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Economic Evaluation of M13 Bacteriophage Production at Large-Scale for Therapeutic Applications using Aqueous Two-Phase Systems

Torres-Acosta MA[a,b], González-Mora A[b], Ruiz-Ruiz F[b], Rito-Palomares M[c], Benavides J[b]*

[a] The Advanced Centre for Biochemical Engineering, Department of Biochemical Engineering, University College London, Torrington Place, London, WC1E 6BT, UK

[b] Tecnologico de Monterrey, Escuela de Ingenieria y Ciencias, Ave. Eugenio Garza Sada 2501 Sur, Monterrey, Nuevo Leon, 64849, Mexico.

[c] Tecnologico de Monterrey, Escuela de Medicina y Ciencias de la Salud, Ave. Morones Prieto 3000 Pte, Monterrey, Nuevo Leon, 64710, Mexico.

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*Corresponding Author:

- Jorge Benavides, Tel: +52 (81) 83582000 Ext. 4821, e-mail: *jorben@tec.mx*

Abstract

BACKGROUND: Bacteriophages are bionanoparticles with several applications in different biotechnology-based products. Among them, vaccines have the potential to treat antibiotic-resistant bacteria and parasitic infections. Traditional methods for their recovery and purification rely on precipitation with polyethylene glycol (PEG) and NaCl. However, applicability of such approach is limited due to large-scale technical constrains. Recently, our research group developed a bacteriophage M13 recovery and purification strategy using Aqueous Two-Phase Systems (ATPS), simplifying the methodology and, potentially, reducing costs. This work aims to develop an economic contrast between ATPS and the traditional PEG precipitation method at different operation scales (10 to 1,000 L bioreactor volume) to determine the applicability of the ATPS methodology at large scale. For this, the effect of ATPS volume ratio (V_R), sample loading and materials discount over production cost were analyzed.

RESULTS: Results indicate that as the discount on material costs increases, ATPS becomes a more affordable unit operation, from a bioreactor scale of 10 L at 0% discount to 410 L at 90% discount (US$ 52.2 to $1.72 *per* gram, respectively). Material cost contribution is a parameter that is of attention when working with ATPS as it is the core for the system construction. Methods for reducing their contribution are highly relevant and should be furthermore investigated.

CONCLUSIONS: Employment of economic analyses help to discover critical parameter for a bioprocess, such as material costs for ATPS. This economic analysis work serves as a platform for new strategies for the recovery of bacteriophage and bacteriophage-like particles using ATPS-based technologies.

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Keywords: *Bacteriophage M13, Aqueous two-phase systems, economic evaluation, vaccines, cost of goods*

Abbreviations list:

- PEG: Polyethylene glycol
- NaCl: Sodium Chloride
- ATPS: Aqueous two-phase systems:
- V_R: Volume ratio
- TLL: Tie-line length
- PFU: Plaque-forming unit
- SAGARPA: Secretaria de Agricultura y Desarrollo Rural
- UF/DF: Ultrafiltration/Diafiltration
- MWCO: Molecular weight cut-off
- CoG/dose: Cost of goods (or production costs) *per* dose
- CoG/batch: Cost of goods (or production costs) *per* batch
- % w/w: percentage in mass

1. Introduction

In recent years, the demand of bacteriophage-based products has increase rapidly due to their uses in a wide range of applications, including vaccines, molecular library screening, drug delivery platforms, cancer treatment, control of foodborne pathogens, gene therapy, nanomaterial production, among others.[1-4] From these bionanoparticles, bacteriophage M13 is one of the most widely used. It belongs to the family of the filamentous non-lytic virus that infects *Escherichia coli*.[5] The non-lytic capacity of M13 phage allows the production of titers ranging from 10^{11}-10^{12}

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PFU/ml in the fermentation media [6, 7] which is equal to only 3-30 mg of protein per liter.[8] This is an advantage for phage display technology as it allows for further amplification and not eliminating its host.

Additionally, the importance of bacteriophage M13 has increased due to its further application in phage display technology for antibody development, bionanomaterial construction, drug delivery, biosensors development, phage therapy for bacterial infections, gene therapy, as well as vaccines development.[6, 9-11] As new applications of bacteriophage M13 are constantly developed, it is expected that in upcoming years a significant production of such bionanoparticle would be needed in order to meet the potential demand.[8] The potential application of bacteriophages in the development of treatments in animals (including humans) may be exploited from two different perspectives. The first one would be using the intrinsic pathogenicity to bacteria as a solution to the growing antibiotic-resistant bacteria crisis. The second perspective is to use the phages as vehicle, through the application of phage display technology, to express antigens or cytotoxic that can trigger an immune response against viruses and other exogenous agents. As a display of a cytotoxic agent, one reported potential application is the use of the S3Pvac-phage vaccine which works as a control of cysticercosis.[6, 12] In this study, each pig received a dose of 1012 PFU of the S3Pvac-phage vaccine, considering that in Mexico there are approximately 16 million pigs, this can represent a demand of 160,000 L is production is typically around 1012 PFU/mL (without considering losses due to purification steps). For bionanomaterial applications, ~3.2 mg of filamentous phages are utilized per square centimeter of an active lithium-ion battery cathode.[2] In that regard, the high demand of bacteriophage M13 has guided to the pursuit of more effective bioprocesses for their production at large scale.

Currently, several methods for the downstream processing of the bacteriophage M13 have been reported. Precipitation by the use of polyethylene glycol (PEG) and NaCl combined with several centrifugation steps is considered the traditional method for the processing of M13 phage.[8] Recently, our research group reported the use of Aqueous Two-Phase Systems (ATPS) as an

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alternative method for the recovery and primary purification of M13 phage from a crude extract with promising results.[12] Although the downstream processing based on the precipitation method is very effective by yielding a large concentration of the bacteriophages, the processing time of the precipitation is four times larger than the ATPS-based strategy and involves several operation units, rendering extended processing times.[13]

From our previous work with ATPS, a PEG 400-potassium phosphate system with a volume ratio (V_R) of 1, tie-line length (TLL) of 25% w/w and sample loading of 10% w/w was an excellent system for the recovery and partial purification of M13 phage from a crude extract, with a recovery yield of 83.3%.[13] Although such system proved to be effective on the recovery of M13 particles, a further optimization of the system parameters such as analysis of V_R and sample loading was done. A recovery yield of 80.1% was achieved using a sample loading of 30% w/w.[12] Although such recovery yield is slightly lower than in the original ATPS, from an economic point of view the improved strategy resulted in a cost reduction of 2.65 times based only on the cost of the materials used to construct the system.[12] However, an in-depth economic analysis through bioprocess modeling is necessary to truly assess the economic feasibility of the ATPS strategy at large scale.

During the design and/or improvement of a new bioprocess, it is important to consider possible scenarios that may occur during real production stages. Bioprocess modelling is a useful and powerful tool that allows to create virtual models to simulate real bioprocess by using reported or experimental data obtained through research. If this modelling is coupled with an economic analysis, this technique allows to determine an approximation of the production costs in each part of the bioprocess to identify where attention is needed, resulting in reduction of time, resources and costs. This tool has been successfully employed to compare the cost of using stainless steel versus single-use equipment,[14] the use of different purification strategies,[15] to determine the best strategy to harvest a perfusion reactor,[16, 17] to optimize the production of monoclonal antibodies,[18] to evaluate the impact of optimizing operation parameters [19] and, in phage technology, to evaluate

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phage application in control of *Salmonella* in poultry at large-scale production.[20] Currently, several software platforms have been developed to perform bioprocess modelling and economic analysis, each with its own strengths and weaknesses. BioSolve Process (BioPharm Services, Chesham, Buckinghamshire, U.K.) is an Excel-based software oriented to biotechnological applications used for designing bioprocess models and performing economic analysis that considers indirect and direct operating costs. This software includes updated costs for materials and equipment from different suppliers and allows modification of most parameters to obtain results of hypothetical scenarios.

