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Optimization of a Bacterial Cultivation Medium via a Design-of-Experiment Approach in a Sartorius Ambr® 15 Fermentation Microbioreactor System

Antonio Baccante [1,*], Pasquale Petruccelli [1], Giovanni Saudino [1], Elena Ragnoni [1], Erik Johansson [2,*], Vito Di Cioccio [1] and Kleanthis Mazarakis [3,*]

[1] AchilleS Vaccines Srl, Via Fiorentina, 1, 53100 Siena, Italy; p.petruccelli@achillesvaccines.com (P.P.); g.saudino@achillesvaccines.com (G.S.); v.dicioccio@achillesvaccines.com (V.D.C.)
[2] Sartorius Stedim Data Analytics AB, Östra Strandgatan 24, 903 33 Umeå, Sweden
[3] Sartorius Stedim UK Ltd., Epsom KT19 9QQ, UK
* Correspondence: a.baccante@achillesvaccines.com (A.B.); erik.johansson@sartorius.com (E.J.); kleanthis.mazarakis@sartorius.com (K.M.)

Abstract: In the evolving landscape of sustainable biopharmaceutical process development, the utilization of bacteria in the production of various compounds via fermentation has attracted extensive attention from scientists. A successful fermentation process and the release of its associated products hinge on the synergy between an efficient bacterial strain and the formulation of a suitable growth medium. Balancing all nutrient levels of a growth medium to maximize microbial growth and the product quality is quite an intricate task. In this context, significant advancements have been achieved via the strategic implementation of design-of-experiment (DOE) methodologies and the utilization of parallel microbioreactor systems. This work presents a case study of the fermentation growth medium optimization of a Gram-negative bacterium of the Neisseriaceae family that releases outer membrane vesicles (OMVs), which represent a potential vaccine platform. To achieve this, the ability of Sartorius MODDE®13 DOE software to explore multiple variables and their interactions was combined with the functionality of a Sartorius Ambr® 15F parallel microbioreactor system. The findings reported in this study have led to the design of a well-suited fermentation medium for a Gram-negative bacterium and an improvement in the quality of the OMVs produced from it.

Keywords: high throughput; biotechnological process; OMVs; small-scale microbiological cultures; experimental design; multiple linear regression modeling

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1. Introduction

Currently, the biopharmaceutical industry is committed to enhancing the speed of process development and reducing the time and the operational costs of testing biological substances in the clinic. A key strategy in meeting these challenges is the application of high-throughput process development (HTPD) tools. HTPD methodologies significantly reduce the time needed for the execution of a large number of experiments in parallel through the application of quality-by-design (QbD) tools, such as the factorial design of experiments (DOEs), which represents an essential instrument that is fully adopted in modern process development and manufacturing organizations [1–3].

Among biological substances, vaccines represent one of the most successful treatments against infectious diseases, with unequivocal evidence of them decreasing morbidity and mortality in the global population [4].

A promising and attractive technology for vaccine development platforms is based on the use of outer membrane vesicles (OMVs). These are biological vesicular structures with a mean size ranging from 20 to 200 nm, and they are released spontaneously during the growth of Gram-negative bacteria. OMVs have been shown to play an important role in

bacterial virulence, as well as in intercellular communication and nutrient acquisition. They are formed via a process that involves the bulging and pinching of the outer membrane. This process can be spontaneous or induced by environmental factors, such as changes in pH or temperature [5].

As OMVs originate from the bacterial wall via blebbing into the extracellular environment, they consist of double-layer membranes and include bacterial outer membrane components and periplasmic space substances, such as membrane proteins, lipopolysaccharides (LPSs), transporters, toxicity factors, DNA, RNA, and enzymes [6–9].

Numerous studies have demonstrated that naturally released OMVs show remarkable biological characteristics, including immunogenicity and adjuvant activity. Furthermore, due to their optimal size, they can be easily taken up by immune cells. Since native OMVs resemble the outer membrane of the bacteria from which they originate, containing the same surface antigens present in the bacterial cell, they have gained attention in the development of new potential vaccines against specific bacterial diseases [8].

The process of producing OMVs involves a microbial cell culture, grown in a stirred-tank bioreactor (STR), which releases the vesicles during growth. Afterwards, the biomass is removed via centrifugation or tangential flow microfiltration, obtaining bacteria-free crude OMVs. These are ready to be further polished via tangential flow ultrafiltration to remove undesired smaller, soluble components.

Among researchers' aims is the optimization of the microbial culture medium and its operative conditions in order to maximize the OMV production yield. Alongside this, unwanted impurities, such as the soluble proteins and nucleic acids released during microbial growth, need to be maintained at the lowest levels. Medium optimization includes the identification of the most suitable nutrients and their appropriate concentrations, resulting in the highest levels of biomass and correlated OMV production [10].

The conventional methods employed to develop and optimize a cell culture medium rely on biochemical studies or a one-dimensional search approach, with individual nutritional factors examined separately. The one-factor approach is inefficient when applied to multiple medium components and may overlook crucial interactions between them, leading to the development of a non-optimal medium [10]. Also, this method entails performing numerous experiments in combination, both in shake flasks and in small-scale stirred-tank bioreactors (STRs), to evaluate the effects of varying the medium composition reagents. With this approach, standard medium development and process definition and optimization require months of intensive and expensive work. Furthermore, bench bioreactors need to be cleaned, prepared, and autoclaved prior to and after each run. To obviate this, substantially shorten the time required, and reduce the overall costs, an alternative method may be used. One such route is via the statistical design of experiment (DOE) associated with a parallel microbioreactor system, such as the Sartorius Ambr® 15F system (Advanced Microscale Bioreactor) [11–13].

A DOE provides a reliable basis for decision making, offering a framework for systematically changing all the important factors but only requiring a limited number of experiments. Such a set of DOE-based experiments does not usually involve more than 10–20 runs, but this number can be further optimized to meet specific requirements. Since the experiments are distributed in a quadratic experimental space, it is possible to identify a design space that will produce a better result. An analysis of the data acquired from the performed experiments will identify the optimal conditions and reveal which key factors influence the results.

Prior to conducting any experiments, the experimenter must specify the experimental objective, the number of responses to some input conditions, and the number of factors and their respective ranges of variation. The experimental design can then be created, and the designated experiments may be conducted either in parallel or sequentially. Each experiment provides some results, i.e., numerical values for the response variables. Once collected, these data are investigated using a regression analysis. This provides a model relating the changes in the factors to the changes in the responses. The model will indicate

which factors are important, explaining how they combine to influence the responses. The modeling results may also be converted into response contour plots, so-called maps, which are used to clarify where the best operating conditions are to be expected [14].

The way this is carried out depends on the problem and the conditions under consideration, meaning that the shape and complexity of the experimental design may vary considerably. A common approach in DOE is to define a standard reference experiment of interest and perform new experiments that are laid out in a symmetrical fashion around the standard reference experiment. Hence, the standard reference experiment is usually called the center point.

