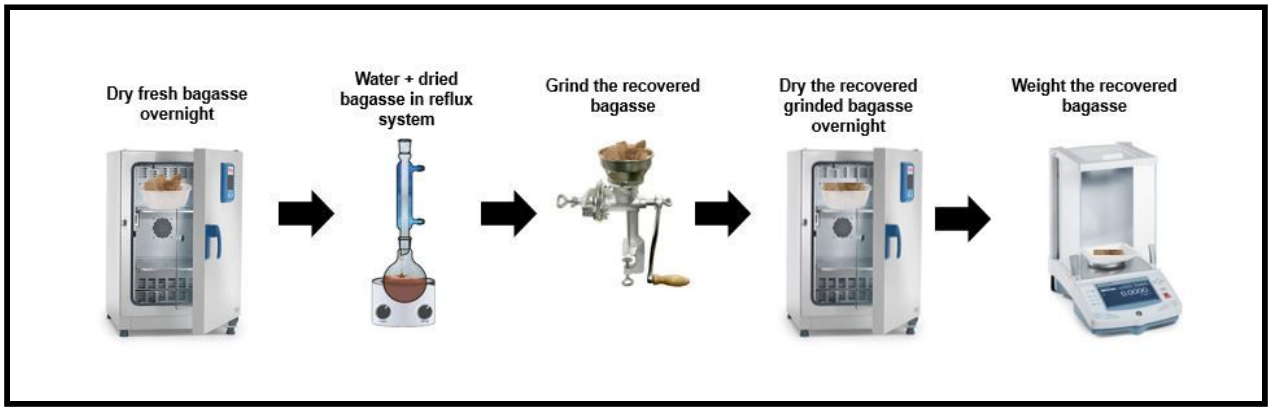
The following data describes the results of our lab work and why our project works.

Glucose can be obtained from bagasse

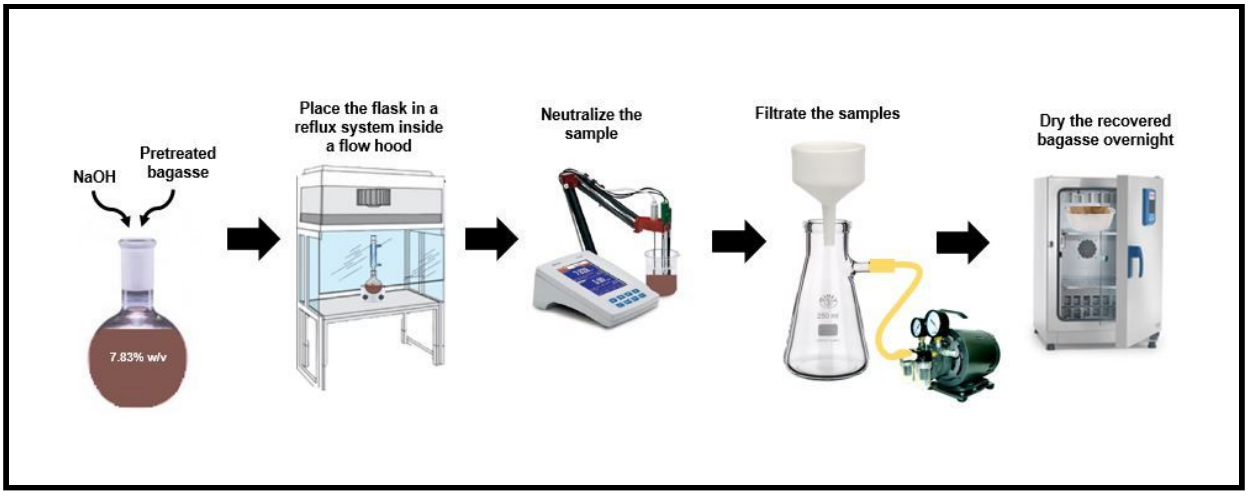
Pseudomona putida KT2440 produces PHA as an energy reserve when feeded with glucose as a carbon source in a nitrogen limited medium. The glucose can be retrieved from a tequila production waste product known as *bagasse*, following the next simple steps:

1. Thermo-hydrolysis



First, 14 g of bagasse were weighed and placed into the ball flask, and 84 mL of water was added to the flask to achieve a $\frac{6\ ml\ water}{1\ g\ bagasse}$ ratio. This was done in triplicate.