The present work uses the experimental data obtained during the evaluation of ATPS for the recovery of bacteriophage M13 [12] in order to perform an economic analysis for the production of bacteriophage M13 at a range of scales and to contrast the production costs of an ATPS-based bioprocess with the traditional precipitation-based method using BioSolve Process software.

2. Model Set-Up and Validation

This study contrasts the production costs of two methodologies for the recovery/purification of bacteriophage M13 for its potential use in phage display technology for vaccines. The first method (traditional method) for phage recovery comprises the induced precipitation of the bionanoparticle by using polyethylene glycol (PEG) and NaCl. The second strategy, recently published,[13] comprises the use of ATPS to achieve the recovery of bacteriophage M13. The first methodology has been extensively used and proved,[9, 21] but the application of ATPS has demonstrated the reduction of production costs by using a more simple but intensive unit operation compared with other techniques [15, 22] (such as filtrations and chromatography). It is important to mention that traditional phage purification is performed by mixing the sample with chemical reagents, then force precipitation by centrifugation, which, also, do not require complex unit operations. This study will allow to determine if ATPS is indeed a less expensive option or how it can improve to be better.

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The present study constructed a model following a previous published [20] strategy using Biosolve Process (Biopharm Services Ltd, Chesham, Buckinghamshire, UK). The model was created by fulfilling 3 categories: 1) the sequence of unit operations and productions parameters, 2) the design of production scenarios and target output and 3) economic datasets. Then variables to be analyzed are discussed.

2.1 Sequence of unit operation and production parameters

For the production of bacteriophage M13 (either for phage display or not), the basic procedure is to have a cell culture of *E. coli*, infect it with the desired phage to allow viral replication until a target concentration of infective particles is achieved in the culture media.

Traditional methods for recovery/purification rely on precipitation. Briefly, after the production of *E. coli* and the phages in a bioreactor, the culture is clarified by centrifugation to remove the biomass. A heat inactivation step (20 min at 70 °C) is added afterwards to kill any possible remaining *E. coli* cells and then remove them by an additional centrifugation. The phages in the supernatant are recovered and concentrated by the addition of polyethylene glycol (PEG) 8000 and NaCl. This process promotes aggregation of the viral particles, which are then forced to precipitate by centrifugation. The pellet formed is collected and re-suspended with a Tris-HCl buffer, but any possible insoluble fraction (particulates formed by insoluble aggregation of bacteriophages and any remaining cell debris [13]) is removed by an additional centrifugation step. To finish this process, a 0.22 µm filtration is performed to assure the removal of any remaining suspended particle. This process at laboratory scale takes approximately 4 hours to complete for a single sample, with the most time-consuming operation being the PEG-NaCl precipitation (2 hours). Alternatively, the application of Aqueous Two-Phase Systems (ATPS) can be done directly from the bioreactor culture media (without clarification or inactivation of *E. coli*) after production. ATPS analyzed partition the bacteriophages to the PEG-rich top phase, while contaminants (such as cells, cell debris and media components) migrate towards the interphase and bottom phase.

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Afterwards, an ultrafiltration/diafiltration (UF/DF) step is required to remove the phase-forming components (primordially PEG, salt and molecular contaminants) in solution (membrane MWCO above 1 kDa, as long as the size is large enough to allow the permeation of PEG and the retention of bacteriophage M13) and exchange the solution in which the phages are suspended. This ATPS-based strategy takes less than 1 hour (at laboratory scale), a significantly shorter time when compared to the traditional method. This makes ATPS an interesting approach by making the recovery process shorter and less complex. Both processes are presented in detail in **Figure 1**. An analysis from a process perspective was conducted before and published by our research group.[13]

2.2 Design of production scenarios and target output

After designing the sequence of unit operations to follow (in this case for 2 possible bioprocesses), is it important to determine which production scenarios are going to be analyzed and the target size of production. This decision will in turn affect the final model, as different scales require different equipment sizes or production parameters.

The target output of the process to be analyzed must be decided based on either the current production levels for the product of interest or the reported data for similar products on the market or research reports. As the orientation of the present work is to analyze the potential use of phage display for production of vaccines, then the target production should be based on other vaccines or phage display products. This can have a wide range of bioprocess sizes as applications or markets can vary widely. Therefore, to simplify the decision and have a more comprehensive data collection, it was decided to analyze different scales for the process, with proper modification of each unit operation (explained in a later section). The scales to be analyzed in this work are for a bioreactor with an operating volume from 10 to 1,000 L, regardless of the recovery option being studied (precipitation or ATPS). These bioreactor sizes, considering data for this model based on a published report for production and recovery, [13] yield a production of 5.72×10^{11} PFU/mL (raw

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extract). This production output is considered equal for both bioprocesses modelled here as this will allow a proper comparison. For most production cost analysis results are presented in cost per mass unit. However, this may be misleading for phages as there is not necessarily a conserved correlation between mass and activity (phage infectivity) at different experimental conditions. Therefore, it was decided to present the results as production cost *per* dose, where a dose is 1 mL containing 10^{12} plate-forming units (PFU) and denoted as CoG/dose.

2.3 Economic datasets

After the unit operations, production parameters and size of the bioprocesses being modelled are set, it is critical to give economic data to every aspect of the model. This is what will populate the model and result in production costs. This model relays mainly on equipment, materials (such as reagents or chemicals) and consumables (as filters or membranes). More complex and commercially applied models can incorporate capital, labor, waste disposal, etc., but as this study is focused on contrasting two recovery strategies, the cost categories studied here were limited in order to maximize attention to other details. Additionally, for both processes these extra cost categories are highly similar and can be discarded in order to only analyze production costs. All the costs are explained in **Table 1**. Briefly, the cost of reagents and chemicals employed in every operation is based on a previous publication.[13] These costs come from standard sizes in Sigma-Aldrich catalog, although their cost is associated with laboratory scale product cost, one of the analysis performed in this study is to analyze the effect of a potential discount on the materials costs (up to 90% discount), therefore this costs were unmodified for the construction of the model but its change (and particularly its decrease) was analyzed afterwards. The costs of consumables were taken from the Biosolve Process database (Biosolve Process version 7) and adjusted automatically as process scale changed. For equipment costs, different sizes were taken from Biosolve database and regressions were calculated to have all possible scales and their respective cost.

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2.4 Analysis Performed to Study the Production Costs

After the completion of the model construction, it is possible to perform an economic analysis and determine the production costs for M13 bacteriophage. Given that the objective of this study is to analyze the use of precipitation against ATPS and its potential commercial application, a strategy for the analysis was designed and presented in **Figure 2**. Results collected from the modelling are the Cost of Goods *per* batch (CoG/batch) considering the duration of each operation as showed in **Figure 1** and the Cost of Goods *per* dose (CoG/dose) – 1 mL containing 10^{12} PFU.

