1.1 UP-STREAM PROCESS

Fermentations has three distinct phases:

1. The inoculum, which is carried out in a flask incubated in a shaker. This first culture is seeded directly from a vial of a deeply characterized Research Cell Bank or Master Cell Bank. Part of the contents of the flask is sterilely transferred inside the fermenter, after about 16 hours.
2. The batch phase, which corresponds to the first part of fermentation where the growth rate of the microorganism is maximal. This first phase ends when the microorganism has used all the available carbon source.
3. A fed-batch phase, where the microorganism growth rate is controlled by the delivery of a substrate inside the fermenter from an external pump, in our case pump number 4.

During the process development period, a total of 28 different fermentation tests were set up and carried out individually or in parallel. It is important to specify that almost all the collected material was used to fine-tune the purification process.
During fermentation, some data were collected by the system in real time, such as the dissolved oxygen value, the stirrer speed, the amount of incoming gas, the pH value, the added liquids such as acid, base and feed.
All these parameters are sampled every minute and collected in an Excel table which are used as a basis for developing artificial intelligence models. In addition to these parameters, it was appropriate to complete the data set with additional parameters measurement generated “off line”:

- The first one corresponds to the optical density measured at 600 nanometers (OD_{600nm}). The measurement is carried out with a spectrophotometer on a suitably diluted aliquot of culture to read a value comprised between 0.2 and 0.6. In this range, the value obtained corresponds to the number of cells present in the fermenter. The conversion factor is 8 10^8 cells per unit of OD_{600nm} and per millilitre of culture. This means that when 50 OD_{600nm} is obtained in one fermentation, the number of cells per millilitre is already 40 billion.

- The second value is the Biomass weight, obtained by centrifugation at the end of the fermentation process. It corresponds to the total weight of bacteria present in the culture medium.

- The inclusion bodies (or IB) weight. This data is one of the most important and corresponds to the material that contains the recombinant protein. These inclusion bodies are formed of recombinant protein aggregates which, being insoluble into the microorganism cytoplasm, accumulates in these structures that we can compare to sand grains. To extract them, it is necessary to "homogenize" the culture, at a pressure of about 750 ± 50 bar, to break the cells and "liberate" the inclusion bodies.

These off-line parameters allowed to complement those collected online and to set-up efficient model capable of predicting the progress of fermentation.

A summary of all the different fermentations performed is reported in the table below containing the description of the different parameters that have been varied during time.

From the fermentations data reported in the table it can be seen that the best yield has been achieved in “run N° 8 ”. These conditions have been repeated in triplicate in runs 22, 23 and 24.

The following table shows, a simplified version of the main parameters monitored in each fermentation carried out during this phase of the project alongside with relevant comments.

Run n.	final OD_{600}	Biomass (gr)	IB (gr)	Comments
1	19,7			Inoculum overgrowth the fermentation has been stopped
2	64,6	59,6	13,5	First fermentation
3	61,4	59,4	13,5	First part of the culture at 30°C, then raised to 37°C for fed batch phase
4	51,6			tested DO_{stat}, but we first need to adjust the culture brought
5	74	64,5	15.1	culture brought modified
6	64,8	70,2	12,6	culture brought modified
7	55,2	59,6	14,1	Less IPTG
8	63,4	66,6	13,5	Best conditions
9	56,4			Problem during the night with utilities - stopped
10	64			Problem during the night with utilities - stopped
11	55	54,4	12	Added 1 hour before induction
12	62,7	59,3	12	4 h of induction
13	96,8	47,3	9,7	OD_{600} and biomass do not correspond, pb in the culture brought
14	40,1			O_2 missing during the night, culture died
15	115,2	38,1	11,3	Same problem than test 13. Glycerol as carbon source
16	56	51	10.8	Increased Inoculum volume
17	28,7			Run with Overnight culture. Only batch phase determination
18	67,4	57,3	13.7	Glycerol as carbon source
19	52,8	124,2		Late induction, high biomass but no recombinant protein production.
20	59,6			Not induced culture
21		48	118	Substrate glycerol and induction too late
22	51.1	53.6	10	Test 8 Replicate
23	50.5	58.4	12.4	Test 8 Replicate
24	52	53.9	10.1	Test 8 Replicate
25	50.3	302	NA	Modified the expression system no inclusion bodies accumulation. Fed-batch phase has been performed at 20°C overnight.
26	55.6	301.1	NA	Same as run 25 with a different inducer concentration (IPTG)
27	44.6	66.3	NA	Same as run 25 with fed-batch phase at 37°C for 3 hours.
28	32.4	69.3	NA	Same as run 25 with fed-batch overnight at 30°C

The first fermentations corresponding to runs 1 to 8, have made possible to fine-tune the protocol and optimize some parameters, while the subsequent fermentations have been set up to explore the design space and verify the influence of some parameters on the process yield. Furthermore, in order to check the program efficiency on very different conditions, the last four fermentations included in the panel have been run in the same medium and with the same *Escherichia coli* strain, but with a different expression system and playing with fed-batch temperature and induction time.

