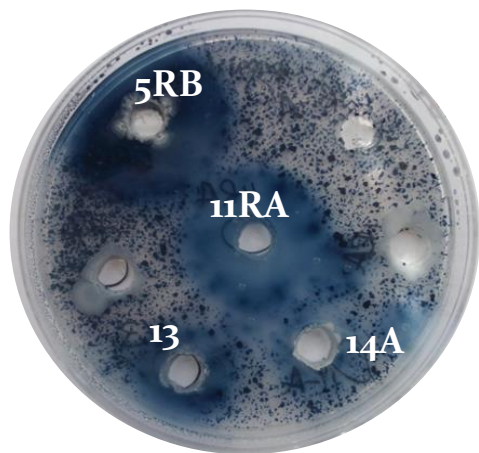
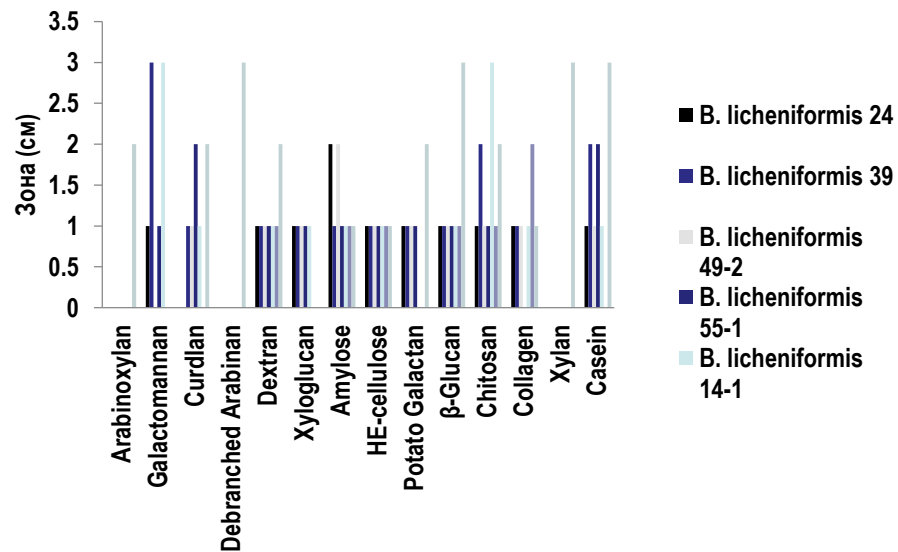
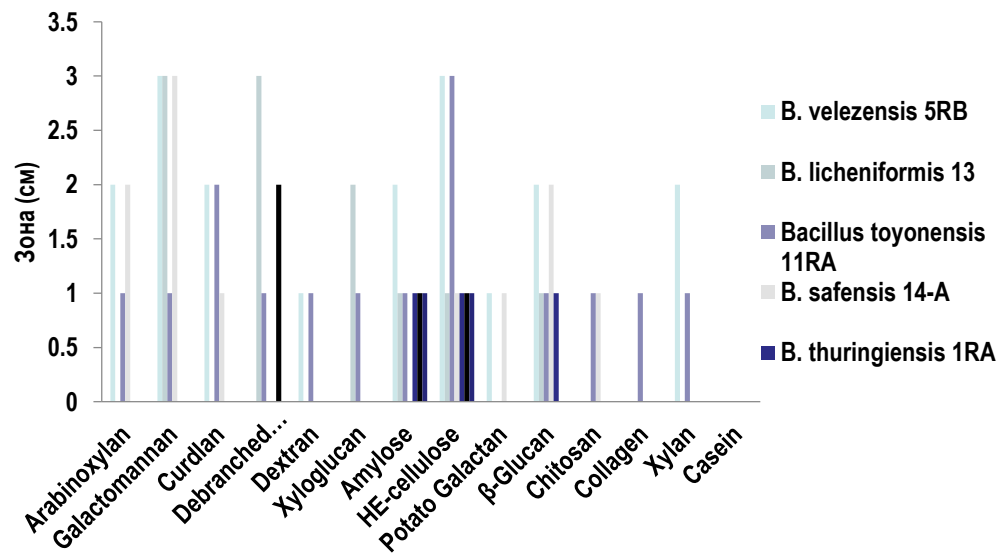


