Insulin production and expression

TERÇA-FEIRA, 18/04/2017

Lactococcus lactis electrocompetent, the first day of the protocol. The microorganism was directly inoculated from stock culture (No. 115, -80 ° C) into 4 glass tubes containing 5mL of MRS medium and erythromycin (3µg / ml)
Growth overnight, 37 without shaking

QUARTA-FEIRA, 19/04/2017

Second day of protocol. Various means were developed to continue the procedure:

- Meio M17
- Regeneration Medium
- Solid M17 medium
- SGM17 Medium
- Glucose solution 50%
- Eletroporation solution
- Wash solution

The tubes containing L.lactis that had been grown the previous day were transferred to erlenmayer flasks. 1mL of the 18/04 culture was inoculated into 200mL of MRS and SGM17 medium.
Growth overnight, 37 °C without shaking

QUINTA-FEIRA, 20/04/2017

The cultures 04/19 have grown.
The protocol to make the microorganism electrocompetent was performed.
Stock in freezer at -80 ° C of L.lactis electrocompetent

SEGUNDA-FEIRA, 24/04/2017

Transformation of the electrocompetent L.lactis using the plasmid pSEUDO_Pusp45_sfGFP (Bs)
Protocol of electroporation:
- 100µL *L.lactis*
-120 ng / µl pSEUDO_Pusp45_sfGFP (Bs)
After electroporation the microorganism was plated on plates containing M17 medium (5µg / mL erythromycin)
Plate: *L.lactis* + pSEUDO-GFP

TERÇA-FEIRA, 25/04/2017

L.lactis + pSEUDO-GFP plates had colony growth! Inoculum was made in liquid M17 medium (5mL) containing 5µg / mL erythromycin.

QUINTA-FEIRA, 27/04/2017

Due to the medium being very dark it was necessary to perform a procedure of centrifugation and resuspension of the pellets in saline solution:
1- Centrifuge 2mL culture, 5 minutes, 4000RPM
2- Resuspension in 1% NaCl solution (10mL water + 0.1g NaCl)

There was fluorescence reading proving the production of GFP!

Stock of crops: Nº128 - L.lactis pSEUDO-GFP

GROWTH CURVE!

QUARTA-FEIRA, 03/05/2017

L.lactis Viability Test under acidic conditions (pH 2.6)

QUINTA-FEIRA, 27/07/2017

Synthetic insulin sequences have arrived from IDT!

IDT gBlock

	Cod.	Name
1	174	galP-sRNA-term rrnB(Bs) - IDT gBlock
2	175	galP-sRNA-rrnB term(Ec) - IDT gBlock
3	176	galP-sRNA-F30Broccoli-rrnB - IDT gBlock
4	177	Pusp45-signal-penetratin-SC-I57-His6 - IDT gBlock
5	178	Pusp45-signal-penetratin-SCI-57 - IDT gBlock
6	179	Pusp45-signal-PenShuf-SCI insulin - IDT gBlock
7	180	Pusp45-signal-SCI-57-penetratin - IDT gBlock
8	181	Pusp45-signal-SCI-57 - IDT gBlock
9	182	PsrfA-yncM-penetratin-SCI57 (B. subtilis) - IDT gBlock
10	183	Pusp45-signal-penetratin - IDT gBlock
11	184	Pusp45-signal-PenShuf - IDT gBlock
12	185	yncM-ASNase GsIDT gBlock

PCR Reaction

	Materials	177	178
1	Dna	1 µL	1 µL
2	Primer 1	2,5 µL	2,5 µL
3	Primer 2	2,5 µL	2,5 µL
4	Buffer	10 µL	10 µL
5	Dna pol	0,5 µL	0,5 µL
6	Water	32,5 µL	32,5 µL
7	Dntps	1 µL	1 µL
8	Total	50 µL	50 µL

Thermocycler program

	Temperature	Time	C
1	98°C	1 min	
2	**98°C**	**50 s**	**30 cycles**
3	**55°C**	**50s**	
4	**72°C**	**4 min**	
5	72°C	7 min	
6	4°C	hold	

Concentrations obtained after purification and reading in nanodrop:
181: 73.7 ng / ml
182: 96.7 ng / ml
183: 99.4 ng / ml
184: 120.2 ng / ml