The rationale of the different fermentations set up is briefly summarized below:

1. This run was unsuccessful because the inoculum, used to inseminate the fermenter, had grown too much and the cells, having entered the stationary phase, have never been able to grow properly.

2. In this fermentation a culture medium for *Escherichia coli* was tested. The medium gives excellent growth and allows for a high production of the recombinant protein. However, being very, if not too rich, the quantity of oxygen dissolved in the medium remains always low. Since oxygen is used by the microorganism to metabolize the substrate, oxidizing it, in the presence of a very rich medium, the microorganism oxygen request is very high. Since the quantity of gas entering the fermenter is limited, a too rich medium result in a too fast microorganism growth, which can have as consequence an oxygen request that is impossible to satisfy and lead the microorganism towards a phenomenon of cell lysis.

Furthermore, the medium preparation is itself is very complicated because some are almost at their solubility limit. It was therefore decided to modify the cultivation strategy in the subsequent fermentations in order to decrease the oxygen demand.
This second fermentation was used as a reference for the following ones.

3. In this run, the culture temperature was decreased to 30 ° C in the first part of the fermentation and brought back to 37 ° C for the fed-batch phase. In the first part, at 30 ° C, the cells have a slower growth rate and, in fact, a lower oxygen demand. In the fed-batch part, the growth rate is "driven" by the delivery of the substrate. It therefore becomes possible to reduce the oxygen demand over the entire fermentation period. This strategy was adopted in all subsequent fermentations.

4. This experiment allowed to test a different substrate delivery system in the fed-batch phase. In previous fermentations, the substrate was dispensed manually and exponentially to compensate the increasing number of cells in the fermenter. Pump number 3 started at 3% of its capacity, and this value was increased by 1% every 30 minutes. Instead, in this test, pump number 3 was directly "driven" by the system referring to the dissolved oxygen concentration. Above 50% of dissolved oxygen the pump was activated by the system and substrate delivered. This was in turn oxidized by the cells, and the oxygen concentration was accordingly decreased. When the cells consumed all substrate, the oxygen was no longer used and its concentration increased within the medium, up to exceeding the value of 50% where the system was automatically activated and again substrate delivered and so on. This system called DO_{stat} (Dissolved-oxygen feedback control) allows to always maintain a certain amount of substrate and to deliver a physiological supply. Unfortunately, in those conditions as the medium was very rich, with probably an excess of yeast extract, the system was very slow and inefficient. It was therefore decided to stop this test, and to adjust the medium composition before trying again this strategy.

5. In this fermentation, as mentioned in the previous point, the composition of the culture medium has been modified. Compared to fermentation number 3 the yeast extract quantity has been halved. Since we observe the same growth, and we get the same amount of final biomass, we can conclude that the remaining amount of yeast extract is enough to allow a good microorganism growth.

6. Again, in this run the concentration of the yeast extract present in the feed was halved compared to test number 5. At this point the concentration corresponds only to 25% of the initial one, and it is again possible to observe a correct microorganism growth, and efficient recombinant protein production. For these reasons, the yeast extract concentration was maintained at this value in all subsequent fermentations.

7. In this run, the inducer concentration was modified. The inducer is a small chemical molecule, corresponding to Isopropyl-β-D-1-thiogalactopyranoside, (IPTG, purchased from Merck), a non-metabolizable molecule, analogue of allolactose. IPTG is used to induce the expression of the genes under the control of the Lac operon in *Escherichia coli*. In our expression system, the addition of IPTG induces, after a series of cascades, the expression of the recombinant protein. In this run, the IPTG concentration was lowered from 1 mM, corresponding to the previously used concentration, to 0.4 mM. This modification had only a minor influence on the expression of the recombinant protein.

8. In this fermentation, the IPTG concentration was triplicated, which had the direct consequence of doubling the production of the recombinant protein. From this point on, the IPTG concentration was maintained at 3mM. In those conditions it is possible to obtain excellent fermentation yields compatible with the project target.