2. Alkaline treatment



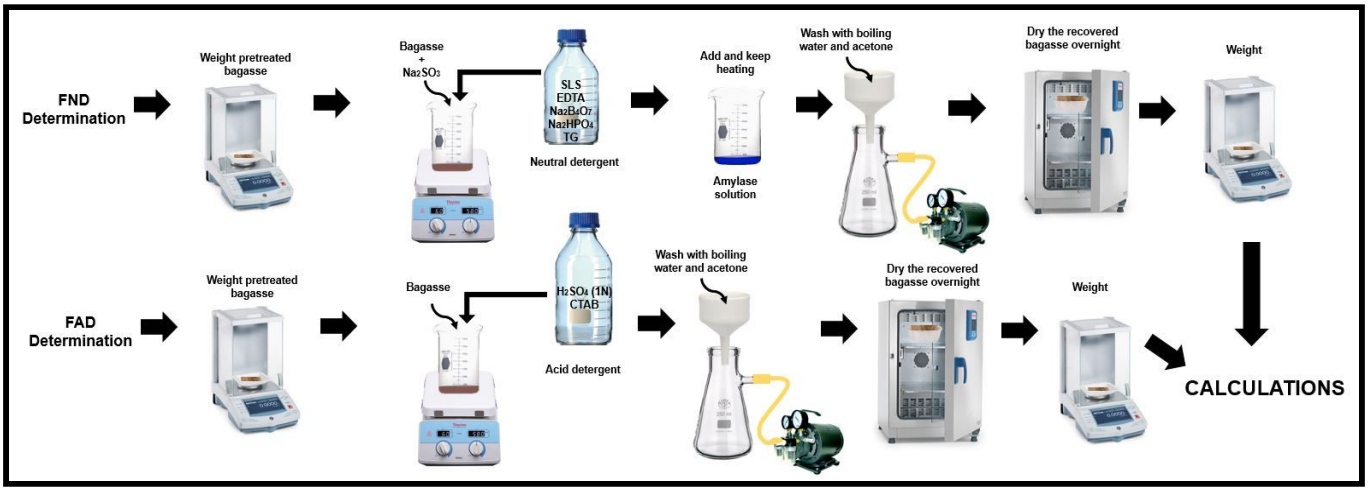
From the bagasse retrieved in the last procedure:

- ➔ 12.6990 g were added to 162.1839 mL of NaOH and placed in a 500mL ball flask.
- ➔ 10.5409 g were added to 134.6220 mL NaOH and placed in a 500mL ball flask.
- ➔ 13.3833 g were added to 170.9260 mL NaOH and placed in a 500mL ball flask.

After the alkaline hydrolysis, the retrieved bagasse was:

- ➔ Sample 1: 10.46 g
- ➔ Sample 2: 10.13 g
- ➔ Sample 3: 7.53 g

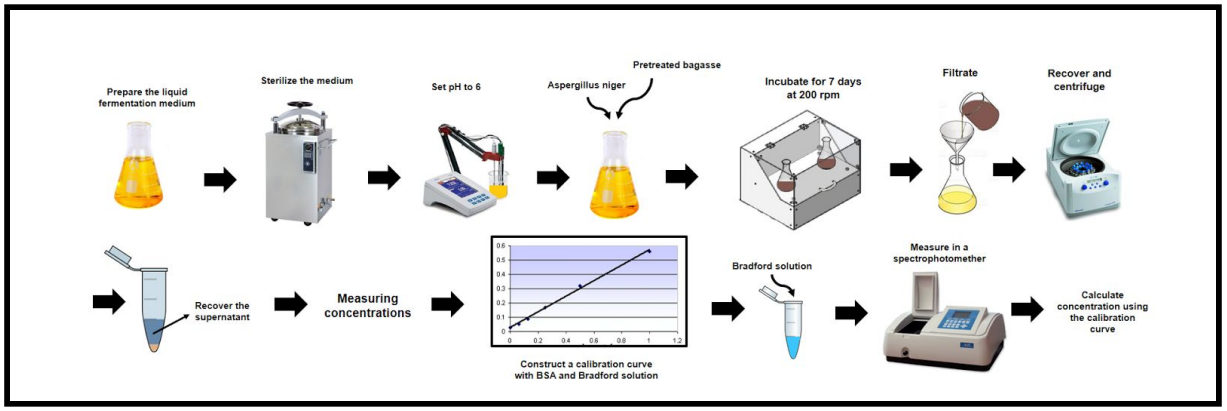
3. Cellulose quantification



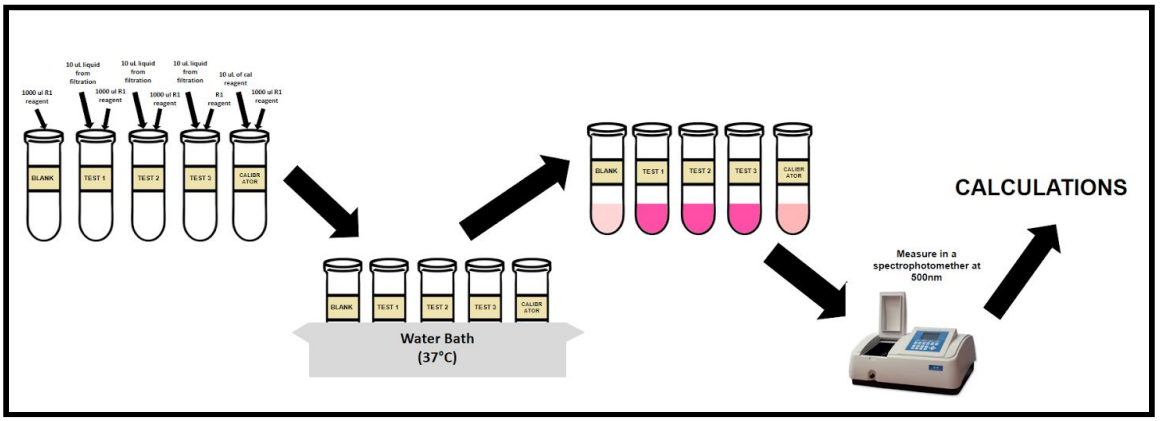
The obtained cellulose after the two treatments was:

Test Number	**Weight of bagasse FAD**	**Weight of bagasse FND**	**Weight of bagasse before treatment**	**% Cellulose**
Untreated Sample				
S1	0.6119 g	0.3423 g	1.117 g	24.13607 g
S2	0.5853 g	0.4297 g	1.1109 14.00666	g
S3	0.6463 g	0.3668 g	1.115 g	25.06726 g
Treated Sample				
P1	0.6771 g	0.2089 g	1.1407 g	41.0449 g
P2	0.8063 g	0.4300 g	1.1102 g	33,894 g
P3	0.8324 g	0.4062 g	1.1441 g	37.251988

4. Obtention of Saccharifying cellulase from *Aspergillus niger* for an enzymatic hydrolysis



5. Glucose obtention and quantification



The obtained glucose was calculated as follows:

Glucose concentration (mg/dl) = A sample x 1000(mg/dl)

Negative control

Glucose concentration (mg/dl) = -0.5 / .181 x 1000(mg/dl) = 0mg/dl

Test 1

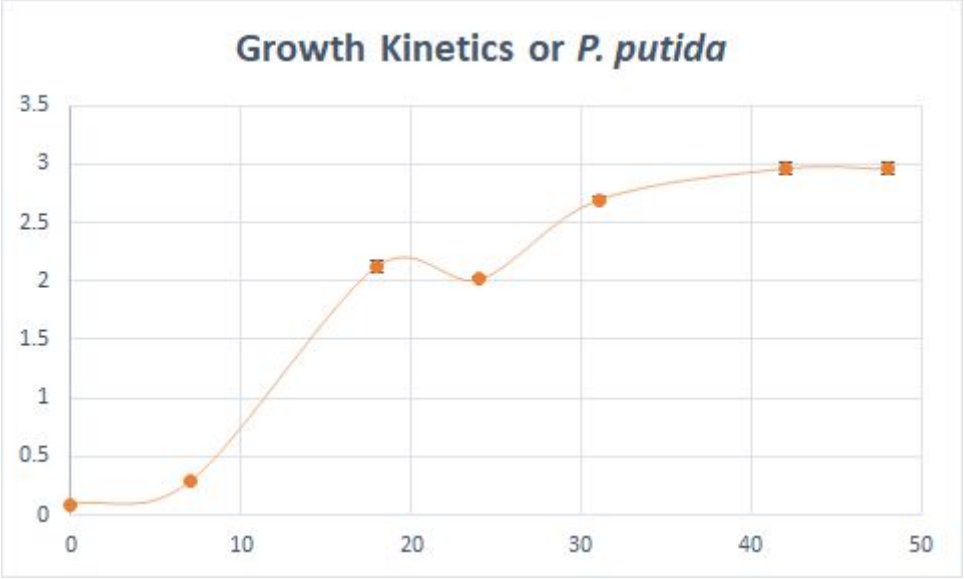
Glucose concentration (mg/dl) = 0.905/0.181 x 1000(mg/dl) =0.0501g/ml