This study analyzed the effect of four variables on the production costs: bioreactor scale, ATPS volume ratio and percentage of sample loading, and materials costs discount. Previously, these variables were studied from a recovery and purification point-of-view.[13] Given the nature of each of the processes evaluated here, not all the variables analyzed can be studied for both process options. For the precipitation methodology, the analysis comprises the effect of the bioreactor scale (10 to 10,000 L) and the materials costs discount (10, 30, 50, 70 and 90% of discount). For the ATPS process, besides process scale and materials discount, it is possible to analyze the impact of changing the relative volume of the systems (V_R; defined as the relation between the top phase volume and the bottom phase volume) between 0.33, 1, and 3, and the amount of sample loading (defined as the percentage in mass that the sample represents from the total ATPS) between experimentally tested percentages (10, 20 and 30% w/w) and projected/theoretical sample loadings (40, 50 and 60% w/w). For the projected sample loading, an extrapolation of the behavior of the experimental results was performed to calculate their corresponding recovery yield. Briefly, the difference on the recoveries between sample loading was obtained, then a linear correlation between the difference and sample loading was obtained (Recovery yield difference [%] = -0.317 x Sample loading [%] + 6.3). This was used to calculate the respective recovery yield for the theoretical 40, 50 and 60% w/w sample loading. These values were used solely for modelling, potential effects on system saturation or over loading of contaminants were not considered. This

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analysis was performed only to study the potential of increasing sample loading and to determine if further experimental work is needed in this area.

Additionally, to increase sample loading can be done in two ways, the first is to maintain the same ATPS size and just increase the amount of sample that is loaded into the system. An alternative is to maintain the same sample size, but the total ATPS size is modified and adjusted so the sample becomes the desired percentage. The second approach (adjust total ATPS size to a fixed sample size) was used in this study. This was a decided as one of the parameters analyzed is the bioreactor scale, which relates directly to the sample being input into the ATPS.

To maximize the modelling capabilities, every variable studied (materials costs discount, ATPS V_R and sample loading) was simulated for all process scales (10 to 1,000 L). The analysis strategy followed in this work (**Figure 2**) analyzed first the effect on the production cost of V_R. An optimal volume ratio was selected for subsequent analyses. Afterwards, the sample input and materials costs discount are analyzed individually and simultaneous to identify their independent and joint impact, respectively. The resulting data of the simultaneous variation is modelled under a response surface methodology to have a unique set of equations that can help predict costs and find an optimal value of operation while giving as input the bioreactor scale, sample loading (for ATPS only) and materials costs discount. Data was processed using the open source software R. Additionally, for every simulation performed the bioreactor size and phage production is registered, in order to be able to exchange data between these two categories.

3. Economic Analysis Results

3.1 Analysis of ATPS V_R on CoG/dose

Previous studies [23, 24] have shown that the V_R of an ATPS can have a critical effect on the partition of a product of interest. This has been theorized to be caused by a saturation effect and volume displacement. These effects generate a phenomenon where a particle is partitioned to one

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of the phases, but the volume of a phase (and the concentration of system components) allows for only a certain amount of product of interest in that specific phase to be contained, which promotes the migration to the opposite phase. This can generate mixed results as it might seems like the particle has no preference for a particular phase but actually the ideal amount of product to be added to the system should be less than the one used in order to have a larger partition coefficient.

From an economic point of view, having a system where the product of interest is collected in the smallest phase from the two formed is convenient. This ultimately translates into a smaller processing volume by subsequent unit operations. Sample loading in a unit operation can determine whether a process is economically viable or not. This has been demonstrated before when analyzing the cost of using ATPS. Typically, after applying ATPS, its components need to be removed as they are now considered contaminant. This is normally accomplished by the use of ultrafiltration and diafiltration.[25, 26] But if the collected phase is large in volume, this can translate to an expensive filtration step by having a large consumption of consumables. Alternatively, a small ATPS phase to be processed will require less consumables.

As mentioned before, the base studies for this work [12, 13] have studied from a bioprocess perspective the variation of V_R. In the present study V_R variations were analyzed for their economic impact. For this, the model recovery yield and output volume of the ATPS were modified to accommodate the changes on the V_R: V_R 1 has a recovery of 83.31% and a volume output of 1/2 of the system, V_R 0.33 has 12.56% recovery and 1/4 output, and V_R 3 has 87.06% recovery and 3/4 for output (**Figure 1**).

After its modelling in Biosolve Process, results for the production costs for each V_R for ATPS and the cost using the precipitation option were graphed in **Figure 3**. From this, precipitation is the best option when considering production cost (CoG/batch and CoG/dose), but ATPS with V_R 3 provides the highest recovery of phages, followed closely by V_R 1 and precipitation. On the other hand, V_R 0.33 is the least optimal option as it yields a very small recovery and a high production cost.

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These results indicate that precipitation is the best option at this stage, but ATPS with V_R 1 or 3 are a promising alternative. During the design of a bioprocess it is important to consider its simplicity as less unit operations typically translate to less operation expenses and product losses. In this context, even with a lower CoG/dose for precipitation, a holistic analysis of the process is relevant to stablish if there are possible scenarios under which lower CoG/dose values are estimated for the ATPS-based strategy when compared to precipitation. Contrasting the two ATPS options, V_R 1 has the highest potential, as one of the main drawback of employing ATPS is the removal of the phase forming chemicals, this is why it is critical to consider the quantity of sample to be processed by the subsequent unit operations. For this reason, given that V_R 1 and 3 have similar results, ATPS with V_R 1 is ideal for having a reduced volume to be processed while having similar costs as V_R 3. Therefore, an ATPS with V_R 1 and the precipitation option were selected for analysis in subsequent sections.

3.2 Analysis of ATPS Sample Loading on CoG/dose

One of the main advantages of using ATPS is the possibility of changing its composition in order to maximize the amount of sample loading (the mass percentage that the processed sample represents from the total mass of the biphasic system), this allows to intensively use this operation. However, this process intensification approach has a limit as saturation problems in one of the phases may negatively affect the partition behavior of the product of interest, decreasing the recovery yield. Usually, an ATPS is constructed by mixing its components, including water as one of them, but potentially, the water content could be supplied by the water present in the sample being added (given that the sample is in an aqueous environment); therefore it is hypothetically possible to remove the added water and supply a larger amount of sample to the system. This approach has already been analyzed in by varying the sample between 10%, 20% and 30% w/w,[13] but this study is now analyzing its cost implications. Additionally, three scenarios were added to understand how the cost behaves, a hypothetical 40, 50 and 60% w/w were used. This can be analyzed as, without the sample, the water content is up to 67.3% w/w, which allows for up to a

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potential 60% of sample with a remaining 7.3% for water. Recovery yield for these hypothetical scenarios is extrapolated (as explained in a previous section) from the data and they are included in **Table 2**.

The increase in sample loading can have two distinct effects on the ATPS construction: 1) the size of an ATPS remains constant, while the sample proportion is modified and 2) the size of an ATPS changes, while the sample size remains constant causing its proportion to change. The first option is straightforward but can hinder industrial applicability by developing a larger unit as the fermenter volume increases. The second option can capture two effects at once, an ATPS can decrease its size while having a constant sample input (total mass/volume) which will cause the proportion to increase, therefore generating a more intensive use of the ATPS. This second approach was used in the present study. Results are summarized in **Figure 4**. Simulations using different sample size but considering their respective recovery yields provided an interesting insight. For the CoG/Batch, it was found that, as expected, an increased amount of sample provided a lower production cost. This is caused by having a smaller ATPS when the sample loading is increased, mainly noticed by the decrease in materials, consumables and equipment costs for ATPS. Although the cost is decreased, the precipitation methodology is the least expensive option, but when analyzing the CoG/dose, ATPS is a better option but only at the 10 and 20 L of bioreactor scales while using a sample loading of 30%. An additional aspect to consider, is that as production scale increases, there is a potential discount on material acquisition, this could impact significantly on the practical implementation of ATPS. This key aspect is analyzed in the next section.