The Ambr® 15F system is an automated mini-bioreactor system capable of running 24 disposable microbioreactors of 15 mL volume capacity, lodged in two culture stations (CS-1 and CS-2) containing 12 microbioreactors each. Each culture station has independent stirring speed control, allowing for 12 microbioreactors of the same stir speed at the same time. Each microbioreactor includes a miniature internal impeller and two optical sensors needed to monitor the pH and optical density (OD). A closed-loop control of dissolved oxygen, pH, and gas supply is present in all microbioreactors. The Ambr® 15F is installed in a bio-safety cabinet to ensure a sterile environment for automated inoculation, sampling, and feeding operations. The culture preparation and inoculation, feed and base addition, and culture sampling can be scheduled by programming the system through the associated software [12,13,15].

This study aims to show how to optimize a semi-defined culture medium utilized for a fastidious Gram-negative bacterium of the Neisseriaceae family and consequently improve the quality and yield of the released OMVs using the Sartorius Ambr® 15F system in combination with a DOE approach study managed using Sartorius MODDE®13 software.

2. Materials and Methods

2.1. Strain, Medium, and Inoculum

Recombinant Gram-negative bacteria of the Neisseriaceae family and OMV release were used for all studies reported in this publication. Bacterial growth was supported by employing a semi-defined medium free of animal components and containing essentially a phosphate buffer, lactic acid (carbon source), yeast extract and casamino acids, salts, trace elements, vitamins, and iron elements.

Although the bacterial strain and the medium components cannot be fully disclosed due to company restrictions related to intellectual property safeguards, the focus of this study was to demonstrate how to simplify a semi-defined medium. The medium in question initially contained high amounts of yeast extract (20 g/L) and casamino acids (10 g/L), employed for the growth of a fastidious Gram-negative microorganism of the Neisseriaceae family and to produce OMVs. We proved that, with the identification of essential key amino acids and the optimal concentrations of trace elements, vitamins, and iron citrate needed to grow this bacterium, it was possible to significantly reduce the quantity of yeast extract used, despite reaching the same level of growth obtained with a high yeast extract concentration. Due to this optimization, the analysis showed an increased quantity of produced OMVs and a noticeable reduction in unwanted contaminants, such as soluble proteins and nucleic acids, in the final purified OMV product.

All reagents used for this study were purchased from Merck, except for the yeast extract and casamino acids, which were bought from Becton Dickinson (BD).

For the inoculum cultivation media and the Ambr® 15F experiments, in addition to the undisclosed reagents, 7.5 g/L of lactic acid, 5 g/L of yeast extract, and 10 g/L of casamino acids without supplements (trace elements, vitamins, and iron citrate) were used. The media were sterilized using a 0.22 µm cut-off filter, Sartorius Sartolab® (Sartorius Biohit Liquid Handling Oy, Helsinki, Finland).

The 200-fold-concentrated trace element stock solutions contained the following substances: $CuSO_4 \times 4H_2O$ at 0.20 g/L, $ZnSO_4 \times 7H_2O$ at 0.15 g/L, H_3BO_3 at 0.5 g/L, and $MnCl_2 \times 4H_2O$ at 0.15 g/L.

The 100-fold-concentrated vitamin stock solutions consisted of 0.3 g/L thiamine chloride hydrochloride, 0.05 g/L nicotinic acid, 0.05 g/L pyridoxine hydrochloride, 0.2 g/L calcium pantothenate, and 0.2 g/L vitamin B12.

The 100-fold-concentrated iron citrate stock solution consisted of 4 g/L of $C_6H_5FeO_7$.

For each amino acid, a 100 g/L concentrated stock solution was prepared.

The above concentrated solutions were sterilized using the 0.22 µm cut-off filter Sartorius Sartolab®.

Prior to this optimization study, the medium used for the 2 L benchtop bioreactor included a yeast extract (20 g/L) with a concentration higher than that of the inoculum, as well as 10 g/L of casamino acids. The medium was sterilized in an autoclave for 30 min at 121 °C. After being processed using the autoclave, the medium was supplemented with 7.5 g/L of lactic acid (carbon source), 5 g/L of the trace element stock solution, 10 g/L of the vitamin stock solution, and 1 g/L of the iron citrate stock solution.

The 24 different fermentation media used for the Ambr® 15F experiments were completed with trace element, vitamin, iron citrate, and amino acid stock solutions in accordance with the concentrations calculated using DOE software. The media were prepared using the Ambr® 15F's liquid handler by programming the associated software.

At the end of this study, the optimized medium used was composed of 5 g/L of yeast extract and 10 g/L of casamino acids. Additionally, it was supplemented with 5 g/L of the trace element stock solution, 10 g/L of the vitamin stock solution, and 10 g/L of the iron citrate stock solution. Furthermore, the media were enriched with the following amino acids: arginine at 0.40 g/L, glutamate at 10.6 g/L, histidine at 0.58 g/L, proline at 3.49 g/L, and glutamine at 0.12 g/L.

The inoculum culture process started with the thawing of one frozen stock research cell bank (RCB) of the strain (1 mL of culture with 20% glycerol stored at −80 °C). The stock was used to inoculate 200 mL of medium (containing only 5 g/L of yeast extract and sterilized via 0.22 µm orthogonal filtration) in a 1000 mL baffled polycarbonate Erlenmeyer flask with a vent cap. The flask was incubated at 37 °C ± 1 on a rotary shaker (orbital diameter 25 mm) at 150 rpm for 8 h. At the end of growth, the culture was placed in an exponential phase and showed a final optical density (OD_{600nm}) of 2.5, as measured using a photometer in 1 cm light path plastic cuvettes at 600 nm. The described inoculum procedure was used to initiate both the 2 L lab-scale benchtop bioreactor and the Ambr® 15F microbioreactors.

2.2. Cultivation in the Bioreactor Systems (Ambr® 15F and 2 L)

The fermentation runs reported in this article were performed using the Sartorius Ambr® 15F and the 2 L benchtop bioreactors (Sartorius UniVessel® Glass 2L, Biostat® B-DCU II MO. Sartorius Stedim Systems GmbH, Guxhagen, Germany). In both cases, the fermentations were conducted in batch mode.

The Ambr® 15F with 24 single-use microbioreactors was operated with an 8 mL working volume for the experiments in this study. The Ambr® 15F was set with an initial volume of 8 mL, and each microbioreactor was filled with the medium and additional solutions using the liquid handler according to the DOE conditions. To prevent foam formation, the medium contained polypropylene glycol 2000 (PPG) at a final concentration of 0.02% (*v*/*v*). The inoculum flask content was transferred to a 24-well plate to allow the Ambr® 15F liquid handler to initiate the inoculation phase for all microbioreactors, which were inoculated to an initial OD_{600nm} of 0.2. The pH was measured online via the sensor's patches embedded in each microbioreactor, and it was held at 7.20 using NaOH 1M or H3PO4 1M. Acid was added using the liquid handler, while the base was pumped into the microbioreactors via pumped liquid delivery. The offline pH reading was monitored using the analysis module (AM), which recalibrates the in-process pH sensor.