*B. velezensis*  
5RB

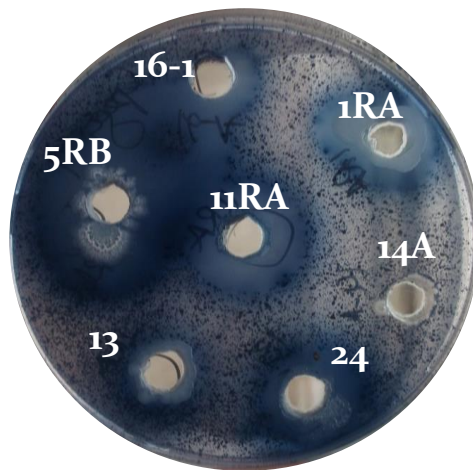


- ✓ Идентифицирани като целулозоразграждащи продуценти на 2,3-БД – 19 щамове (15 новоизолирани + 4 колекционни)

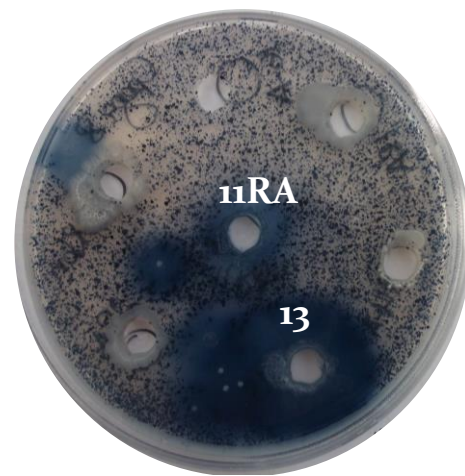
# ❖ РП4 – Изследване на новоизолираните продуценти за активност при разграждане на полизахариди



Целулазна активност



Амилазна активност



Ксило-глюканазна активност

## ❖ РП4 – Изследване на новоизолираните продуценти за активност при разграждане на полизахариди

	5RB	11RA	14A	13	24	14-1	16-1	39	49-2	55-1	1RA	1RB
Арабиноксилан	+	+	++	-	-	-	-	-	-	+	+	+
Галактоманан	+++	+	+++	+++	+	+	+	+	+	-	-	+
Курдлан	+	++	+	-	-	-	-	-	-	-	-	-
Разклонен арабинан	+/-	++	-	+	+	+	+	+	+	-	-	+
Декстран	+	+	-	-	-	-	-	-	-	-	-	-
Ксилоглюкан	-	+	-	++	+	+	+	+	+	-	-	+
Амилоза	++	+	-	+	+++	+	+	+	+	+	+	+
НЕ-целулоза	+++	+++	+	+	+	+	+	+	+	-	+	+
Картофен галактан	+	-	+	-	+	+	+	+	+	+	+	+
Бета-глюкан	++	+	++	+	+	+	+	+	+	-	+	+
Хитозан	-	+	+	-	-	-	-	-	-	-	-	-
Колаген	+			+	+	+	+	+	+	+	+	+
Ксилан	++	+	-	-	-	-	-	-	-	+	+	+

✓ Като най-перспективни са определени щамовете 5RB, 11RA, 13, 24 и 14A.