9. Immediately after the conclusion of this fermentation, as for number 10, a problem occurred with the breaking of a cooling water pipe, which did not allow the biomass to be collected and processed correctly. Consequently, these data do not need to be taken into account.

10. Same as fermentation number 9.

11. The fermentation protocol has been modified. It was chosen to produce more biomass before induction of the recombinant protein, assuming that starting from a higher biomass, more recombinant protein would have been obtained at the end of fermentation. IPTG has been added an hour later than usual, and the induction phase was maintained for the canonical three hours. However, the results showed that in these conditions, the recombinant protein production is lower than expected.

12. This fermentation was set up to evaluate the influence of the induction time on the production of the recombinant protein. The induction was in fact extended by one hour (four hours instead of three). Also, in this case, the lengthening of the induction time was detrimental to the recombinant protein production yield.

13. In this run during culture medium preparation, as for test number 15, a problem was encountered with the solutions sterilization. At the beginning of the cycle, the autoclave had difficulties in reaching its operating temperature. In fact, the overall sterilization time was much longer than usual, and some components of the culture medium were damaged. The medium obtained was darker than usual, which gave higher values to the 600nm readings on the spectrophotometer. The final OD_{600nm} is not reliable, as it is too high as compared to the collected biomass.

14. In this run the fermenter was inseminated the night before, the culture was continued over-night to obtain, the next morning, a high cell density, almost at the end of the batch phase. The culture would then be carried out with the fed-batch phase throughout the day. Unfortunately, pure oxygen went down during the night and the culture went in anaerobiosis. However, the data has been collected and can be used to evaluate the influence of oxygen shortage on bacterial growth.

15. In this culture, glucose was replaced by glycerol, as carbon source. This experiment aims to replace glucose, in a powder form, with glycerol which is commercially available as liquid solution. This could be an advantage for medium preparation. Glucose must be prepared separately and sterilized as a 60% solution, after which it can be added to the separately sterilized culture medium. When added directly to the culture medium, glucose oxidizes (Maillard reaction) and “caramelizes”. This approach could simplify the preparation of the culture medium in the GMP Plant. Unfortunately, having been prepared together with trial 13 where a prolonged autoclaving time was encountered, the collected OD_{600nm} were overestimated.

16. In this run, the inoculum volume was evaluated, and increased by 33%. Fermentation times have been reduced.

17. This fermentation was inoculated the day before and carryon over-night. The purpose of this experiment was to accurately determine the end of the fermentation batch phase and, determine when all substrate was consumed.

18. Retest the conditions of the fifteenth fermentation, with glycerol as a carbon source.

19. In this fermentation, the moment of induction was postponed to the maximum. Normally the system is induced when the OD_{600nm} reaches a value of around 30, in this case the addition of IPTG occurred when the culture had exceeded the value of 56 OD. The amount of biomass collected at the end of the culture was almost twice the normal (124.2 g) but in those conditions the microorganism was unable to produce the recombinant protein. This lack of production is probably due to a too high metabolic stress of the microorganism.

20. This culture was not induced to verify the influence of the inducer addition on the fermentation kinetics, or in other words on the metabolism of the microorganism.

21. In this experiment the carbon source was again glycerol and induction has been postponed when OD_{600nm} reached a value of about 50. As in test 19, in these conditions the system was not able to produce the recombinant protein.

22. 23 and 24 runs, are replicates of conditions used in run 8. Those three fermentations have shown that the process was very robust and that with this medium, and all parameters a high Biomass is not needed to obtain a very high production of the recombinant protein.