Dissolved oxygen (DO) was measured online using the sensor's patches embedded in each microbioreactor, and it was set at 30%. To maintain this value, a cascade (air–oxygen–

stirrer) was set as follows: air flow rate from 8 to 30 mL/min; oxygen enrichment flow rate from 0 to 30 mL/min; and stirrer from 750 to 3000 rpm.

The growth temperature of each bioreactor was set and maintained at 37 °C. Cell growth was monitored online using the Ambr® 15F's sensor technology, consisting of cell scattering and an analysis of reflectance-associated signals. By applying user calibration (performed earlier with the same microorganism employed in this study), the reflectance value was converted into a measure of optical density (OD_{600nm}). For the offline OD measurements at 600 nm, an external photometer in 1 cm light path plastic cuvettes was used.

The cultures were run in batch mode and ended when the growth stationary phase was reached. This was recognized when the online OD measurement graphical trend either remained constant or decreased.

The 2 L bioreactor contained 1.5 L of sterile medium, and it was assembled with baffles and two Rushton six-blade disc turbines (φ = 5.5 cm) used for mixing, one located 3.5 cm from the bioreactor bottom and the other 7 cm from the first turbine. The bioreactor operating conditions were as follows: temperature at 37.0 °C; dissolved oxygen (DO) at 30%; air flow rate from 1.5 to 3 L/min (1 to 2 vvm); and a stirrer from 200 to 1000 rpm. Air flow and agitation were set in cascade to maintain the DO value.

The pH was set at 7.2 and measured using an online sterilizable electrode (HAMILTON EasyFerm BIO HB Arc 225. Hamilton, Bonaduz, Switzerland). NaOH at 2M and H_3PO_4 at 1M solutions were used to maintain the pH setpoint. The DO concentration was detected using an online optical electrode (HAMILTON VISIFERM DO ECS 225 H0. Hamilton, Bonaduz, Switzerland). The 100% point was calibrated before the bioreactor inoculation with the same operative conditions.

The inoculum flask contents were used to inoculate the benchtop bioreactors. The volume of inoculum required for use was calculated to have an initial OD_{600nm} of 0.2 units in the bioreactor. Foam was controlled by adding PPG in the sterile medium to a final concentration of 0.02% (v/v). The bioreactors were run in batch mode, and the OD of the cultures was monitored during growth using the photometer at 600 nm in 1 cm light path plastic cuvettes.

During the batch fermentation, microbial biomass and OMVs were formed. The fermentation ended when the growth stationary phase was reached. The culture stationary phase was recognized when the OD_{600nm}, measured each hour, was equal to (or lower than) the previous measure. Furthermore, a DO spike associated with a stirrer drop and a reduction in the acid addition confirmed the stationary phase and the end of fermentation.

2.3. Purification of OMVs

OMVs were purified from the fermentation broth through a purification process that is extensively described in a number of articles [16,17]. At the end of fermentation, 1 L of culture was collected in 20 × 50 mL Falcon tubes and centrifuged at 4120× *g* for 30 min at +4 °C. The biomass was discarded, and the supernatant containing OMVs was filtered using a 0.22 µm filter (Sartorius Sartolab® RF 1000 PES) in order to reduce the bioburden. The obtained material was ultrafiltrated via tangential flow filtration (TFF) on a Sartoflow® Smart system from Sartorius (Sartorius Stedim Systems GmbH, Guxhagen, Germany), equipped with two Sartocon® Slice 200 Hydrosart® Cassettes (Sartorius Stedim Biotech GmbH, Goettingen, Germany) at a 300 kDa molecular weight cut-off (MWCO). The OMVs were repeatedly ultrafiltered using Tris and PBS buffers to remove all the residual small contaminants [18].

2.4. Analytical Methods: OMV and DNA Quantification

The OMV concentration was calculated as the total protein content through a Lowry protein assay [19], purchased from Bio-Rad (DC™ Protein Assay Kit II. Bio-Rad Laboratories Inc., Hercules, CA, USA), in accordance with the kit procedure. The protein

concentration was estimated in comparison to a reference curve obtained using bovine serum albumin, as previously described in the literature [20].

Nucleic acid impurities were detected and estimated via a Picogreen assay, purchased from Thermo Fisher (Quant-iT™ PicoGreen™ dsDNA Assay Kits. Life Technologies Corporation, Eugene, OR, USA), according to the manufacturer's instructions [21]. The DNA concentration was estimated in comparison to a reference curve obtained with the calf thymus DNA standard provided with the kit.

2.5. Sartorius MODDE®13 Software

Sartorius MODDE®13 (MODeling and DEsign) is a Windows program for the generation and evaluation of statistical experimental designs, being part of the Umetrics® Suite of Data Analytics Solutions. MODDE®13 converts the experiments into information using a graphical data representation.

2.6. Statistical Analysis

The Lowry and the Picogreen assays were conducted in triplicate, and the data were averaged and are presented as the mean ± standard deviation. The statistical parts related to the DOE experiments are reported in the Supplementary Materials (Tables S2 and S3).

3. Results

Most fastidious microorganisms rely on a variety of trace elements, vitamins, and amino acids, which are typically not present in standard mineral media. Therefore, chemically undefined media containing complex components derived from natural sources are frequently used. Some examples of the complex nutrients frequently used are yeast extract, peptone, meat extract, and hydrolysates derived from either casein or soybeans. One of the most used complex supplements is yeast extract, particularly due to its rich composition of carbohydrates, amino acids, peptides, vitamins, trace elements, and diverse oligomeric compounds [16].

Initially, the bacterial strain was cultivated in a 2 L benchtop bioreactor with a semi-defined medium containing only 5 g/L of yeast extract. With this condition, it was possible to reach a final OD_{600nm} of the culture equivalent to 5 units. To increase the final OD_{600nm} value to an average value of 10 and, thus, increase the OMV production yield, the yeast extract concentration was raised up to 20 g/L. In this way, protein concentrations (corresponding predominantly to the OMVs' protein concentrations) were obtained after ultra-tangential flow filtration (TFF) equal to 0.23 mg/mL (fermentation run A) and 0.30 mg/mL (fermentation run B).

A possible approach to preventing the use of such a high yeast extract concentration and the consequences of the foregoing statement involved identifying the unknown auxotrophies of the bacteria, with the aim of obtaining a simplified and tailor-made medium. This, in turn, led to a reduction in medium expenses and prevented excessive nutrient residues at the end of the fermentation process.

The rapid time- and labor-saving identification of the essential nutrients is based on the application of the DOE associated with the 24-microbioreactor Sartorius Ambr® 15F system.

The first DOE experiment was based on the auxotrophy identification of various elements, including all amino acid, trace element, vitamin, and iron citrate solutions.

Essential amino acids can be divided into five distinct groups according to metabolic pathways [22–24]. The synthesis of amino acids commences with the intermediates derived from glycolysis, the citric acid cycle, or the pentose phosphate pathway.