This result (lower cost of ATPS than precipitation at a sample loading of 30% instead of having it at 60%) can be counterintuitive, but it is important to consider the experimental and projected recovery yields. First, as scale increases ATPS becomes more expensive for its intensive use of materials. Second, as sample loading increases, the recovery yield will decrease, this is due to saturation effect (critical amount of product in a particular phase or excess contaminants that prevent correct partition). This causes that even if the CoG/batch decreases when increasing the

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sample loading, the CoG/dose will be affected by the amount of product being generated per batch. When the sample loading increases (as it was decided to keep the same amount of sample, but adjust the ATPS size), less product will be generated per batch. This can make the CoG/dose be higher as the sample loading increases (and consequently recovery yield decreases). With the current recovery yields projected, this is the case.

Contrasting only ATPS among the sample loadings evaluated here, using the bioreactor scale as analysis variable, it can be seen in **Figure 4d** that as the scale increases, the sample loading with the lowest CoG/dose also changed (although not enough to be below precipitation). At high scale, the least expensive ATPS are the ones operating at 60% of sample loading. This effect, given that at low scales 30% sample loading is more efficient, is caused by the contribution of materials. As scale increases, the cost contribution of materials increases as well, but the overall amount of product being generated at large scales compensate this and overcomes it to become the least expensive ATPS.

It is important to consider that given the method followed to extrapolate the potential recovery yields for the sample loading 40, 50 and 60% w/w, actual experimental results could be completely different. The results presented here are to be followed as a glance to the cost implications that optimizing this parameter can have. Additionally, if the actual recovery yield is less than the projected here, then the 30% of sample loading potentially will be the least expensive ATPS configuration. To study the effect of potentially having a less favorable recovery yield on the sample loading of 60%, the CoG/dose for a range of recovery yields were calculated. These results are presented in **Figure 5**. This figure shows which sample loading (30 or 60% w/w) is the least expensive given that the recovery yield for 60% varies. Results indicate that a recovery below approximately 45% would be need for it to be more expensive than 30% sample loading at any given bioreactor scale.

As mentioned before, current research focuses on using low sample loading [27, 28] and this parameter is not frequently optimized, but from an economic perspective it might be relevant to

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study its effect. Results presented here show that recovery yield decreases as sample loading increase, but this effect can be product- or sample-related. Therefore, it is critical to maximize its potential in order to decrease production costs and commercial applicability. Recent large-scale work are scarce and ATPS should be pushed more strongly into commercial implementation.[29] These results are important as a base for the future study on the increase on sample loading and how to make ATPS a more intensive unit operation. This is of critical importance as the water content can be displaced to capture more product of interest, potentially decreasing production costs, as demonstrated here as long as recovery and purity are not significantly compromised.

3.3 Analysis of Materials Discount on CoG/dose

One of the main advantages of using ATPS as a separation technique is the possibility of using samples that contain large particles, like biomass, directly after fermentation. This is the case in the bioprocess designed here (**Figure 1**). Although it is a promising alternative, one of the major contributors to the production costs when employing ATPS are materials used for their construction.[22] This is due to the essence of their typical construction where the sample being separated, usually, represents a 10 to 30% w/w (sample loading), this means that system components constitute approximately 70 to 90% w/w of a system, as this has been reported before [27, 28, 30] and employed in this study. In an industrial scale, bioprocesses where a 1,000 L bioreactor is operated, if the culture media is used directly after fermentation into ATPS as sample (**Figure 1**), this mean that the system can have a size around 10,000 L (it is important to note that ATPS are constructed in weight relationships).

Although this can be discouraging, it is important to note that large-scale bioprocesses can acquire materials with a discount because of the quantities that are acquired. This is where the concept of economy of scale becomes relevant for ATPS technology. The present study analyzed several potential discounts in order to understand if it is possible for ATPS to achieve the production costs that precipitation has. The discounts analyzed here comprise a 10, 30, 50, 70 and

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90% discount on costs for the materials employed, for the precipitation and ATPS alternatives. This analysis consisted in performing the simulation of the production from 10 to 1,000 L while applying each of the possible discounts, this resulted in a 5,000 data point collection. Results for this analysis are summarized in **Figure 6** and **Table 3**. Particularly, **Figure 6c** represent the difference between the CoG/dose calculated for the precipitation and ATPS alternatives. Negative values for the CoG/dose denote scenarios where ATPS is less expensive than precipitation. It can be noted that as the discount for the materials costs increases there is a larger range for ATPS to be less expensive than precipitation. This is expected as one of the major contributors for ATPS constructions are materials. **Table 3** was constructed to highlight the maximum scale at which ATPS is still less expensive than precipitation (for each discount percentage evaluated) and the particular amount of product generated at that scale.

3.4 Simultaneous Evaluation of Sample Loading (ATPS Only), Process Scale, and Materials Discount

To understand the overall behavior of the analyses performed here additional simulations were performed. This was done by generating all possible scenarios for the combination of the 3 analyzed variables, this means that each production scale (from 10 to 1,000 L) had the five materials discounts possibilities (10, 30, 50, 70 and 90% of discount) and, for ATPS only, each of the sample loading levels (10, 20, 30, 40, 50 and 60% w/w). This translates into 500 combinations (scenarios) for the precipitation recovery approach and 2,500 for the ATPS-based process.

After the simulations were performed, the data was used to create a mathematic model following a response surface methodology. This resulted in an equation that has as independent variables the production scale, the discount for materials and the sample input, and as a dependent variable the production cost. Additionally, the model considered the two-way interactions of the variables and the quadratic terms. To create this mathematic model, the software R was employed by using the RSM package. As part of the simulations, the amount of phages produced at each

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scale per bioreactor run was calculated, with this information it was possible to create a set of equations to pass from the bioreactor scale to production quantities as doses of phages per batch. This data results in four equations (**Table 4**), two of them for the determination of production cost and two for interchange between bioreactor scales and doses produced, one of each for ATPS and precipitation process alternatives. Given the nature of the data, production cost per dose was converted into natural logarithm in order to maximize the R^2 value and have a proper correlation.

Given the results from this simultaneous variation analysis, the least expensive scenario for ATPS yields a CoG/dose of US $0.92 *per* dose at a scale of 1,000 L, 90% material discount and 50% sample loading. In contrast, at the same conditions, the precipitation alternative can decrease up to US $0.85 (90% discount with a bioreactor 1000 L). As expected, the higher the scale and material discount, the lower the production costs. Surprisingly, but in accordance to what has been discussed in previous sections, when sample loading increases there is a relationship between the decrease in recovery yield (and therefore product generation) and CoG/batch. Depending on the scale being analyzed, the amount of product generated can compensate the quantity (and costs) of ATPS construction. This is evident at large scales, where higher sample loading, regardless of their lower recovery yields, they grant a lower production cost.

Altogether, results from this work help to have an initial platform for the application of ATPS-based bioprocess on the recovery and purification of bacteriophage products. It has been possible to determine which parameter affect the most the production costs and which ATPS options were the best.