## ❖ РП4 – Изследване на новоизолираните продуценти за активност при разграждане на захари от състава на лигноцелулозата и други отпадни субстрати

Щам	(D+) Целобиоза	(D+) Рафиноза	(D+) Ксилоза	(L+) Арабиноза	(D+) Галактоза	(D+) Малтоза	(D+) Маноза	Глицерол	ФОЗ
<i>B. velezensis</i> 5RB	++	+	+	+	-	+	+	+	++
<i>B. toyonensis</i> 11RA	++	+	+	+	+	+	+	+	++
<i>B. licheniformis</i> 13	+	-	-	+	-	+	+	+	+
<i>B. licheniformis</i> 14-1	+	-	-	+	-	+	+	+	-
<i>B. safensis</i> 14A	+	+	+	+	+	+	+	+	++
<i>B. licheniformis</i> 16-1	+	-	-	+	-	+	+	+	-
<i>B. licheniformis</i> 24	+	-	-	+	-	+	+	+	+
<i>B. licheniformis</i> 39	+	-	-	+	-	+	+	+	+
<i>B. licheniformis</i> 49-2	+	+	+	+	+	+	+	+	+
<i>B. licheniformis</i> 55-1	+	+	+	+	-	+	+	+	-
<i>B. thuringiensis</i> 1RA	+	+	+	++	+	+	+	+	++
<i>B. toyonensis</i> 1RB	+	+	+	+	+	+	+	+	++
<i>B. subtilis</i> 32	+	+	-	+/-	-	+	+	+	-
<i>B. subtilis</i> 35	+	+	-	+/-	-	+	+	+	-

✓ Като най-перспективни са определени щамовете 5RB, 11RA, 14A, 1RA и 1RB.

## ❖ РП4 – Установяване на количествените възможности на новоизолираните щамове да продуцират 2,3-БД

- Общ брой изследвани щамове – 65 (57 новоизолирани + 8 колекционни)

✓ Изследването е проведено на пет различни хранителни среди с въглероден източник глюкоза.

✓ Продуктите са селектирани на базата на получените количества 2,3-БД при нарастваща концентрация на субстрата.

Щам	Усвоена глюкоза (g/L)	Скорост на усвояване (g/Lh)	Концентрация 2,3-БД (g/L)	Продуктивност 2,3-БД (g/Lh)	Добив (g/g)
<i>B. safensis</i> 14A	100.0	0.82	38.05	0.31	0.38
<i>B. licheniformis</i> 24	100.0	1.18	34.70	0.48	0.35
<i>B. licheniformis</i> 13	100.0	1.23	34.37	0.48	0.34
<i>B. velezensis</i> 5RB	100.0	0.82	27.01	0.23	0.27
<i>B. subtilis</i> 18	88.9	0.64	20.51	0.17	0.23
<i>B. subtilis</i> 35	100.0	1.33	18.22	0.24	0.18
<i>B. subtilis</i> 32	100.0	1.38	17.94	0.24	0.18
<i>B. subtilis</i> 47	100.0	1.21	16.84	0.22	0.17
<i>B. toyonensis</i> 11RA	63.1	0.65	15.29	0.31	0.24