The glutamate family includes the amino acids proline, glutamate, glutamine, and arginine, which are derived from α-ketoglutarate. As histidine can also be broken down into glutamate and enters the citric acid cycle through α-ketoglutarate, it is placed in this family. Serine is synthesized from 3-phosphoglycerate and can be converted into glycine and cysteine, making them part of the serine family. Pyruvate, which is the end

product of glycolysis, can be converted into alanine, leucine, lysine, and valine, which fall into the pyruvate family. Oxaloacetate, an intermediate in the citric acid cycle, serves as a precursor for aspartate. Methionine, isoleucine, asparagine, and threonine can be produced from aspartate, categorizing them in the aspartate family. Finally, aromatic amino acids, including tryptophan, tyrosine, and phenylalanine, are synthesized from phosphoenolpyruvate and erythrose-4-phosphate, constituting the aromatic family [24].

Using a classification based on the intermediates or their precursors, which had no more than four groups of amino acids for the purpose of this study, a grouping scheme was devised as outlined in Table 1 (including the corresponding concentrations). For each amino acid, the related concentration was equivalent to the sum of the quantity contained in 20 g/L of yeast extract and in 10 g/L of casamino acids, as reported in the supplier technical manual [25]. These amino acid concentrations were established before this study to perform experiments aiming to completely remove the yeast extract and the casamino acids. These investigations demonstrated that our bacterial strain was unable to grow in the total absence of yeast extract and/or casamino acids but that it needed a minimal concentration of both. The minimal concentration of yeast extract was 5 g/L, while that of the casamino acids was 10 g/L. These concentrations ensured a minimal growth of the bacterial strain equal to 4 OD_{600nm} units.

Table 1. Classification and concentration of amino acids in four groups (g/L).

Group 1 [1]	Group 2 [1]	Group 3 [1]	Group 4 [1]
Arginine (0.77)	Serine (0.55)	Aspartate (1.32)	Lysine (1.44)
Glutamine (0.06)	Glycine (0.72)	Asparagine (0.24)	Alanine (1.38)
Histidine (0.32)	Valine (1.38)	Threonine (0.47)	Tryptophan (0.1)
Proline (1.18)	Leucine (1.44)	Methionine (0.3)	Phenylalanine (1.08)
Glutamate (3.6)	Cysteine (0.05)	Isoleucine (1.16)	Tyrosine (0.2)

[1] Group 1: glutamate family; group 2: serine family and part of pyruvate family; group 3: aspartate family; group 4: aromatic family and part of the pyruvate family.

3.1. First DOE Experiment

In the initial medium, which contained 20 g/L of yeast extract and 10 g/L of casamino acids, the strain revealed a growth up to an average value of 10 OD_{600nm} units. As mentioned earlier, the first DOE experiment was based on the identification of strain auxotrophies for all amino acids and the effects of the trace element, vitamin, and iron citrate solution concentrations. The aim was to determine the optimal growth conditions that would enable the bacterial strain to maintain the minimum concentrations of yeast extract (5 g/L) and (10 g/L) casamino acids.

Following the amino acid group division reported in Table 1, four medium mixes were identified as being among the factors of this DOE, in addition to the trace elements, vitamins, and iron citrate solution. Each medium mix included the base medium (composed of a phosphate buffer, salts, lactic acid at 7.5 g/L, yeast extract at 5 g/L, and casamino acids at 10 g/L) and the five amino acids shown in Table 1; i.e., Mix 1 contained group 1 amino acids, Mix 2 contained group 2 amino acids, etc. Furthermore, minimum and maximum concentrations were established for each additional solution (trace elements, vitamins, and iron citrate). In this case, the value "1" corresponded to the minimum concentration and "20" to the maximum concentration of each solution (Table 2).

The concentrations of each amino acid are reported in Table 1.

The first design had three quantitative factors, illustrated as a cube with eight corners. There were also four formulation (mixture) factors, illustrated as a pyramid in each corner and in the center of the cube (see Figure 1). From the 160 potential experiments, 24 were chosen using a D-optimal algorithm integrated into MODDE software. The D-optimal design was only constrained by the number of experiments and the settings of the factors (see Table S2, Supplementary Materials).

Table 2. Factor settings of the first DOE.

Name	Abbreviation	Units	Type	Settings
Trace elements	Trace	mg/kg	Quantitative	1 to 20
Vitamins	Vitam	mg/kg	Quantitative	1 to 10
Iron citrate	Iron	mg/kg	Quantitative	1 to 10
Mix 2	Mix 2	g/kg	Formulation	0 to 1
Mix 3	Mix 3	g/kg	Formulation	0 to 1
Mix 4	Mix 4	g/kg	Formulation	0 to 1
Mix 1	Mix 1	g/kg	Formulation	0 to 1

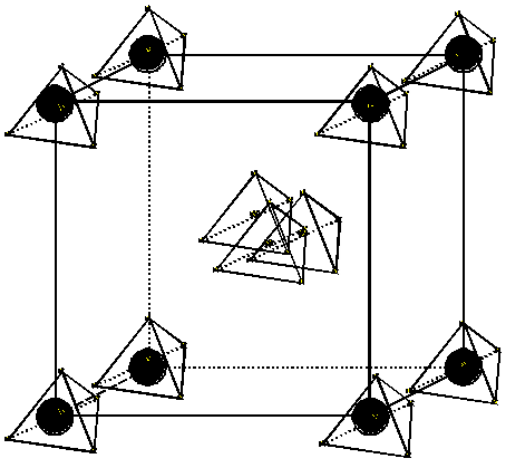


Figure 1. First DOE design, which was established as pyramids in each corner and in the center of a cube design. Each pyramid indicates the mixture (Mix 1, Mix 2 Mix 3, and Mix 4).

As a result of this DOE, we established the need to consider the maximum OD_{600nm} and the minimal value of doubling time (Td) among the 24 bioreactors (Table 3).

Table 3. Response settings of the first DoE.

Name	Abbreviation	Units	Condition	Objective	Min	Target
OD	OD	-	Required	Maximize	4	10
Duplication time	Td	h	Required	Minimize	-	1

The microbioreactor OD values were recorded by the Ambr® 15F system every 1 min and exported to an Excel sheet.

Td is defined as the time needed for the bacterial cells to divide, and it is expressed in hours. A high doubling time value indicates slow growth, while a short doubling time is equivalent to quick growth. Td is related to the bacterial growth rate as shown in the following equation [26]:

$$Td = Ln(2)/\mu, \quad (1)$$

where μ is the specific growth rate of the bacteria culture.

The Td value for each microbioreactor culture was calculated mathematically using Excel. We drew a semi-log plot of several collected points of OD_{600nm} vs. time, in which the *y* axis (OD) was scaled logarithmically. The Td value was computed by considering only the values included in the exponential phase and fitting them in the exponential mode. Specifically, we first obtained the following exponential function:

$$Y = Ae^{Bx}, \quad (2)$$

where Y represents the OD_{600nm} of bacteria at a given time $\times$ during the exponential growth phase; constant A represents the initial bacteria OD_{600nm} at the start of the exponential

growth phase; *e* is Euler's number; and constant B determines the growth rate of the bacteria during the exponential phase.

In Functions (1) and (2), as B is equivalent to the specific growth rate (μ) of the bacterial population, Td was obtained as follows:

$$Td = Ln(2)/B \quad (3)$$

where Ln(2) is equal to 0.693.