Additional to the analysis presented in the present work, a holistic approach considering factors such as the potential environmental implications and public perception of the proposed biotechnological application (use of bacteriophages as vaccine vehicles). Other reported process economy studies have performed this type of analysis, once the fully bioprocess has been developed and optimized.[22] As mentioned before, the scope of the present work is to contrast two different recovery strategies and set the ground for future developments. As a perspective, the use of ATPS relies heavily on material

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consumption, but improvement have been made to recycle materials by a continuous back-extraction through the use of a secondary ATPS.[31] This research work represent the first step in understanding, from a economic point-of-view, the factors that have significant influence on the recovery of novel phage-based vaccines.

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4. Conclusions

The present work performed an economic analysis of the implementation of ATPS into a large-scale bioprocess and its contrast against a traditional methodology. It was possible to observe that ATPS has the potential to achieve a reduced production cost for phage products, but still has areas of opportunity in terms of process optimization.

Results obtained in this work show that for ATPS to achieve a lower cost than precipitation, it needs to maintain a sample input around 30% w/w or/and increased discount for materials, which could only be possible at large scales. The combined effect (sample loading and material discount) becomes more pronounced making the gap smaller. Given the potential for phage therapy products, it is highly relevant to develop cost-efficient technologies to reduce production costs and, therefore, prices for costumers. An ideal scaled-up bioprocess must have few unit operations with high recovery yields without compromising the purity and retaining its cost-effective attributes. The ATPS strategy developed by our research group for the recovery of bacteriophage M13 demonstrated to be an attractive alternative when compared to the traditional recovery method based on PEG-NaCl precipitation from an economical point-of-view, particularly at large scale. The present work serves as a platform for the future commercial development of phage-based products.

Further work is needed to capture all aspect of phage production, such as environmental impact, energy consumption, CO_2 balance and societal implications. Although these concepts are

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outside the scope of the present work, they should be considered for further analysis, once a fully optimized bioprocess for the generation of phage-based vaccines is designed.

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Acknowledgements

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TABLES

Table 1. Economic datasets employed for model construction and composition of solutions used for both process options described in Figure 1.

Item	Cost or Cost/g (US $)
Equipment	
Bioreactor	Obtained from Biosolve Process database (reference costs and scale-up factors) and scaled accordingly using the formula:
Centrifuge	
Stirred Tank	

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Filtration	$Cost = Reference\ Cost * \left(\frac{Size}{Reference\ Size}\right)^{Scale-up\ Factor}$
Materials	
For fermentation media	
Yeast Extract (20 g/L)	0.26
Tryptone (30 g/L)	0.15
Kanamycin (0.07 g/L)	21.6
3-(N-morpholino)propanesulfonic acid) (MOPS) (10 g/L)	0.8
For ATPS (composition in Figure 1)	
K2HPO4	0.05
KH2PO4	0.03
PEG 400	0.2
For Precipitation	
NaCl (30 g/L)	0.11
PEG 8000 (40 g/L)	0.21
Buffer for Phages (TBS Buffer)	
Tris-HCl (50 mM)	0.17
NaCl (150 mM)	0.11
Consumables	
Vessel Filters (used before entry into a vessel)	Taken from Biosolve Process database and interpolated following the next equation for the desired vessel volume: $Vessel\ Filter\ Cost\ (US\ \$) = 0.3058 * Vessel\ Volume\ (L) + 45.334$
Filtration (0.45 µm)	Automatically adjusted by Biosolve Process; base cost is US $630 for 0.6 m2
UF/DF Filter	Automatically adjusted by Biosolve Process; base cost is US $5,269 for 1.1 m2

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Table 2. Recovery yields for each sample loading utilized in this study. Recovery yields for sample loading 40%, 50% and 60% w/w were extrapolated utilizing the behaviour of the difference of the recovery yields for loadings 10% to 30% (*Recovery yield difference [%] = -0.317 x Sample loading [%] + 6.3*).

Sample Loading (% w/w)	Recovery Yield (%)
10	83.31
20	83.27
30	80.06
40	73.68
50	69.41
60	64.62

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Table 3. Summary of results for materials discount analysis. Range of bioreactor volumes at which ATPS (VR 1 and 30% w/w of sample loading) is less expensive than precipitation and their corresponding production of doses at the given bioreactor scale (at the highest volume still less expensive than precipitation method).

Material Discount (%)	**Range of Bioreactor Volumes (L)**	**Production (Doses per batch)**	
		Precipitation	ATPS
10	10 – 20	924.4	897.6
30	10 – 30	1386.5	1346.4
50	10 – 50	2310.9	2243.9
70	10 – 100	4621.8	4487.8
90	10 – 410	18949.3	18400.2

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Table 4. Equations derived from simultaneous analysis for precipitation and ATPS while varying bioreactor scale, material discount and, only for ATPS, sample loading. Response variable (CoG/dose) is calculated as a natural logarithm. First two equations are employed for the calculation of CoG/dose. The second set of equation are used for interchange between bioreactor scale (dependent variable) and doses per batch (independent variable).

Equations for CoG/dose using ATPS and Precipitation			
	Variables	**ATPS[+]**	**Precipitation[#]**
	Intercept – β0	3.7662	2.6254
First Order	Bioreactor Scale (L) – β1	-3.8404e-3	-6.1691e-3
	Materials Discount (%) – β2	-5.3842e-3	-1.2139e-4*
	Sample Loading (% w/w) – β3	-5.1424e-2	N/A
Two-Way Interaction	Bioreactor Scale (L) x Materials discount (%) – β4	-1.0082e-5	-3.9742e-6
	Bioreactor Scale (L) x Sample Loading (% w/w) – β5	-2.1412e-5	N/A
	Materials Discount (%) x Sample Loading (% w/w) – β6	1.6925e-4	N/A
Quadratic	Bioreactor Scale (L) 2 – β7	3.4177e-6	4.0339e-6
	Materials Discount (%) 2– β8	-5.4297e-5	-3.7134e-6*
	Sample Loading (% w/w) 2– β9	5.4538e-4	N/A
	R^2	**0.8999**	**0.9089**
Equation for bioreactor scale (L) conversion from doses/batch and, only for ATPS, sample loading (% w/w)			
	Variables	**ATPS[^]**	**Precipitation[&]**
	Intercept – β10	-8.833e1	-1.593e-9
	Production (Doses/Batch) – β11	-2.332e-2	2.164e-2
	Sample Loading (% w/w) – β12	2.668	N/A
	R2	**0.99**	**1**

*Not statistically significant.

[+]Equation for CoG/dose for ATPS option with the form: *Ln (CoG/dose) = β0 + β1 x Bioreactor Scale [L] + β2 x Material Discount [%] + β3 x Sample Loading [% w/w] + β4 x (Bioreactor Scale [L] x Materials Discount [%]) + β5 x (Bioreactor Scale [L] x Sample Loading [% w/w]) + β6 x (Materials Discount [%] x Sample Loading [% w/w]) + β7 x (Bioreactor Scale [L])^2 + β8 x (Materials Discount [%])^2 + β9 x (Sample Loading [% w/w])^2*

[#]Equation for CoG/dose for precipitation option: *Ln (CoG/dose) = β0 + β1 x Bioreactor Scale [L] + β2 x Material Discount [%] + β4 x (Bioreactor Scale [L] x Materials Discount [%]) + β7 x (Bioreactor Scale [L])^2 + β8 x (Sample Loading [% w/w])^2*

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[^]Equation for Bioreactor scale using ATPS option: *Bioreactor Scale [L]* = β10 + β11 x Production [Doses/batch] + β12 x Sample Loading [% w/w]
[&]Equation for Bioreactor scale using precipitation option: *Bioreactor Scale [L]* = β10 + β11 x Production [Doses/batch]