Test 2

Glucose concentration (mg/dl) = 0.911/0.181 x 1000(mg/dl) =0.0512g/ml

***Pseudomona putida* KT2440 is able to grow when fed with the obtained glucose**

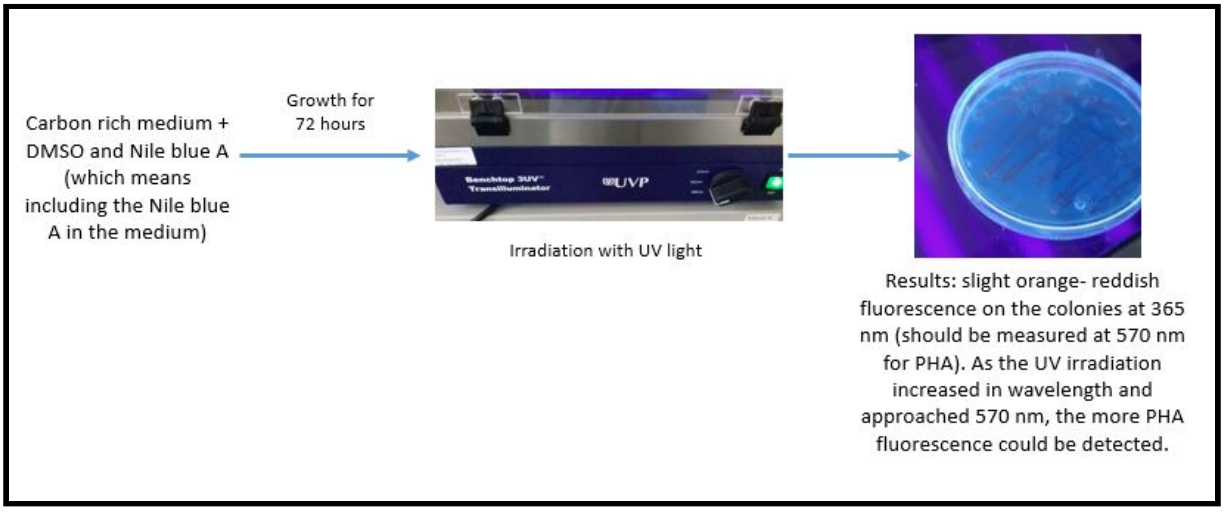
Pseudomona putida KT2440 can grow in a carbon rich medium supplied with glucose obtained from agave bagasse. Growth kinetics in a medium supplied with 10 g/L of this glucose, show the following absorbances at different times, resulting in a long exponential phase, represented in *Graph 1*.