Figure 2d shows the exponential growth curve related to culture station 2—bioreactor 2 (N14 experiment) and the related Td calculation.

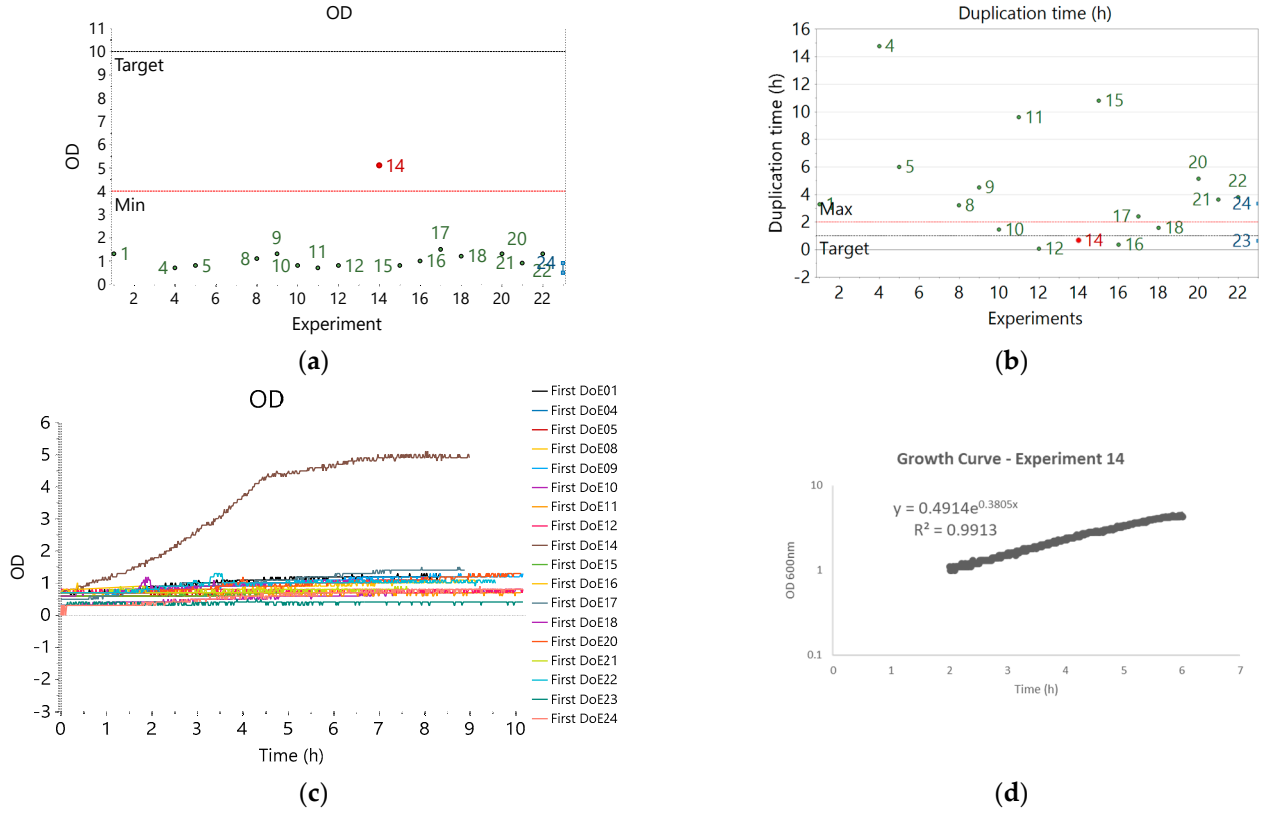


Figure 2. Results analysis: (**a**) OD_{600nm} results of first DOE, where experiment 14 shows the highest OD_{600nm} value; (**b**) duplication time results of first DOE, where experiment 14 shows low Td; (**c**) measured OD_{600nm} chart from Ambr® 15F software. In gold is the N14 experiment that reached the highest OD_{600nm} value and the lowest Td value. Some of the bioreactors are missing, as they did not reach the minimum threshold value for the OD_{600nm} reading. (**d**) Example of Td calculation: culture station 2—bioreactor 2 (N14 experiment).

MODDE®13 software was used to obtain the model fit and to perform an analysis of the results. As shown in the screenshots below (Figure 2), experiment 14 (including only Mix 1) showed higher OD_{600nm} values and a lower duplication time.

The results of the first DOE clearly showed that experiment 14 was by far the best experiment for OD_{600nm}. The model that was obtained with those results was not reliable enough due to the large number of experiments where the minimum OD_{600nm} was not reached. As such, we decided to focus only on experiment 14 and investigate this region further.

Experiment 14 included Mix 1 (composed of arginine, glutamine, histidine, proline, and glutamate), the minimal concentrations of trace elements (5 g/L of the 200-fold stock solution) and vitamins (10 g/L of 100-fold stock solution), and the maximum concentration of the iron citrate solution (10 g/L of 100-fold stock solution).

At this point, a second DOE was set to further optimize the process. We decided to focus on the composition of Mix 1's amino acids and to evaluate their optimal concentrations in a separate DOE.

3.2. Second DOE Experiment

The aim of the second DOE was to explore the different concentrations of the five amino acids included in Mix 1. For each of the five amino acids, we set the following minimum and maximum concentrations: arginine (from 0.4 to 2.3 g/L); glutamate (from 1.8 to 10.7 g/L); histidine (from 0.16 to 1 g/L); proline (from 0.6 to 3.5 g/L); and glutamine (from 0.03 to 0.2 g/L) (Table 4). The trace elements, vitamins, and iron citrate solutions concentrations were kept at the same values, defined in the previous N14 experiment, as well as lactic acid at 7.5 g/L, yeast extract at 5 g/L, and casamino acids at 10 g/L.

Table 4. Factor settings of the second DOE.

Name	Abbreviation	Units	Type	Settings
Arginine	Arg	g/L	Quantitative	0.4 to 2.3
Glutamate	Glu	g/L	Quantitative	1.8 to 10.7
Histidine	His	g/L	Quantitative	0.16 to 1
Proline	Pro	g/L	Quantitative	0.6 to 3.5
Glutamine	Gln	g/L	Quantitative	0.03 to 0.2

The maximum OD_{600nm} and the minimum duplication time (Td) values were considered as the results of this second DOE.

MODDE®13 software was used to generate the second DOE (Table 5) using an optimization design with five factors and two responses. The optimization design was primarily based on a fractional factorial design with 16 experiments, shown in rows 1–16 in Table 5. This was equivalent to half of the black corners in the graph. In addition, there were ten axial star points (rows 17–26 in the Worksheet). Half of the axial points in red were excluded in order to bring down the number of experiments to 21. Axial experiments with the highest factor setting for each factor were excluded. There were also three center points (see rows 27 to 29 in Table 5) (see Figure 3).