FIGURES

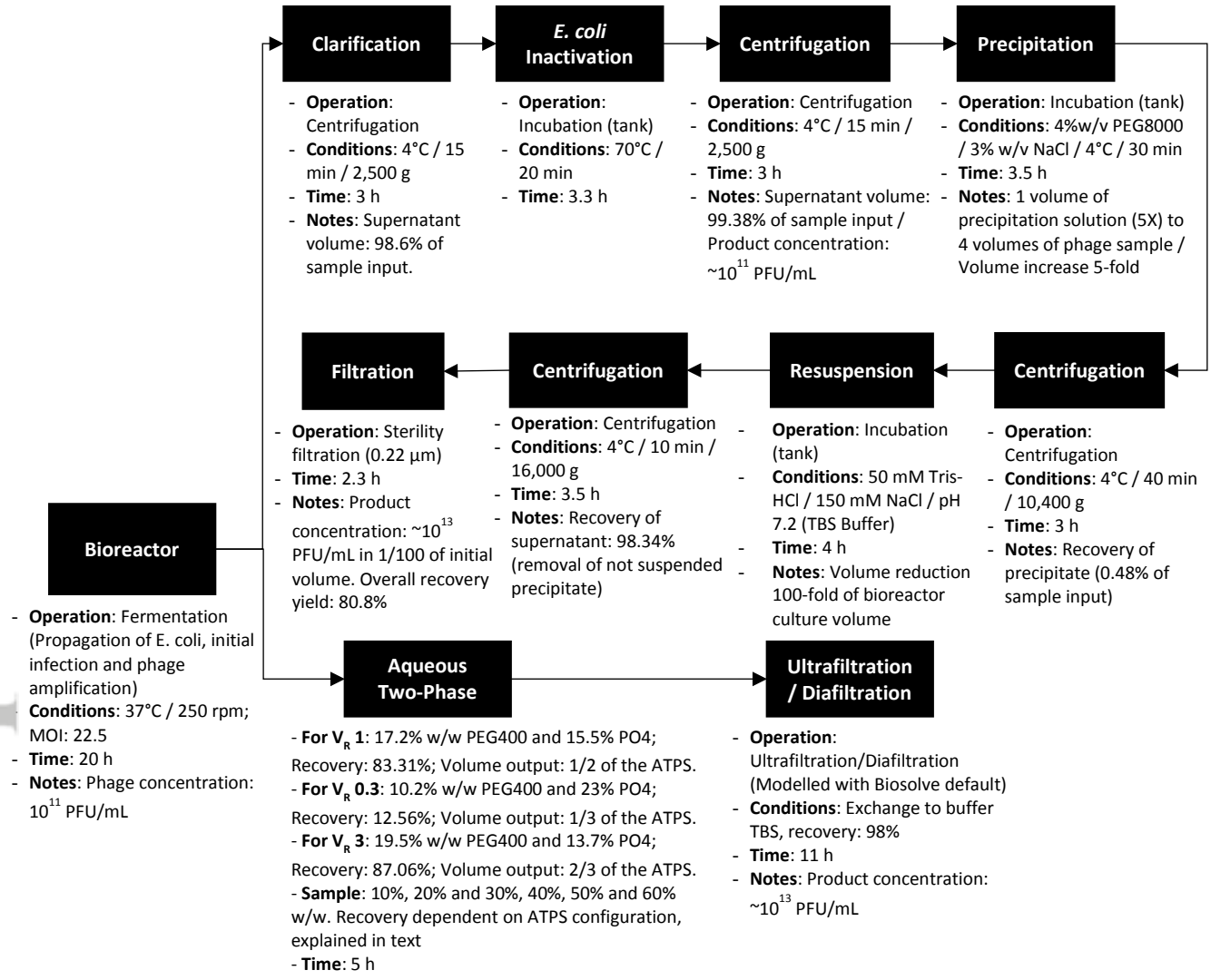

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Figure 1. Bioprocesses modelled for Aqueous Two-Phase System (ATPS) and Precipitation. Diagram indicates principal characteristics of each unit operation incorporated into Biosolve Process (version 7). The basis for the analysis performed in this work was to contrast the precipitation-based process with the ATPS-based version. Both bioprocesses have variable production scales ranging from 10 to 1,000 L for the working volume in the fermenter.

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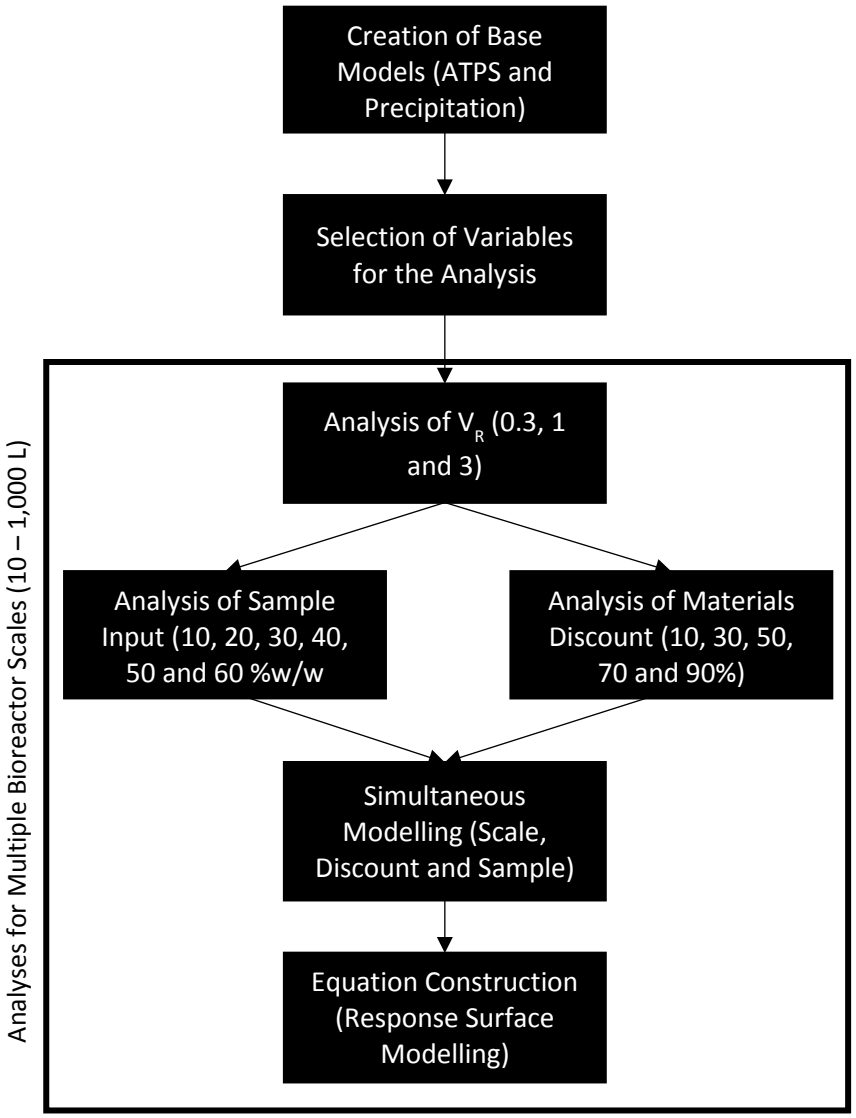

Figure 2. Analysis strategy followed in the present study. Briefly, models were created using the base reference (processes described in detail in Figure 1 and Table 1), and the same variables (volume ratio and sample input), now including materials costs discount, are studied from an economic perspective. Afterwards, the impact of V_R was studied. Then sample input and materials discounts were analyzed independently. Afterwards, they both were varied simultaneously (along with bioreactor scale) to obtain an equation that could describe the three variables at the same time. Sample input was analyzed only for ATPS.