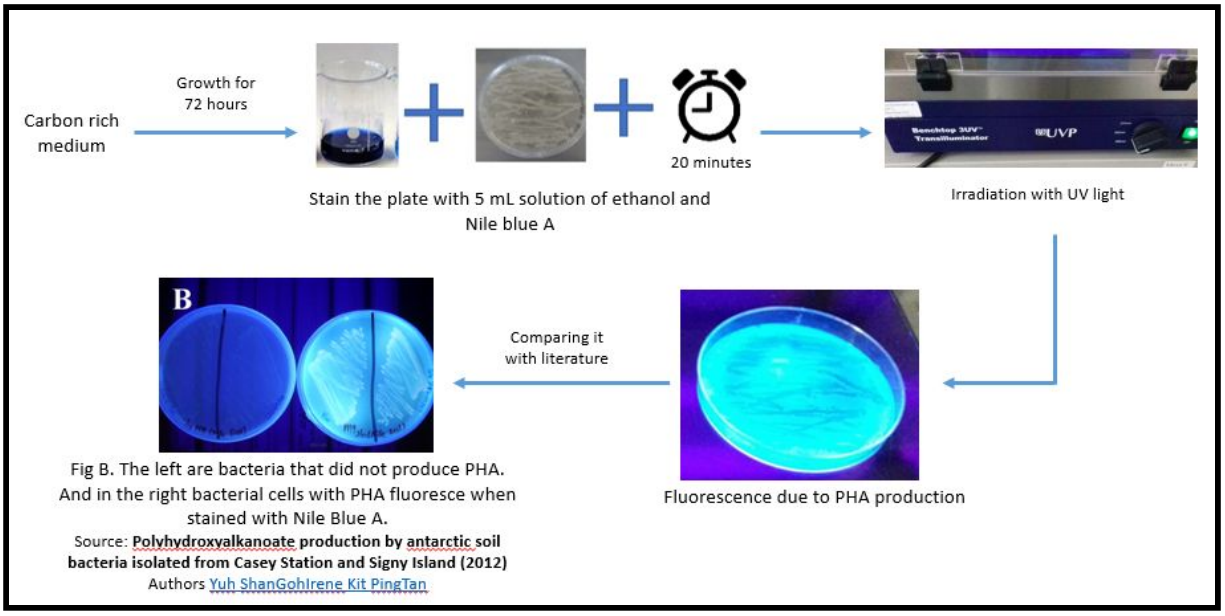
Graph 1. Growth kinetics of P. putida in a medium supplied with bagasse's glucose (10%).

Pseudomona putida **KT2440 can produce PHA**

Pseudomona putida KT2440 is capable of producing PHA in a carbon rich medium. By plate staining with Nile Blue, PHA can be easily detected using UV light irradiation. The following steps show PHA detection using different staining methods: 1) using staining directly in the medium and 2) staining in a slide before growth.