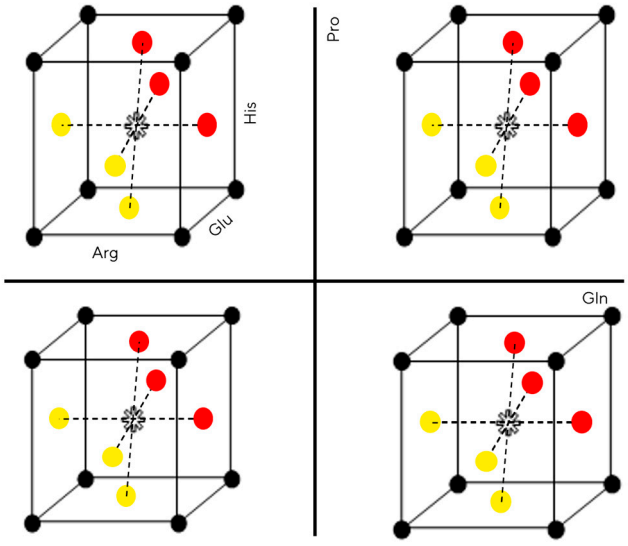


Figure 3. Second DOE design illustrated as cubes of 3 factors (Arg, Glu, His) in the high and low levels of the other 2 factors (Gln and Pro).

Table 5. Worksheet of experiment settings together with the results of the experiments of the second DOE. The conditions that were not run are displayed in red.

Exp No.	Exp Name	Run Order	Incl/Excl	Arg	Glu	His	Pro	Gln	OD	Td
1	N1	21	Incl	0.4	1.8	0.16	0.6	0.2	4	2.13
2	N2	23	Incl	2.3	1.8	0.16	0.6	0.03	3.2	2.36
3	N3	24	Incl	0.4	10.7	0.16	0.6	0.03	3.1	1.91
4	N4	7	Incl	2.3	10.7	0.16	0.6	0.2	3.1	2.58
5	N5	20	Incl	0.4	1.8	1	0.6	0.03	2.5	1.80
6	N6	13	Incl	2.3	1.8	1	0.6	0.2	2.3	2.60
7	N7	12	Incl	0.4	10.7	1	0.6	0.2	3	1.91
8	N8	14	Incl	2.3	10.7	1	0.6	0.03	3.2	3.04
9	N9	9	Incl	0.4	1.8	0.16	3.5	0.03	3.6	2.95
10	N10	8	Incl	2.3	1.8	0.16	3.5	0.2	3.6	2.05
11	N11	10	Incl	0.4	10.7	0.16	3.5	0.2	4	2.20
12	N12	3	Incl	2.3	10.7	0.16	3.5	0.03	2.3	2.11
13	N13	5	Incl	0.4	1.8	1	3.5	0.2	3.1	2.11
14	N14	15	Incl	2.3	1.8	1	3.5	0.03	2.8	1.80
15	N15	1	Incl	0.4	10.7	1	3.5	0.03	4.2	1.84
16	N16	26	Incl	2.3	10.7	1	3.5	0.2	3	1.92
17	N17	28	Incl	0.4	6.25	0.58	2.05	0.115	5.5	1.80
18	N18	18	Excl	2.3	6.25	0.58	2.05	0.115	-	-
19	N19	6	Incl	1.35	1.8	0.58	2.05	0.115	3.3	2.60
20	N20	25	Excl	1.35	10.7	0.58	2.05	0.115	-	-
21	N21	27	Incl	1.35	6.25	0.16	2.05	0.115	2.1	2.69
22	N22	16	Excl	1.35	6.25	1	2.05	0.115	-	-
23	N23	29	Incl	1.35	6.25	0.58	0.6	0.115	4.1	1.91
24	N24	11	Excl	1.35	6.25	0.58	3.5	0.115	-	-
25	N25	4	Incl	1.35	6.25	0.58	2.05	0.03	2.7	2.84
26	N26	22	Excl	1.35	6.25	0.58	2.05	0.2	-	-
27	N27	2	Incl	1.35	6.25	0.58	2.05	0.115	2.6	2.67
28	N28	19	Incl	1.35	6.25	0.58	2.05	0.115	2.4	2.24
29	N29	17	Incl	1.35	6.25	0.58	2.05	0.115	3.7	1.65

The condition numbers (quality) of the two models were 7.5 for the OD model and 10.3 for the Td model [27].

MODDE®13 software was used to analyze the model fit and the results. A multiple linear regression model was fitted and optimized for each response, which resulted in the coefficient models shown in Figure 4 (see Table S3, Supplementary Materials).

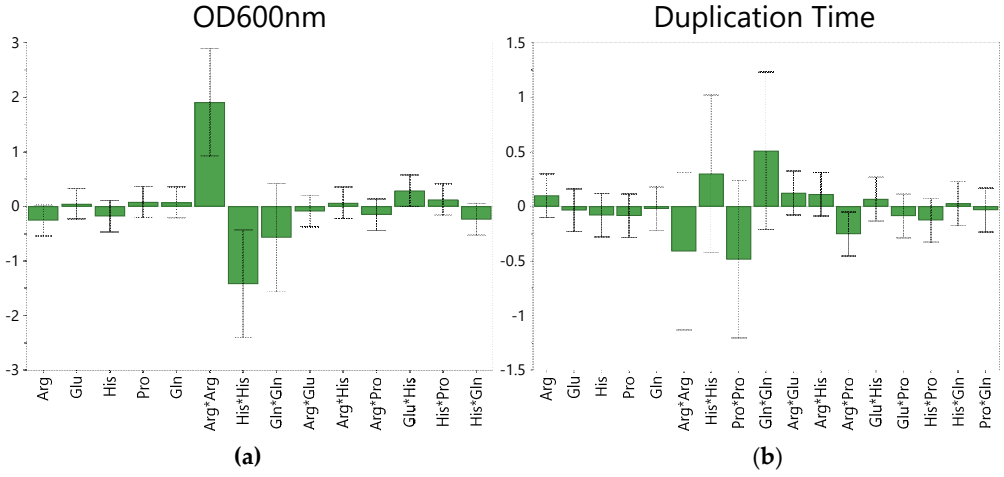
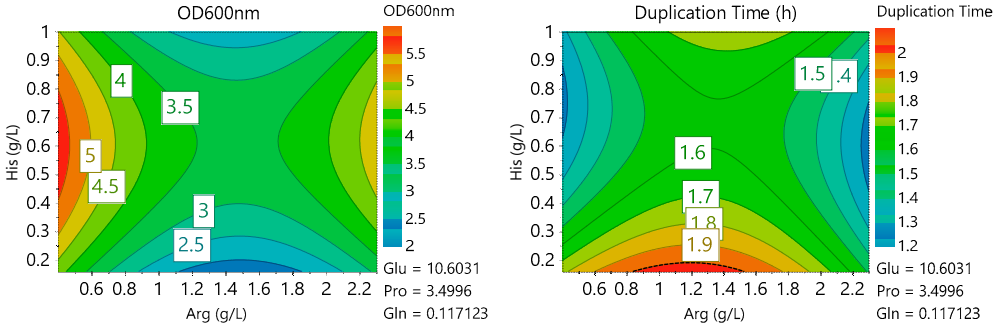


Figure 4. (**a**) Refined coefficient plot of OD_{600nm}; (**b**) refined coefficient plot of duplication time.