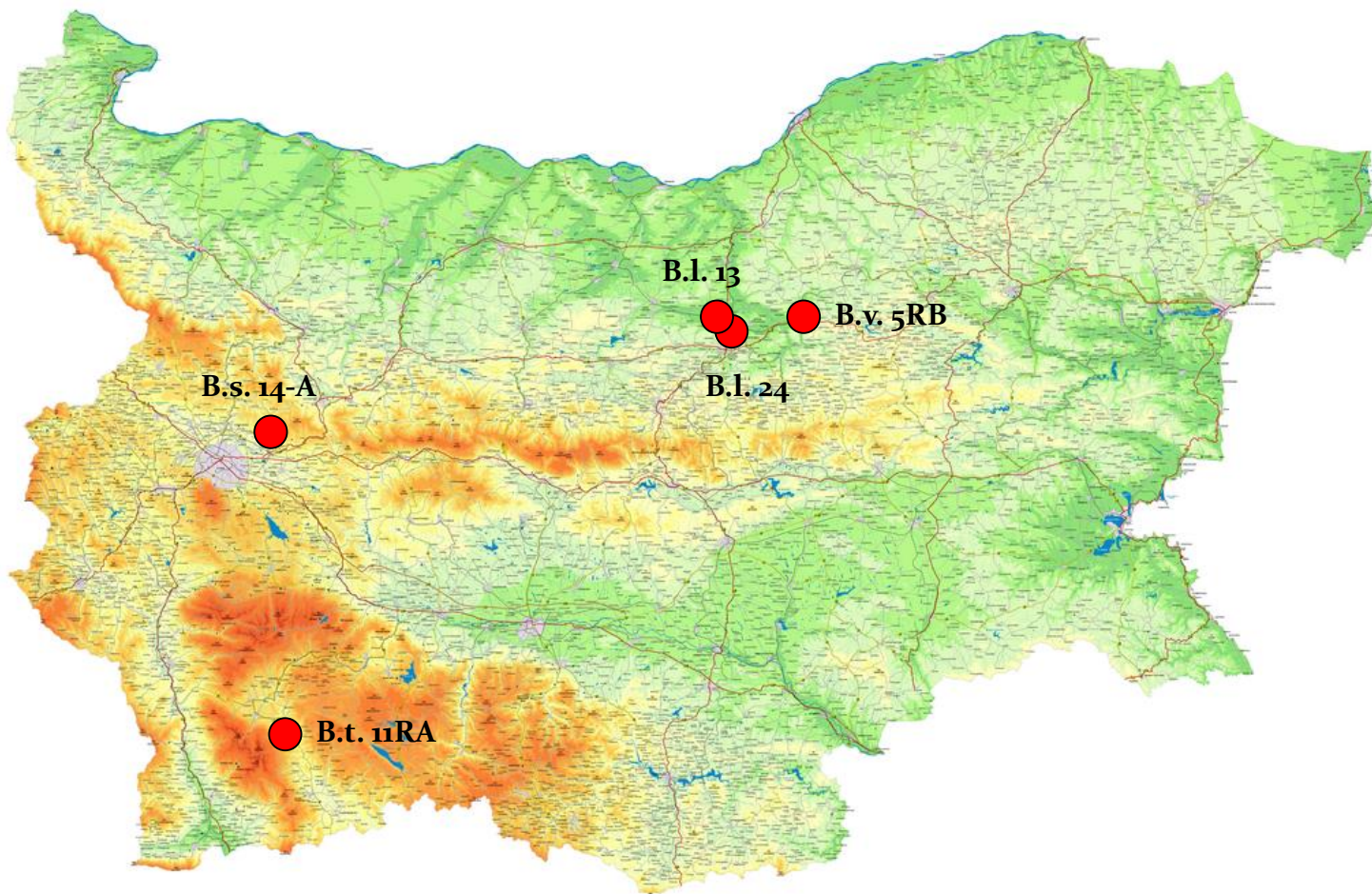
- Получаване на 2,3-БД от новоизолирани непатогенни щамове, култивирани на среда № 3 (Okonkwo et al. 2017) с начална концентрация на глюкоза 100 г/л. Култивиране на клатачка при температура 37 °С и разбъркване 200 rpm.

- ✓ Идентифицирани като свръхпродуценти на 2,3-БД от глюкоза – 5 щамове (14A, 13, 24, 5RB, 32 и 35)

## ❖ РП1-РМ4: Избор на щам

### Селектирани щамове за получаване на 2,3-БД от отпадни продукти:

- *Bacillus velezensis* 5RB (добър продуцент, целулазна, амилазна, инулиназна активност)
- *Bacillus licheniformis* 13 (сврџхпродуцент, амилазна активност)
- *Bacillus licheniformis* 24 (сврџхпродуцент, изключителна амилазна активност)
- *Bacillus safensis* 14A (сврџхпродуцент)
- *Bacillus toyonensis* 11RA (целулазна, амилазна, инулиназна активност)