A digestion reaction was performed:
Sequences 181, 182, 183, 184 and pSEUDO-bbp plasmid

Digestion reaction

	Material	181	182	183	184	pSEUDO-bbp
1	DNA 1000 ng	13,6 uL	10,4 uL	10 uL	8,32 uL	6,41 uL
2	SpeI	1.0 uL	1.0 uL	1.0 uL	1.0 uL	1.0 uL
3	EcoRI	1.0 uL	1.0 uL	1.0 uL	1.0 uL	1.0 uL
4	CIP	-	-	-	-	1.0 uL
5	Buffer	5 uL	5 uL	5 uL	5 uL	5 uL
6	Water	29,4 uL	34,6 uL	33 uL	34,68 uL	36,6 uL
7	TOTAL	50 uL	50 uL	50 uL	50 uL	50 uL

Incubação a 37°C, por 1 hora.

SEGUNDA-FEIRA, 31/07/2017

Ligation Reaction:

- Insert: sequences 177, 178 and 179
- Vector: pSEUDO-bbp-RFP

Ligation Reaction - 31/07					
	Material	177	178	179	Negative Control
1	Plasmid	5,8 uL	5,8 uL	5,8 uL	5,8 uL
2	Insert	3,48 uL	3,73 uL	6,5 uL	-
3	Buffer	2 uL	2 uL	2 uL	2 uL
4	Ligase	1 uL	1 uL	1 uL	1 uL
5	Water	7,72 uL	7,47 uL	4,7 uL	11,2 uL
6	Total	20 uL	20 uL	20 uL	20 uL

Incubation for 25 ° C, 1 hour
Inactivation: 65 ° C, 10 minutes

TERÇA-FEIRA, 01/08/2017

PCR - Phusion Protocol of the sequences 177, 178, 179 and 180

PCR Reaction - Phusion Protocol - 01/08			
	Materials	177	178
1	Dna	5 µL	5 µL
2	Primer 1	2,5 µL	2,5 µL
3	Primer 2	2,5 µL	2,5 µL
4	Buffer	10 µL	10 µL
5	Dna pol	0,5 µL	0,5 µL
6	Water	28,5 µL	28,5 µL
7	Dntps	1 µL	1 µL
8	Total	50 µL	50 µL

QUARTA-FEIRA, 02/08/2017

Digestion Reaction of the sequences 177, 178, 179 and 180 using the enzymes EcoRI and SpeI Concentrations:
177: 130.3 ng / µL
178: 150.8 ng / µL
179: 171.6 ng / µL
180: 164.8 ng / µL

Digestion Reaction 02/08

	Materials	177	178	179	180
1	DNA (2000ng)	15,4 µL	13,3 µL	11,65 µL	12,13 µL
2	Buffer	10 µL	10 µL	10 µL	10 µL
3	EcoRI	2 µL	2 µL	2 µL	2 µL
4	SpeI	2 µL	2 µL	2 µL	2 µL
5	Water	70,6 µL	72,7 µL	74,35 µL	73,87 µL
6	Total	100 µL	100 µL	100 µL	100 µL

Incubation at 37 ° C for 1 hour.
Ligation Reaction: A Binding Reaction was performed between inserts 177, 178, 179 and 180 and the plasmid pSEUDO-bbp

Ligation Reaction 02/08

	Materials	177	178	179	180	Negative Control
1	Insert	3,66 µL	2,4 µL	2,32 µL	2,03 µL	-
2	Plasmid	1,32 µL	1,32 µL	1,32 µL	1,32 µL	1,32 µL
3	Buffer	2 µL	2 µL	2 µL	2 µL	2 µL
4	T4 Ligase	1 µL	1 µL	1 µL	1 µL	1 µL
5	Water	12,02 µL	13,28 µL	13,36 µL	13,65 µL	15,68 µL
6	Total	20 µL	20 µL	20 µL	20 µL	20 µL

The samples were incubated at 22 ° C for 1 hour.