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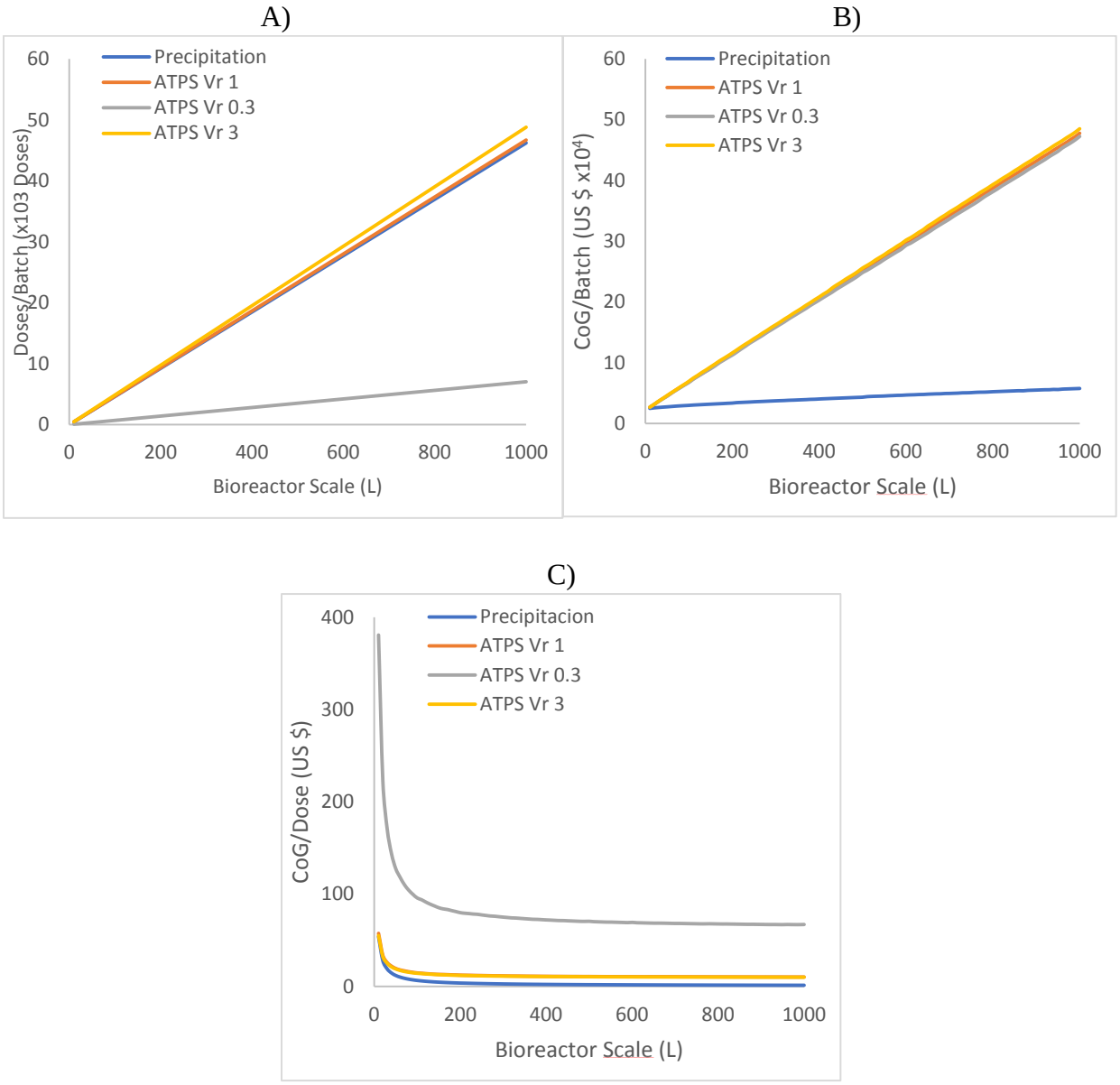

Figure 3. Results for V_R analysis for ATPS (0.3, 1 and 3) and precipitation. A) Doses produced per batch, B) CoG/Batch and C) CoG/dose. Legend: Blue (precipitation), orange (V_R 1), gray (V_R 0.3) and yellow (V_R 3). Data indicates that ATPS-based process at V_R 0.3 is the most expensive and least productive option, while the precipitation-based option is the least expensive. Additionally, the ATPS-based option at V_R 1 and 3 are highly productive and have a similar CoG/dose than precipitation.

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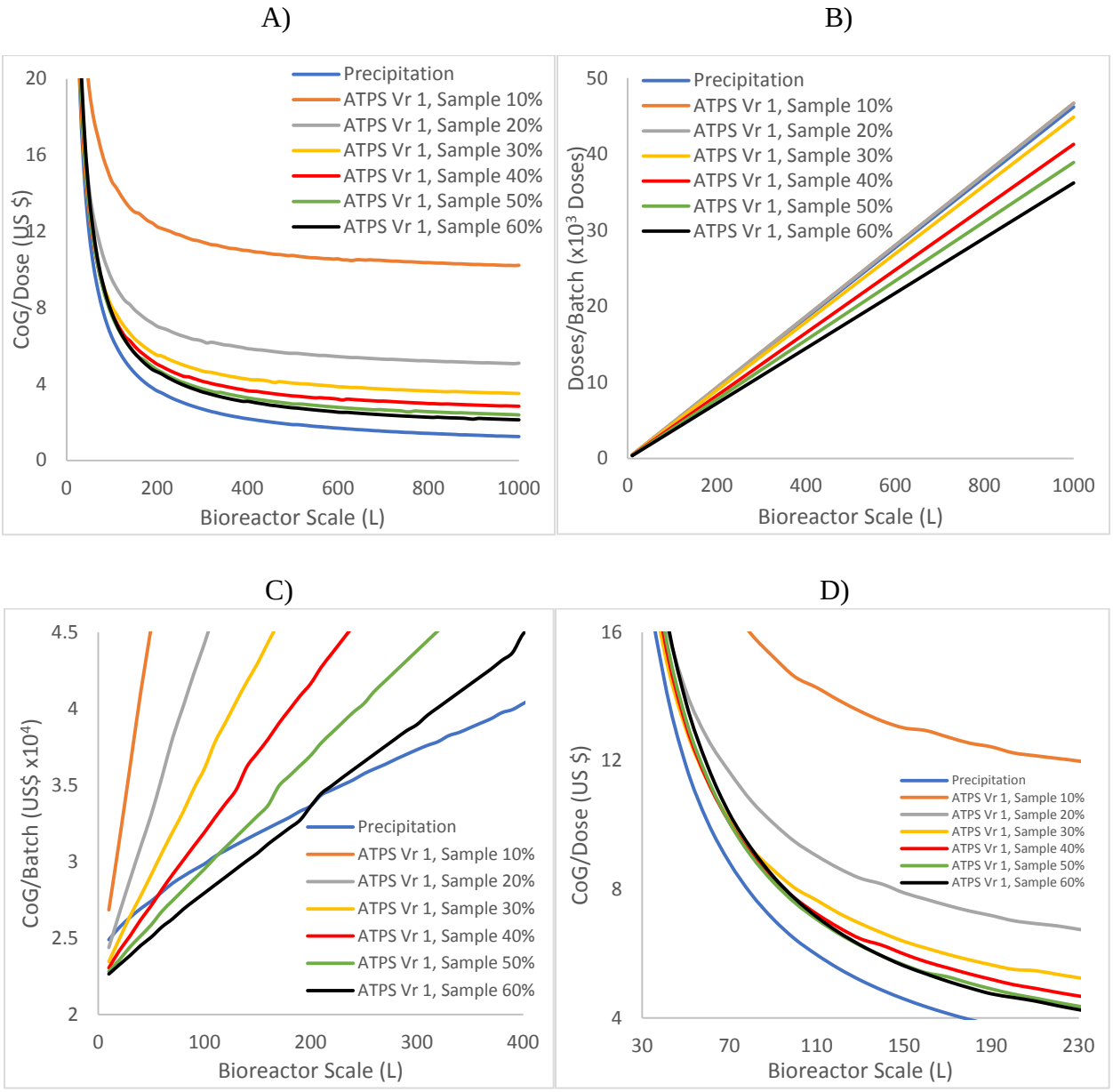