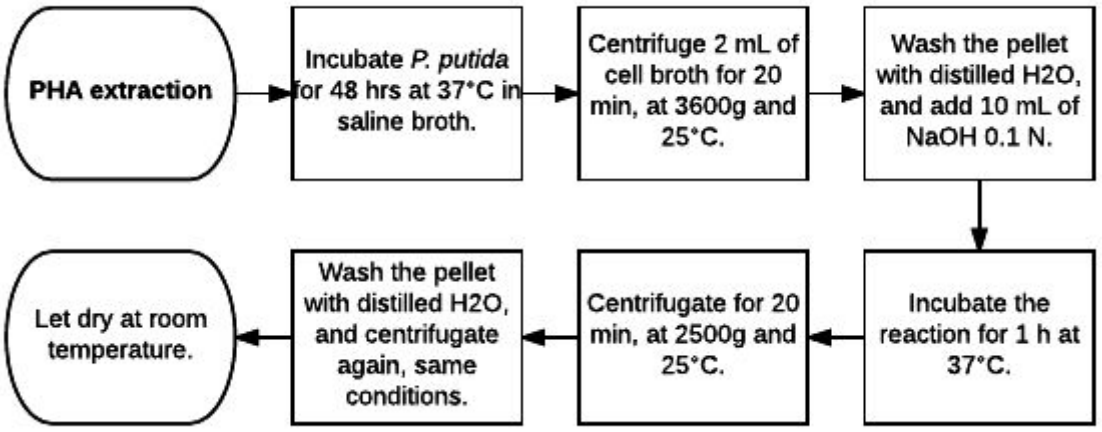


1) Plate staining using Nile Blue A in a carbon rich medium

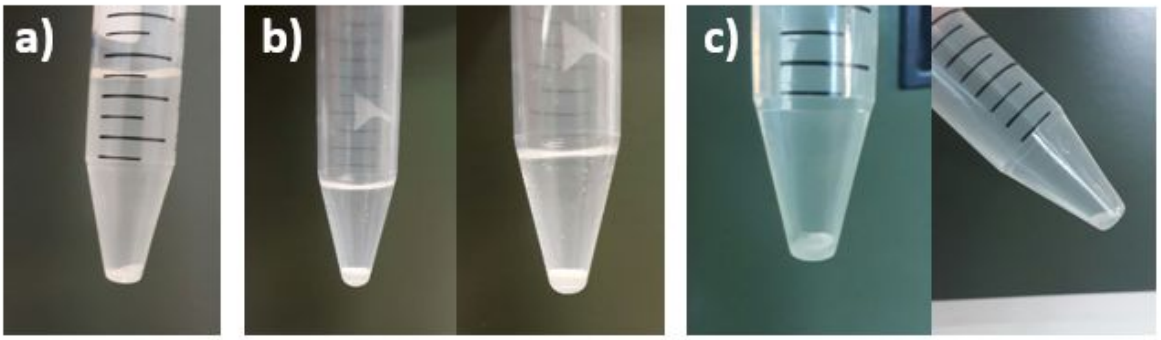


2) Nile blue + ethanol staining before 72 hours of growth in a carbon rich medium

By following the next steps, PHA was recovered from the carbon rich medium containing *Pseudomona putida* KT2440.



The amount of recovered PHA at different incubation hours is shown below.



Recovered PHA after different times of incubation in a carbon rich medium: a) 24 hours; b) 48 hours; c) 72 hours.

In a carbon rich medium supplied with bagasse's glucose, the amount of PHA produced by *Pseudomona putida* KT2440 is similar than the produced with commercial glucose. The difference lays in the time it takes to produce it, as shown below. The highest production of PHA was after 18 hours of growth; 6 hours later (24 hours of growth) the bacteria keeps producing PHA but in a less quantity.

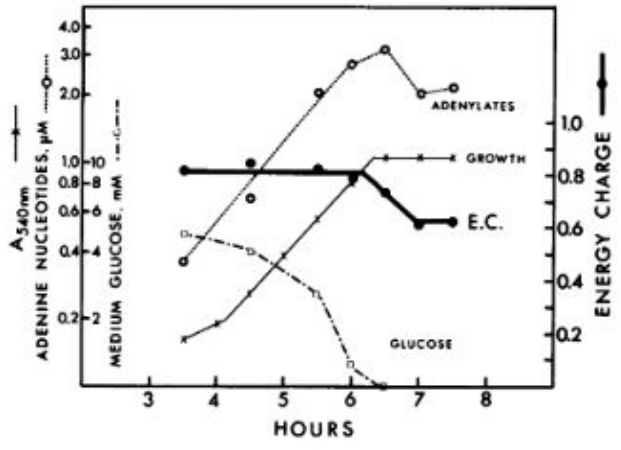
Time	PHA dry weight
18 hours	0.0051
24 hours	0.0006

Recombinant E. coli

The following is expected from the recombinant E. coli that we designed.

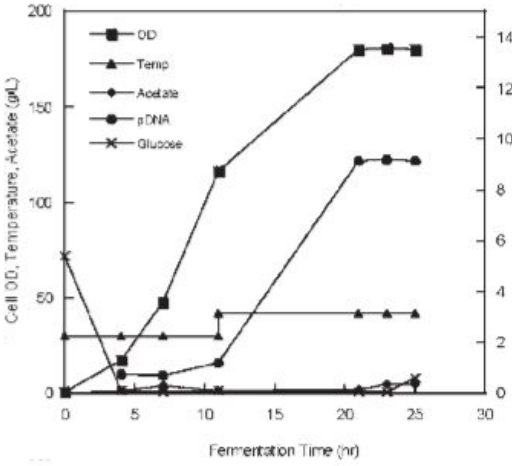
Depending on the glucose concentration, *E. coli* DH5α reaches its stationary phase between the 10th and 20th hour of incubation, as shown in different literature:

➔ With a 5.5 mM concentration, it takes approximately 7 hours:



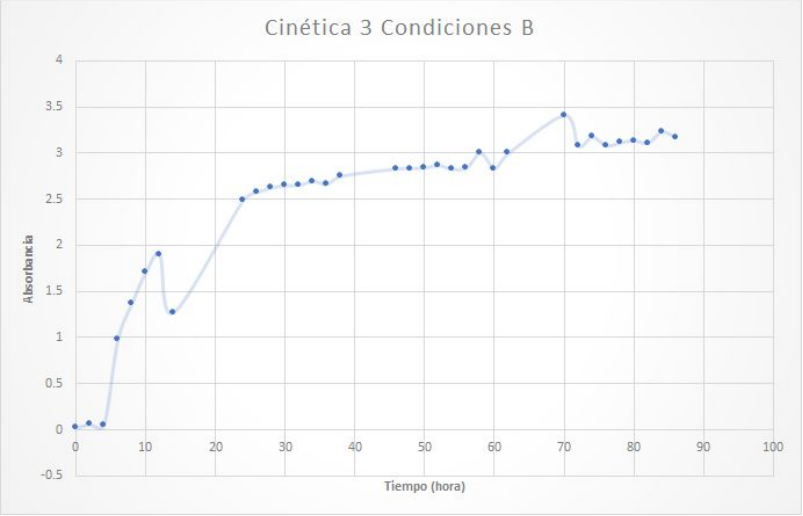
Chapman et al., 1971

➔ And with a 5 g/L glucose concentration, it takes approximately 20 hours:



As shown before, Pseudomona putida KT2440 takes considerately more time to grow, making the process slower and less efficient.

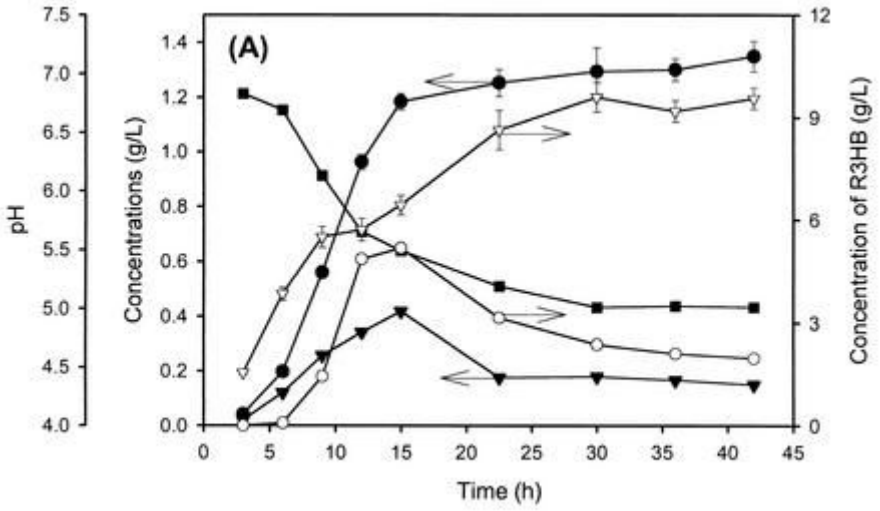
- ➔ With a 20 g/L concentration, it takes approximately 50 to 60 hours to reach stationary phase:



- ➔ And even with a much higher glucose concentration (35 g/L), it takes approximately 30 to 40 hours:

Even though the times reduces a little, it is still a lot more than with *E coli*, so the ideal PHA recovery time could reduce from 24 hrs to 5-10 hours.

With 20 g/L of glucose concentration (as our *Pseudomona putida* KT2440 kinetic), Lee et al. (2003) needed approximately 15 to 20 hours to achieve its maximum PHB (which is a type of PHA) concentration, using a recombinant *E coli* with phaC and phaB genes.



Where: Time profiles of cell concentration (•), PHB concentration (▾), pH (▪), and R3HB concentration before (○) and after (▽) alkaline heat treatment.

The literature proves how our process will be faster and better with our recombinant bacteria than the one with the wild type, making it cheaper and more efficient. There's no reason why the process would not work, hence its validated.

References

Chapman, A.; Fall, L.; and Atkinson, D. (1971). A*denylate Energy Charge in Escherichia coli During Growth and Starvation*. Journal of bacteriology. 108(3). Retrieved from: http://jb.asm.org/content/108/3/1072.full.pdf

Lee, S; and Lee, Y. (2003). *Metabolic Engineering of Escherichia coli for Production of Enantiomerically Pure (R)-(−)-Hydroxycarboxylic Acids*. Applied and environmental microbiology. 69(6). Retrieved from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC161469/

Phue, J.; Jun, S.; Trinh, L.; and Shiloach, J. (2008). M*odified Escherichia coli B (BL21), a Superior Producer of Plasmid DNA Compared With Escherichia coli K (DH5a)*. Biotechnology and Bioengineering. 101(4). Retrieved from: http://0-onlinelibrary.wiley.com.millenium.itesm.mx/doi/10.1002/bit.21973/epdf