SEXTA-FEIRA, 04/08/2017

Digestion Reaction using SpeI, EcoRI and CIP from the pSEUDO-bbp and pSEUDO (original) plasmids obtained by PCR

Digestion Reaction 04/08

	Materials	pSEUDO-bbp	pSEUDO
1	DNA	4,03 µL	5 µL
2	EcoRI	2 µL	2 µL
3	SpeI	2 µL	2 µL
4	CIP	1 µL	1 µL
5	Buffer	10 µL	10 µL
6	Water	80,97 µL	80 µL
7	Total	100 µL	100 µL

Incubated samples at 37 ° C, 1 hour.

TERÇA-FEIRA, 08/08/2017

A Digestion Reaction was performed to confirm if the insulin sequence was actually attached to the plasmid pSEUDO (binding made on 02/08)

QUARTA-FEIRA, 09/08/2017

Ligation Reaction 09/08

	Materials	177	178	189
1	Insert	2,2 µL	1,46 µL	1,23 µL
2	Plasmid	1,2 µL	1,2 µL	1,2 µL
3	Buffer	1 µL	1 µL	1 µL
4	Water	5,2 µL	5,94 µL	6,17 µL
5	T4 Ligase	0,4 µL	0,4 µL	0,4 µL
6	Total	10 µL	10 µL	10 µL

A binding reaction was performed between the sequences 177, 178 and 180 and the pSEUDO-bbp plasmid
The ratio used for this connection is 1: 3
A binding reaction was performed between the sequences 177, 178 and 180 and the pSEUDO-bpp plasmid The ratio used for this connection is 1: 3

sequencing 09/08

	Materials	179-pSEUDO-bbp1	179-pSEUDO-bbp2
1	DNA 500ng	3,5 µL	2,7 µL
2	Big Dye Mix*	1 µL	1 µL
3	Primer (3,2 µM)	2 µL	2 µL
4	5X Buffer	3,5 µL	3,5 µL
5	Water	10 µL	10,8 µL
6	Total	20 µL	20 µL

QUINTA-FEIRA, 10/08/2017

Connection Purification Reaction performed on 09/08 Concentrations obtained:
-177: 453.4
-180: 345
-180: 341.1

Digestion of the ligations for electrophoresis technique in order to confirm if the insert was correctly attached to the plasmid:

Digestion

	Materials	177	180	180`
1	DNA 600ng	1,33 µL	1,74 µL	1,76 µL
2	SpeI	0,2 µL	0,2 µL	0,2 µL
3	EcoRI	0,2 µL	0,2 µL	0,2 µL
4	Buffer	3,5 µL		3,5 µL
5	Water	7,27 µL	6,86 µL	6,84 µL
6	Total	10 µL		10 µL

SEXTA-FEIRA, 11/08/2017

Materials:

- Sequences insert:
  - **178**: Pusp45-signal-penetratin-SCI-57 - IDT gBlock
  - **180:** Pusp45-signal-SCI-57-penetratin - IDT gBlock
- Plasmid:
  - pSEUDO

Ligation Reaction (pSEUDO and 178 or 180)

	Materials	178	180
1	Insert (36,3ng)	1,452 µL	1,23 µL
2	Plasmid (100ng)	1,2 µL	1,2 µL
3	Buffer	1 µL	1 µL
4	Ligase T4	0,4 µL	0,4 µL
5	Water	5,5 µL	6,2 µL
6	Total	10 µL	10 µL

The sequences

TERÇA-FEIRA, 12/09/2017

Digestion Reaction (Insulin 182 and plasmids)

	Materials	pBs1c3	pBs1c	pLike	182
1	DNA	4 µL	10 µL	10 µL	10 µL
2	EcoRI	0,2 µL	10 µL	10 µL	10 µL
3	PstL	0,2 µL	10 µL	10 µL	10 µL
4	CIP	0,1 µL	1 µL	0,5 µL	-
5	Buffer	1 µL	10 µL	10 µL	10 µL
6	Water	9,5 µL	10 µL	10 µL	10 µL
7	Total	15 µL	100 µL	50 µL	50 µL

After that the ligation reaction of this samples were plated. But the colonies didn't grow.