# De novo геномно секвениране на *Bacillus Velezensis* 5RB

- Геномът на *B. velezensis* 5RB е депозиран в базите данни DDBJ, ENA и GenBank NCBI под номер QXJL00000000
- Съдържа гените *alsS*, *alsD* и *bdhA*, кодиращи ацетолактат-синтаза, ацетолактат-декарбоксилаза и бутандиол-дехидрогеназа, осигуряващи синтез предимно на (2R, 3R) изомера на 2,3-БД
- 225 гена са отговорни за конверсията и транспорта на въглехидрати, сред тях са гените, кодиращи гликозид-хидролази *amyE*, *mall*, *sacA*, *xynA*, *xynB*, *xynD*, *xynC*, и *eglS*
- Генетичната база на *B. velezensis* 5RB разкрива възможностите на щама да конвертира целулоза, лигноцелулоза, нишесте и инулин в 2,3-БД
- В генома присъстват седем пълни клъстера за синтез на антибиотици (макролактин, бацилаен, дефицидин, фенгицин, бацилбактин, бацилисин и сърфактин), което е предпоставка за разработване на биотехнологичен процес за синтез на 2,3-БД в нестерилни условия



GENOME SEQUENCES



## Genome Sequence of *Bacillus velezensis* 5RB, an Overproducer of 2,3-Butanediol

Penka Petrova,<sup>a</sup> Petya Velikova,<sup>a</sup> Kaloyan Petrov<sup>b</sup>

<sup>a</sup>Institute of Microbiology, Bulgarian Academy of Sciences, Sofia, Bulgaria

<sup>b</sup>Institute of Chemical Engineering, Bulgarian Academy of Sciences, Sofia, Bulgaria

**ABSTRACT** *Bacillus velezensis* 5RB is capable of producing large amounts of 2,3-butanediol. Whole-genome sequencing revealed that the strain contains one circular chromosome of 3.91 Mbp without plasmids. A large part of the genome is devoted to carbohydrate metabolism, encoding an abundance of enzymes participating in polysaccharide utilization pathways.

### ACKNOWLEDGMENT

This study was supported by grant DH 17/1 from The National Science Fund, Ministry of Education and Science, Republic of Bulgaria.

Released *Bacillus* sp. 5RB (QXJL00000000 ; BioSample SAMN09947506)

genomes@ncbi.nlm.nih.gov

To pepipetrova@yahoo.com

Dear Penka Petrova,

We have processed and released the following submission:

SUBID	BioProject	BioSample	Accession	Organism
SUB4485361	PRJNA488987	SAMN09947506	QXJL00000000	<i>Bacillus</i> sp. 5RB

Please cite the accession number QXJL00000000 like this:

This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession QXJL00000000. The version described in this paper is version QXJL01000000.

The master record will be available from our various Entrez servers within a few days. The individual sequences are available from a hyperlink at the bottom of the WGS master record QXJL00000000.

We will upload the list of accession numbers to the Submission Portal, QXJL01\_accs.

# Публикации и представления

## Публикации:

- Petrova P., Velikova P., Petrov K. (2019) Genome sequence of *Bacillus velezensis* 5RB – an overproducer of 2,3-butanediol. *Microbiology Resource Announcements*, vol. 8(1), e01475-18. (<https://doi.org/10.1128/MRA.01475-18>) (SJR 0.553 - 2017)

## Постери:

- Kaloyan K Petrov, Flora V Tsvetanova, Petya V Velikova, Penka M Petrova (2018) Novel Bacilli sp. isolates producing 2,3-butanediol have the potential to degrade lignocellulose. 21 th European Biotechnology Congress, 11-12 Oct. 2018, Moscow, Russia (J Biotechnol Biomater 2018 vol. 8)
- Penka M Petrova, Petya V Velikova, Flora V Tsvetanova, Kaloyan K Petrov (2018) De novo whole genome sequencing of 2,3-butanediol producing Bacillus sp. strain 5RB. 21 th European Biotechnology Congress, 11-12 Oct. 2018, Moscow, Russia (J Biotechnol Biomater 2018 vol. 8)