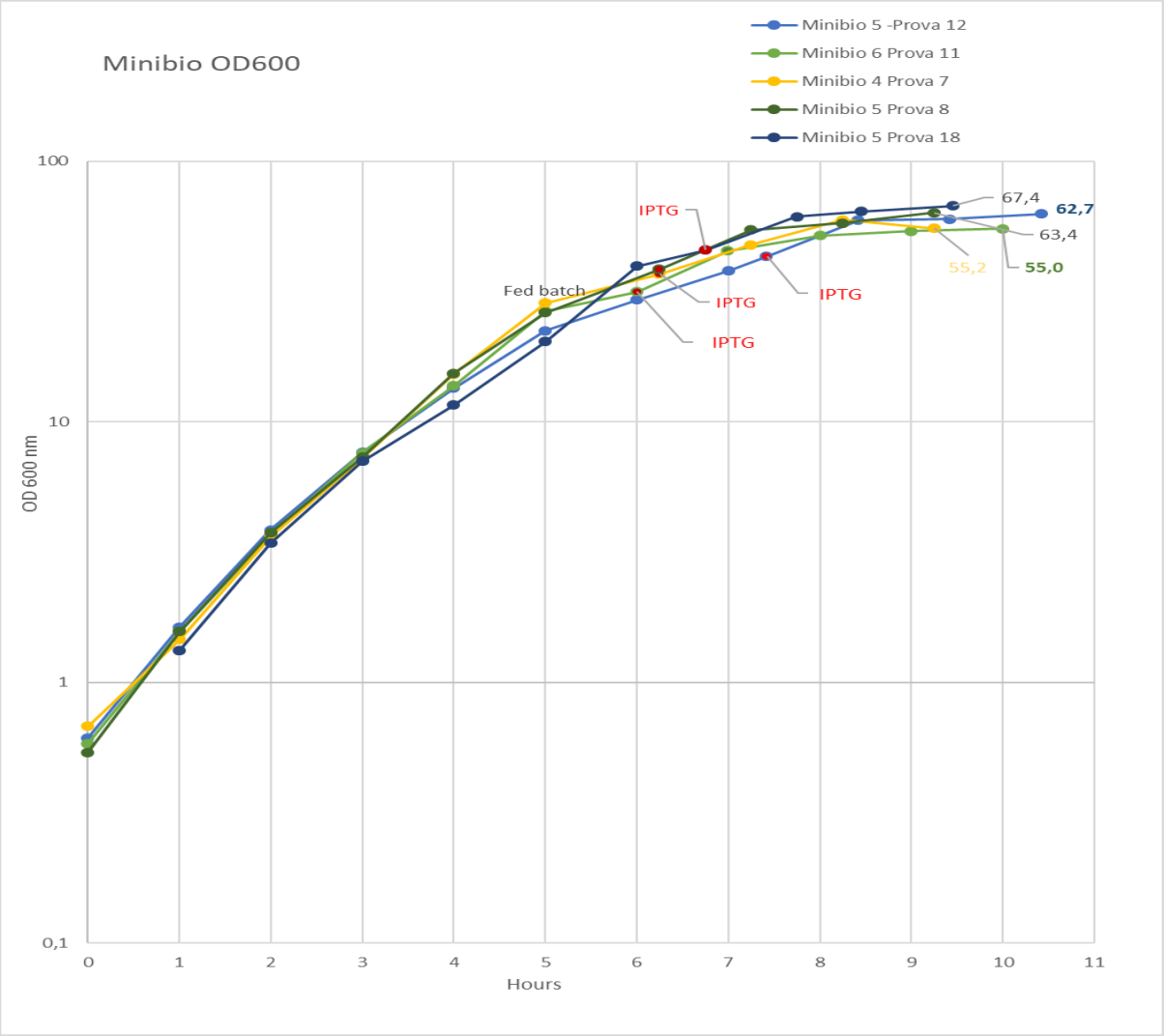
25. In this fermentation the expression vector has been modified in order to try to avoid inclusion bodies formation. As medium and strain haven't been changed it was a good opportunity to test our model on a different type of fermentation. Model has been applied on the batch phase and on the first three hours of induction. In order to slow down recombinant protein synthesis, the temperature of the fed-batch phase has been set at 20°C. This was a further challenge for the model. The long fed-batch phase can explain the high value of the harvested biomass.

26. Same run as 25 with a higher concentration of IPTG as inducer.

27. Same expression vector as in run 25 but with a "classical" fed-batch phase at 37°C with 3 hours of induction.

28. Same conditions than run 25 with an overnight fed-batch phase at 30°C instead of 20°C. The lower final OD_{600} and recovered biomass values are due to some culture lysis due to the long fed-batch phase at 30°C.

Relevant MiniBio growth curves are reported below. As we can observe, all the curves overlap and indicate an excellent reproducibility of the developed process.



Conclusions (Up-Stream):

From the overall evaluation of the obtained results, it can be concluded that the culture conditions have been identified permitting the recombinant protein production in a sufficiently robust manner to allow the project to proceed.
It is possible to replace glucose by glycerol as carbon source, this can be considered a *plus* for the culture medium preparation.
In the range of the identified conditions, some parameters have been varied in order to verify their influences on the recombinant protein production.