By inspecting the contour plots below, it was determined that using a lower amount of arginine can lead to better results, in terms of both maximizing OD_{600nm} and minimizing the duplication time, while keeping the concentration of the other amino acids around their average value settings.

3.3. Fermentation at 2 L Scale

After identifying the ideal amino acid concentrations based on the response contour plot (Figure 5) and to further confirm the data produced by the Ambr® 15F, two fermentations were run at the 2 L scale (runs C and D) using a Sartorius UniVessel Glass 2L BioSTAT B-DCU II MO with the operative conditions delineated earlier in Section 2. Briefly, there was 1.5 L of medium; a temperature of 37.0 °C; a DO of 30%; a pH of 7.2; an air flow rate from 1.5 to 3 L/min (1 to 2 vvm); and a stirrer with a range from 200 to 1000 rpm. Air flow and agitation were set in cascade to maintain the DO value.



(**a**) (**b**)

Figure 5. (**a**) Contour plot of OD_{600nm}; (**b**) contour plot of duplication time. The color in both plots goes from blue (low) to red (high). As OD should be maximized and duplication time minimized, it is shown in both plots that better results can be achieved by decreasing the concentration of Arg.

The medium consisted of a phosphate buffer, lactic acid at 7.5 g/L, salts, yeast extract at 5 g/L, and casamino acids at 10 g/L, as well as the following five amino acids: arginine at 0.40 g/L, glutamate at 10.6 g/L, histidine at 0.58 g/L, proline at 3.49 g/L, and glutamine at 0.12 g/L.

With the optimized media, cultures C and D reached final OD_{600nm} values of 10 units (Td = 1.27 h) and 10.2 units (Td = 1.10 h), respectively. The final OD_{600nm} values at the 2 L scale were higher than the OD units recorded in the Ambr® 15F under the same conditions. The higher OD values were mainly expected due to the better operative conditions that prevailed in the 2 L bioreactor, promoting the growth of bacterial cells.

The OMVs were recovered after fermentation and further purified as detailed in the Section 2.

3.4. OMV Characterization

To more accurately assess and define the beneficial effects of the optimized media on bacterial growth and, subsequently, on OMV production, a panel of analytical assays was carried out. The fundamental characteristics of OMVs suitable for vaccine development include low-impurity concentrations in terms of nucleic acids and soluble proteins, and a high OMV yield. For this reason, purified OMVs were characterized using colorimetric assays such as Picogreen [28] for DNA quantification and a Lowry protein assay to estimate the protein concentration and, thus, the OMV yield [20,29]. The procedure used to separate the analyzed OMVs from the fermentation broth, which entails centrifugation and ultrafiltration steps, is described in Section 2. Comparing the DNA and protein content produced during the fermentation process using the two distinct media (the initial standard medium pre-optimization and the optimized medium), the starting material (SM, hereinafter), which

was the material before the ultrafiltration step, was examined first. The results reported in Figure 6 demonstrate that the SM obtained using the optimized medium showed a lower DNA concentration than the SM produced using the standard medium (Figure 6A, first four columns). Additionally, the SM comprising the optimized material had a protein content that was still lower than that of the other SM (Figure 6B, first four columns). By combining these two findings, it was evident that the bacteria grown in the optimized medium caused the release of considerably lower quantities of contaminants, making OMV purification easier and improving its suitability for vaccine production. Additionally, the final OMV product (FP, hereinafter) was characterized to evaluate the impact of the bacterial medium used on the OMV yield and contaminant concentration. Because the DNA concentration in the SM was lower than that of the optimized media, the DNA concentration in the FP was also lower than it was in the FP from the standard media (Figure 6A, second four columns). A reduction in the SM contaminant concentration allowed for a more efficient ultrafiltration step, which resulted in a significantly larger reduction in impurities in the FP. The Lowry test, which is routinely used to assess OMV yield, showed that the OMV concentration in the FP purified from the bacteria grown in the optimized medium was higher than that of the FP from the standard medium, as reported in the second four columns of Figure 6B. This shows that, by using the optimized medium, it is possible to reach a higher OMV yield, which is an essential parameter for a vaccine candidate. In conclusion, growing bacteria on an optimized medium significantly reduces the concentrations of contaminants while also significantly increasing the OMV yield.

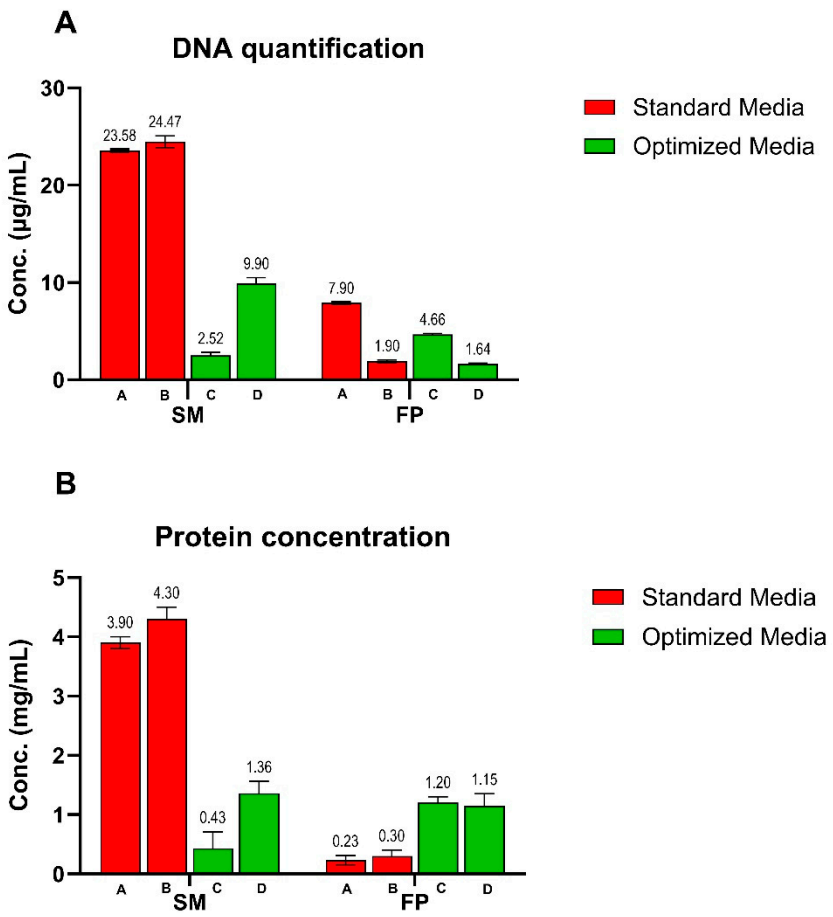


Figure 6. Colorimetric assays for the (**A**) DNA and (**B**) protein quantification of the starting material (SM) and final product (FP). Green bars are related to material produced from fermentation (runs C and D) in the optimized media, while red bars are related to material produced from fermentation in the standard media (runs **A**,**B**).