QUINTA-FEIRA, 14/09/2017

We repeat the ligation reaction in the following conditions:

Repetition of Ligation Reaction

	Materials	pLike	pBs1C	pLike (Control)	pBs1C (Control)
1	DNA (100ng)	4,739 µL	2,9	5,98 µL	2,9 µL
2	Insert (182)	1,429 µL	1,56 µL	-	-
3	Buffer	1 µL	1 µL	1 µL	1 µL
4	Ligase T4	0,4 µL	0,4 µL	0,4 µL	15 µL
5	Water	2,432 µL	4,14	2,61 µL	5,7 µL
6	Total	10 µL	10 µL	10 µL	10 µL

Thereafter, the Ligation reaction of these samples was transformed and plated into LB medium + Kanamycin or Chloramphenicol according to resistence marker into plasmid. And our colonies grow up!

SEGUNDA-FEIRA, 18/09/2017

Master Mix

	Materials	By reaction	22 Reactions
1	EcoR1	0,2 µL	4,4
2	PstI	0,2 µL	4,4
3	Buffer	1	22
4	Total	1,4	30,8

Using this mix plus plasmids into a concentration of 600 ng according to table:

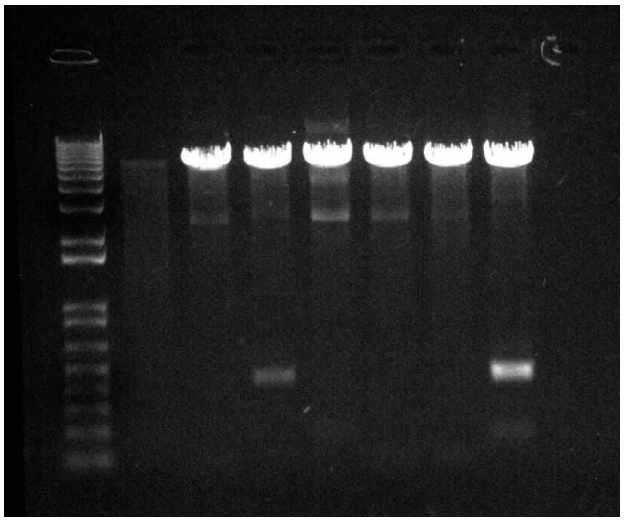
Data for Digestion Reaction

	pBs1c	pBs1c (100)	pLike	pBs1c (Control)	pBs1c (100) (Control)	pLike (Control)
1	78,5 µL	94,7 µL	495,6 µL	7,6 µL	6,7 µL	1,2 µL
2	43,6 µL	87,4 µL	401,2 µL	7,6 µL	6,7 µL	1,5 µL
3	87,9 µL	63,1 µL	126,9 µL	7,6 µL	7,6 µL	6 µL
4	74 µL	88,4 µL	205,7 µL	7,6 µL	6,7 µL	3 µL
5	81,7 µL	78,4 µL	73,2 µL	7,6 µL	7,6 µL	7,6 µL
6	66,4 µL	50,1 µL	253,4 µL	7,6 µL	7,6 µL	3 µL

So we did make a electrophoresis reaction to confirm the binding of insulin number 182 into plasmids PBS1C and pLIKE.

pLIKE:
Lether-Control- Sample 1 - Sample 2 - Sample 3 - Sample 4- Sample 5- Sample 6

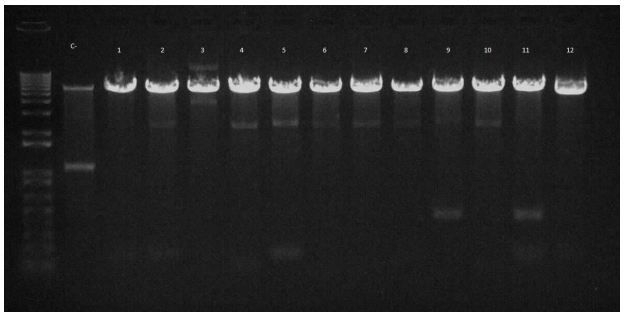
eletroforese-18-setembro-pLIKE-182 OK.jpg



Samples number 2 and 6 were positive!

pBs1C:

eletroforese-18-setembro-PBS1C-OKnumber.jpg



The samples numer 3 and 5 were positive!

Samples for positive cultures were stored in the freezer -80.