## Награди:

- Награда за най-добър постер на 21-ви конгрес по Биотехнология – Октомври 2018, Москва, Русия (за постера Novel Bacilli sp. isolates producing 2,3-butanediol have the potential to degrade lignocellulose) (сертификат)

# Благодаря за вниманието



GENOME SEQUENCES



## Genome Sequence of *Bacillus velezensis* 5RB, an Overproducer of 2,3-Butanediol

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<sup>a</sup>Institute of Microbiology, Bulgarian Academy of Sciences, Sofia, Bulgaria

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**ABSTRACT** *Bacillus velezensis* 5RB is capable of producing large amounts of 2,3-butanediol. Whole-genome sequencing revealed that the strain contains one circular chromosome of 3.91 Mbp without plasmids. A large part of the genome is devoted to carbohydrate metabolism, encoding an abundance of enzymes participating in polysaccharide utilization pathways.

The organic chemical 2,3-butanediol (2,3-BD) is the starting reagent in chemical syntheses and an ingredient in foods and pharmaceuticals (1). Biotechnological approaches for 2,3-BD production have progressed over the past decade, turning 2,3-BD into a major product of mixed-acid fermentations (2, 3). Currently, the aims are to use nonpathogenic *Bacillus* strains (4) and convert renewable raw materials (5).

*B. velezensis* 5RB was isolated in the Veliko Tarnovo region of Bulgaria from lake sediment containing plant roots. Single colonies of the strain were grown in nutrient broth (Oxoid) at 30°C. Genomic DNA was extracted using a GeneJET genomic DNA purification kit (Thermo Fisher Scientific). The TruSeq DNA PCR-free kit was used for library construction; the sequencing was performed on an Illumina HiSeq 2500 instrument with FastQC quality control (Macrogen, Inc., South Korea). Quality-filtered data contained 43,639,513,900 total bases and 289,794,196 read counts. The assembly was done using SOAPdenovo2 software (6) yielding 26 contigs with a total length of 3,910,395 bp, 134.22× genome coverage, an  $N_{50}$  value of 394,584 bp, and a 46.5% G+C content. The NCBI Prokaryotic Genome Annotation Pipeline (7) detected 4,605 genes, 3,745 of them encoding proteins, 81 tRNAs, and 8 rRNAs.

Strain 5RB belongs to the *Bacillus amyloliquefaciens* operational group (8), with a 99% similarity with soy isolate *B. velezensis* YJ11-1-4 (GenBank accession number NZ\_CP020874) (9). *In silico* DNA-DNA hybridization (DDH) (10) resulted in a DDH value of 90.20% with the *B. velezensis* FZB42 genome (CP000560) and a relatively lower DDH of 85.7% with that of the type strain NRRL B-41580 (LLZC0000000).

*B. velezensis* 5RB contains genes which are typical for plant-associated rhizobacterial genomes (11–13). The metabolic model of Rapid Annotations using Subsystems Technology (RAST) (default settings) (14) built by ModelSEED v2.3 predicted a 2,3-BD synthesis pathway engaging *ilvB*, *ilvS*, and *ilvH* (encoding  $\alpha$ -acetylactate synthase), *alsD* ( $\alpha$ -acetylactate decarboxylase), and *bdhA* [(R)-2,3-butanediol dehydrogenase; EC 1.1.1.4]. The last enzyme was identical to the 2,3-butanediol dehydrogenase of *B. amyloliquefaciens* KHG19 (GenBank accession number CP007242) (15) but different from those of *B. velezensis* FZB42 and NRRL B-41580<sup>T</sup>, which may explain the overproduction of 2,3-BD by *B. velezensis* 5RB.