Figure 4. Results for contrast of increasing percentage of sample added (at V_R 1) against base precipitation. A) Doses produced per batch, B) CoG/Batch and C) CoG/dose. D) Zoom in of 5c in order to see the crossover of lines. Legend: blue (precipitation), orange (ATPS 10%), gray

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(ATPS 20%), yellow (ATPS 30%), red (ATPS 40%), green (ATPS 50%) and black (ATPS 60%). Data shows that as sample input increases there is a larger range of bioreactor sizes that have a lower CoG/batch than the precipitation-based option, but when considering the CoG/dose, only the 10 and 20 L scales at a sample input of 10% w/w are less expensive.

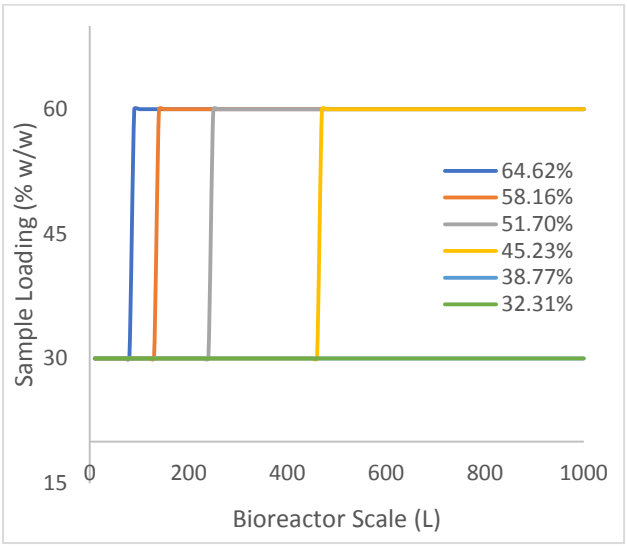

Figure 5. Effect of projected recovery yield on 60% w/w of sample loading. A decrease on the recovery yield of the theoretical 60% w/w scenario will increase the range of bioreactor scales at which 30% sample loading is the least expensive ATPS. Recovery yields presented are a decrease from 100% (original 64.62% - dark blue), 90% (58.15% - orange), 80% (51.70% - gray), 70 (45.23% - yellow), 60% (38.77% - light blue), and 50% (32.31% - green) from the projected recovery yield for sample loading 60% w/w. (Light blue and green lines are overlapped). Given the potential effect on saturation and product displacement, potential

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recovery yields for a 60% w/w sample input are likely to be low, a recovery yield of approximately 38% will make it a not viable option at any bioreactor scale.

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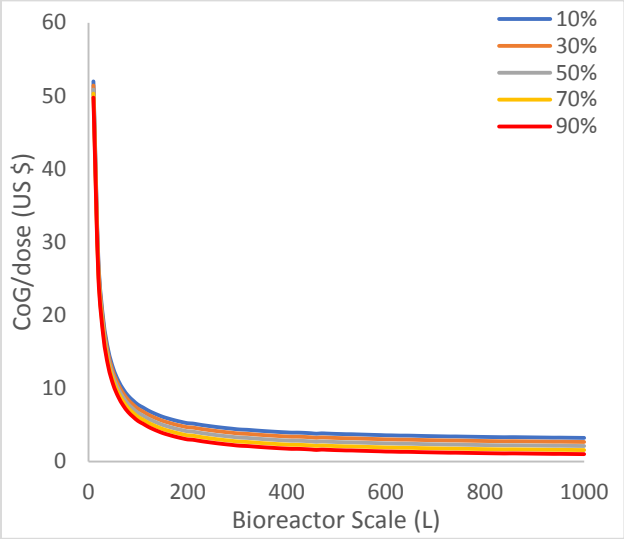
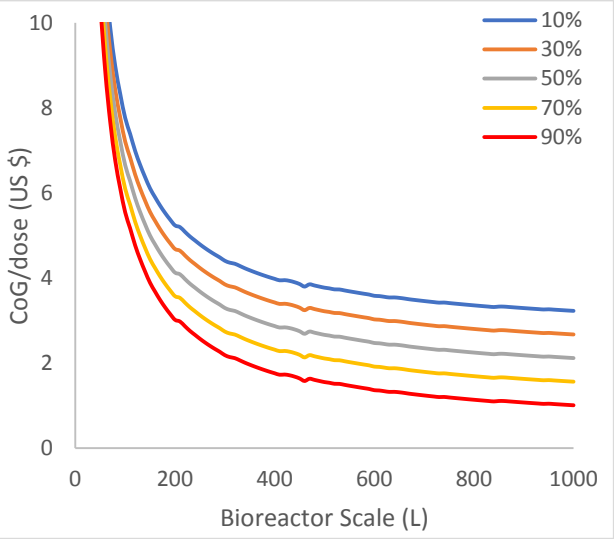
A)



B)



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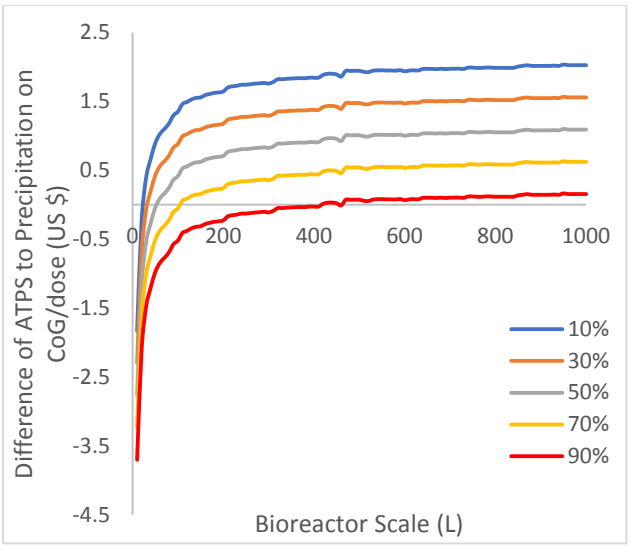

Figure 6. Results for the evaluation of materials discount (10, 30, 50, 70 and 90%). A) Overall results for all the discounts and base precipitation scenario. B) Zoom in for ATPS costs for less than US $10. C) Difference between precipitation and ATPS, negative values represent scales at which ATPS is more expensive than precipitation. Legend: blue (10%), orange (30%), gray (50%), yellow (70%) and red (90%). Data suggest that material discount does have an effect on reducing all production costs, but Figure 6c presents that this effect is reduced and the impact of increasing the production scale has a deeper effect.

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