QUARTA-FEIRA, 20/09/2017

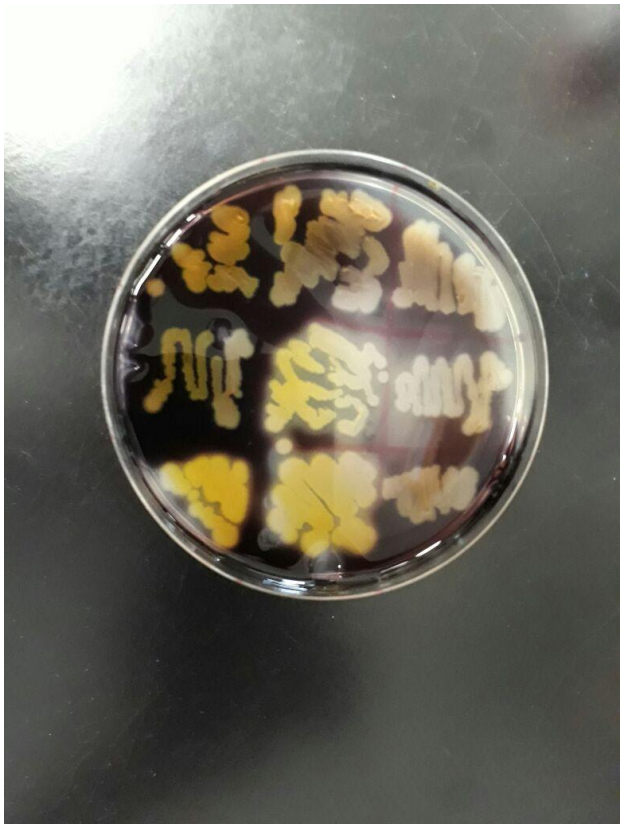
With the constructs containing insulin in the hands we were able to make the transformation in *Bacillus subitilis* K07.

Transformation of Bacillus

	Sample	Concentration (500 ng/ul)
1	pBs1C (100) 3	8 µL
2	pBs1C (100) 5	6,34 µL
3	pLike 2	1,3 µL
4	pLike 6	2 µL

After the transformation we made a plate with starch to do the lugol test:

WhatsApp Image 2017-10-28 at 12.58.14.jpeg



This test shows us that *Bacillus subtilis* K07 integrated the plasmid into its chromosome. So, in order to confirm that an integration went well we sequenced their genome to see if the insulin is now part of it. But the sequence was not integrated

QUARTA-FEIRA, 27/09/2017

On that day we did the repetition of the steps of digestion reaction until electrophoresis. When we tried again twice, we had some problems during the reactions and they kept going wrong. We believe that errors were associated with the conditions of the digestion reaction. So we did a review on the calculations.

TERÇA-FEIRA, 03/10/2017

Correct reactions:

Digestion Reaction

	Materials	pLike-rep	pBs1c
1	DNA (1000ng)	7,7	6,8
2	EcoRI	1	1
3	SpeI	-	1
4	XbaI	1	-
5	CIP	0,5	0,5
6	Buffer	5	5
7	Water	34,8	35,7
8	Total	50	50

Ligation reaction

	Materials	pLike-rep	pBs1C	Negative Control pLike	Negative Control pBs1c
1	Insert	1,31	1,31	-	-
2	Plasmid	5,43	2,71	5,43	2,71
3	Buffer	2	2	2	2
4	Ligase T4	1	1	1	1
5	Water	10,26	12,98	11,57	14,39
6	Total	20	20	20	20

After the Ligation reaction of these samples was transformed and plated into LB medium + Kanamycin or Chloramphenicol according to resistence marker into plasmid. And our colonies grow up! So, we make the cultive of this cells and prepare to analyse :

- Steps:
    - **Growing:** 1 mL of cells into 50 mL LB medium plus erythromycin at 37°C and 220 rpm overnight;
    - **Centrifugation**: 5000 rpm at 30 minutes;
    - **Separation of insulin**: Transfer the supernatant to a Falcon with a cutting membrane of 5 KDa and centrifugation 4000rpm for 30 minutes

QUARTA-FEIRA, 18/10/2017

We did qualitative / quantitative analyses for:

- Bradford;
- SDS-Page;
    - We junst found a very small band with the size of Insulin;