A large portion of the genome of *B. velezensis* 5RB is devoted to carbohydrate metabolism (225 genes). The following genes encode glycoside hydrolases: *amyE*, *malL*, *sacA*, *xynA*, *xynB*, *xynD*, *xynC*, and *eglS*. This rich enzyme spectrum enables the conversion of cellulose, hemicellulose, starch, and inulin and is promising for the use of *B.*



## Novel *Bacillus* isolates producing 2,3-butanediol have the potential to degrade lignocellulose



Kaloyan K. Petrov<sup>1</sup>, Flora V. Tsvetanova<sup>1</sup>, Petya V. Velikova<sup>2</sup>, Penka M. Petrova<sup>2</sup>

<sup>1</sup>Institute of Chemical Engineering, BAS, Bulgaria

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## Introduction

2,3-Butanediol (2,3-BD) is valuable bulk-chemical with industrial applications as fuel additive and reagent in manufacturing of moistening and softening agents, perfumes, fumigants, insecticides, explosives, plasticizers and printing inks. The present work is dedicated to the development of bio-based process for its production by non-pathogenic strains from renewable, waste, abundant, and inexpensive substrate as the lignocellulosic biomass.

## Results

Here we report a comprehensive study of the ten *Bacillus* sp. strains. They were isolated from different soil, rhizosphere, and yogurt samples and selected for their ability to produce 2,3-butanediol from glucose. Based on 16S rRNA gene sequences, seven of them (13, 14, 15, 24, 39, 49, and 55) were affiliated to *B. licheniformis*, two (1RA, 1RB) – to *B. cereus* group, and one strain (5RB) belonged to *B. amyloliquefaciens* group (Fig. 1). Considering the strains' potential to degrade lignocellulose, their hydrolytic enzyme activities were tested using AZCL (azurine cross-linked) substrates. Nine strains were able to degrade cellulose, since they liquefied HE-, DEAE-cellulose, and  $\beta$ -glucan (Fig. 2). Several strains degraded the hemicellulose polysaccharides xyloglucan, xylan and arabinoxylan (Fig. 3). Importantly, the strains fermented the main lignocellulose monosaccharide components D-xylose, L-arabinose, D-mannose, and D-galactose. Eight of the strains utilized branched arabinan, 7 of them – galactomannan, and 7 – inulin (all spread in the plant biomass). Disaccharides utilization profiles revealed that all novel strains were able to ferment sucrose, lactose, maltose, and cellobiose (Fig. 4).

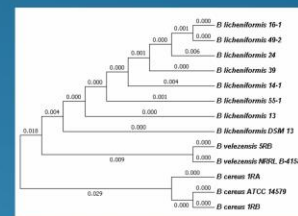


Fig. 1. Phylogenetic tree of the isolates.

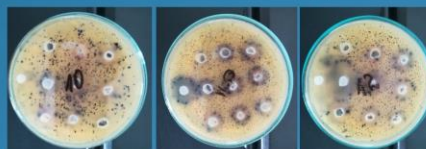


Fig. 2. Lignocellulosic hydrolytic activity.

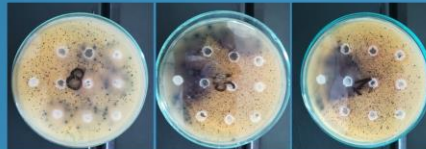


Fig. 3. Hemicellulosic hydrolytic activity.

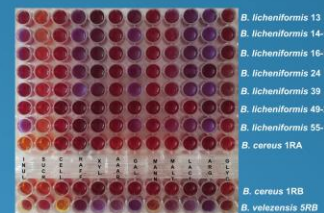


Fig. 4. Utilization of lignocellulosic monosaccharides and disaccharides demonstrated by growth in medium containing bromocresol purple.

## Conclusions

- Promising non-pathogenic producers of 2,3-BD were isolated.
- Wide spectrum of active hydrolytic enzymes were displayed.
- The established enzyme activities could be successively used in a new biotechnology for 2,3-BD production from lignocellulose.

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