4. Discussion

In this study, the results show the suitability of the DOE approach in terms of optimizing a standard bacterial culture medium for the growth of a Gram-negative bacterium and consequently improving the yield and quality of the released OMVs. The OMVs need to meet the compendial standards for key quality attributes after the upstream and downstream production phases in terms of the presence of residual impurities, such as soluble proteins and nucleic acids [16,17].

The cultivation of the fastidious microorganism in question, before this optimization work, was carried out in a semi-defined medium with high concentrations of yeast extract (20 g/L) and casamino acids (10 g/L). Both of these complex compounds are particularly important, as they are the main nitrogen sources for bacterial growth and allow the cultivation to reach an average OD_{600nm} of around 10 units. However, the use of high concentrations of yeast extract results in the presence of elevated numbers of soluble proteins and nucleic acids after fermentation, as well as in the final product after the downstream steps.

At first, a preliminary study based on the classical one-factor-at-a-time (OFAT) optimization method was carried out by varying one medium component (factor) at a time while maintaining the other factors at a constant level [30]. The removal, the supplementation, and the replacement (data not reported) of some components (i.e., yeast extract, casamino acids, trace elements, vitamins, and iron citrate) were tested by running several experiments at flask scale and by using benchtop bioreactors. The main outcome of these tests was to determine the condition that ensured the minimal growth of the bacterium, which included the presence of at least one of the two complex components (yeast extract and casamino acids). Additionally, when only the two compounds were employed together, it was possible to achieve moderate growth. The drawback of running these tests in shake flasks and a benchtop bioreactor was the amount of time required to run a large number of experiments and the high costs in terms of the raw materials and work time needed. Furthermore, the OFAT method utilized could not be used to examine the interactions among the medium components of these studies.

To better identify a suitable medium composition, a statistical experimental method approach was employed. By knowing the most critical nutrients (factors) needed for the growth of the microorganism and entering them into the DOE software Sartorius MODDE®13, it was possible to delineate the experimental design space [14]. The execution of the experiments defined using the software was carried out on the microbioreactor system Sartorius Ambr® 15F, which allowed us to run 24 microbioreactors in parallel. Research of the key nutrients and their related concentrations for the optimization of the medium was performed by executing a first DOE based on a design constructed in MODDE®13 using the D-optimal algorithm, which resulted in 24 experiments (see Table S1 in Supplementary Materials). D-optimal designs are computer-generated designs, tailor-made for a specific problem. As detailed in Section 3, seven different factors were examined in the software in this DOE, and 24 conditions were run on the Sartorius Ambr® 15F. A significant number of experiments did not reach the minimal value of OD. Hence, the model was not reliable, and it was decided to explore the region of condition 14 (see Figure 2, Section 3), which showed the best results in terms of OD and Td. Experiment N14 included Mix 1 (composed of arginine, glutamine, histidine, proline, and glutamate), trace elements and vitamins at their minimum concentrations (5 g/L and 10 g/L, respectively), and the iron citrate solution at its maximum concentration (10 g/L). To further optimize the media and determine the correct concentration of each amino acid in Mix 1, a second DOE was set (see more details in Table 4 and Figure 3, Section 3) based on a central composite face-centered (CCF) optimization design with five factors. This allowed us to perform 29 experiments derived from MODDE®13. CCF designs are composed of a full or fractional factorial design and axial points placed on the faces of the factor range sides. As described in Section 3, 5 of the 10 axial points were excluded from the design to bring down the total number of experiments to 24. This second cluster of 24 conditions was tested on the Ambr® 15F. In this case, the results of the contour plots showed that the best medium conditions,

resulting in a maximum OD and minimal Td values, included a lower amount of arginine and average concentrations of the other amino acids.

The process was tested on a bench-scale 2 L bioreactor, using the selected medium composition to confirm the results obtained using the Ambr® 15F. The starting material recovered after the fermentations and the final product obtained after the ultra-TFF were analyzed using a Lowry protein assay and Picogreen tests. The results clearly showed (see Figure 6) an improvement in the final optimized media compared with the standard media. The analytical data confirmed the optimization of this process, which led to a relevant improvement in the key quality attributes of the OMVs produced while maintaining a consistent bacterial biomass growth.

The DOE approach, in the context of fermentation medium optimization and the possibility to run all tests in parallel using a multi-microbioreactor system, allowed us to considerably shorten the time compared to the use of shake flasks. Furthermore, the Sartorius Ambr® 15F enabled better control of the physical culture parameters, such as pH and DO, than the shake flasks.

5. Conclusions

Currently, in the research and development of many promising pharmaceutical products like OMV-based vaccines, fermentation medium optimization represents a crucial step in terms of maximizing production yield and meeting key quality attribute standards, hence lowering the final manufacturing costs.

In this work, the suitability of the use of DOE software, such as Sartorius MODDE®13, coupled with a microbioreactor system like the Sartorius Ambr® 15F, is demonstrated in terms of optimizing the fermentation medium for the growth of a Gram-negative bacterium of the Neisseriaceae family. This capacity significantly increases the final product quality of OMV-based vaccines when applied to either the starting material post-fermentation or the final product post-purification.

As demonstrated via a quantitative analysis and compared to a traditional medium employed for the same microorganism, the yield and purity of the OMVs considerably increased. However, additional DOE and experiments are planned to further assess the microorganism cultivation and the associated OMV characteristics. This will ensure that the OMV production process has sufficient robustness to be fully scaled-up in an industrial manufacturing environment for large-scale production while also maintaining its high-quality features.

The DOE approach, combined with a multi-microbioreactor system, allowed us to considerably shorten the production time compared to the use of shake flasks. In fact, with the Sartorius Ambr® 15F, four days of work were needed to perform all the conditions defined with the DoE method. Given that, if all experiment conditions from the two DoEs were to be carried out using 125 mL or 250 mL shake flasks, considering the required time to prepare all the different medium combinations (which, in the Sartorius Ambr® 15F, is performed by the liquid handler autonomously), it would have taken at least two full weeks of work.

It is also important to highlight the fact that the DOE approach employed in this work contributed to our company's sustainability efforts by reducing resource consumption and waste generation.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/fermentation9121002/s1, Table S1: Worksheet of experiment settings together with the results of the experiments of the first DOE; Table S2: ANOVA table of the two responses of the first DoE; Table S3: ANOVA table of the two responses of the second DoE.

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Abbreviations

AM	analysis module
Ambr®	advanced microscale bioreactor
CCF	central composite face
CS	culture station
DNA	deoxyribonucleic acid
DOE	design of experiment
DO	dissolved oxygen
FP	final product
HTPD	high-throughput process development
LPS	lipopolysaccharide
MODDE	modeling and design
MWCO	molecular weight cut-off
OD600nm	optical density at 600nm
OFAT	one factor at a time
OMVs	outer membrane vesicles
PPG	polypropylene glycol
QbD	quality by design
RCB	research cell bank
RNA	ribonucleic acid
rpm	rotation per minute
SM	starting material
STR	stirred tank bioreactor
Td	doubling time
TFF	tangential flow filtration
vvm	vessel volume per minute

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