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Simplified Method to Optimize Enzymatic Esters Syntheses in Solvent-Free Systems: Validation Using Literature and Experimental Data

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Abstract: The adoption of biocatalysis in solvent-free systems is an alternative to establish a greener esters production. An interesting correlation between the acid:alcohol molar ratio and biocatalyst (immobilized lipase) loading in the optimization of ester syntheses in solvent-free systems had been observed and explored. A simple mathematical tool named Substrate-Enzyme Relation (SER) has been developed, indicating a range of reaction conditions that resulted in high conversions. Here, SER utility has been validated using data from the literature and experimental assays, totalizing 39 different examples of solvent-free enzymatic esterifications. We found a good correlation between the SER trends and reaction conditions that promoted high conversions on the syntheses of short, mid, or long-chain esters. Moreover, the predictions obtained with SER are coherent with thermodynamic and kinetics aspects of enzymatic esterification in solvent-free systems. SER is an easy-to-handle tool to predict the reaction behavior, allowing obtaining optimum reaction conditions with a reduced number of experiments, including the adoption of reduced biocatalysts loadings.

Keywords: immobilized lipases; solvent-free reactions; enzymatic esterification; esters

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1. Introduction

Solvent-free systems (SFS) are becoming popular for enzymatic esterifications. These systems have many advantages because the reaction media is formed only by the reactants, increasing the volumetric productivity of the process and avoiding complex downstream and hazardous wastes [1–3]. The adoption of solvent-free systems may contribute to achieving the feasibility of biocatalytic ester syntheses on a large scale in both technical and economic aspects, in consonance with the principles of Green Chemistry.

Immobilized lipases have been utilized successfully for esterification reactions in SFS [4–9]. Enzyme immobilization enables enzyme recovery and reuse, associated with the possibility of improving enzyme stability, activity, selectivity, or specificity [10–12]. Moreover, it may enlarge the window of operating conditions (reducing inhibitions or inactivation by chemicals) and be coupled to the purification processes [13–16]. Immobilized lipases are extensively studied for esters syntheses [10,12,17–20], potentially addressing demands in many different sectors such as energy and transport [21–23], food industries [24–26], cosmetics and personal care [19,27], and chemical industries [28–30].

Molar ratio and biocatalyst loading are two of the main parameters studied in solvent-free enzymatic esterifications because this reaction is thermodynamically controlled and, thus, the concentration of the catalyst determines the conversion rate [31–33]. The reaction media and the experimental conditions have major influences on these aspects, and, in

solvent-free systems, the medium is determined by the substrates and the molar ratio of the substrates [1]. The evaluation of molar ratio in SFS gains additional importance, considering that the use of a surplus reagent is required to obtain high conversions in the synthesis of the other substrate, and the surplus reagent, i.e., its nature and its quantity in the system, will define critical physicochemical characteristics of the reaction media in the different steps of the reaction [1,31,34–36]. A dynamic environment is presented in SFS esterification; initially, the media is formed exclusively by the mixture of the substrates, and at the end, it will be formed by the ester product, the co-product water (if is not removed), the remaining excess substrate, and some traces of the minority substrate [1,37]. The final reaction media is generally more hydrophobic than the initial system. The elimination or capture of the formed water is a way to shift the reaction thermodynamic equilibrium towards synthesis [37,38]. However, even if water is eliminated in the reaction medium when the enzyme activity is very high, the accumulation of water inside the biocatalyst particle (when the enzyme activity is very high) can generate a water phase in the enzyme environment, adversely affecting the enzyme performance [39,40]. The use of very hydrophobic supports or ultrasounds may reduce these adverse effects [41–48]. On the other hand, biocatalyst loading in the reactor defines the kinetics aspects of the reaction, such as reaction rate and the occurrence of inhibition/inactivation, with a substantial impact on reaction time, productiveness, and process costs [42,49–52]. As immobilized lipases are still expensive incomes, the definition of an optimized biocatalyst loading is a critical parameter for any applied biocatalytic process [44,49–52].

The classical approach of evaluating independent variables once a time is still commonly used for enzymatic esterification reaction optimization, even in recent studies [7–9,53–56]. However, it has been shown that correlation among some of the studied variables makes the independent optimization incomplete, and, thus, statistical tools, such as response surface methodology (RSM), became popular for optimization studies [49,57–64]. Although interactions between variables may be discovered using RSM, the molar ratio of reagents and biocatalyst loading are generally considered independent variables. Nonetheless, in a previous study, we have found an interesting correlation between both variables for the synthesis of octyl octanoate catalyzed by Novozym 435 in a SFS [6]. Our findings led us to develop a simple mathematic tool, that we named SER (Substrate-Enzyme Relation) that correlates the mass of reagents and immobilized lipases, as described in Equation (1):

$$\text{SER} = \frac{\text{m alcohol} - \text{m acid}}{\text{m biocatalyst}} \quad (1)$$

where "m alcohol" and "m acid" are the masses (grams) of alcohol and acid in the reaction, respectively, and "m biocatalyst" is the mass of immobilized lipase (enzyme + support). SER is a dimensionless number that expresses a certain reaction condition—a mass of reagents and biocatalyst in the system. This reaction condition will generate a conversion result in the reaction, i.e., the percentual degree of conversion of the reagents (in general carboxylic acid) into products (ester). Considering that lipases are specific catalysts that catalyze only esterification/hydrolysis reactions, the conversion of the reagents means the yield of the reaction. Thus, a SER number may be associated with a conversion result. In our first approach, the study of the interaction between molar ratio and biocatalyst loading in the reactor and their influence on the process performance resulted in high conversions (above 96.0%), using relatively low enzyme loading (1.5% *wt*/*wt* acid mass) and 30% stoichiometric excess of alcohol [6]. SER possible fundamentals were based on the hypothesis of a shift of chemical equilibrium by using stoichiometric excess of alcohol until an estimated level in which the yields are not further improved because lipase suffers inhibition or inactivation. The simple combination of the masses of reagents and biocatalyst enabled us to obtain practical information about the reaction thermodynamics and kinetics, using the outcome to establish a range of reaction conditions in which high conversions could be achieved (in this case, SER between 0 and 65).

Thus, considering that SER can be an easy-to-handle tool to predict the enzymatic esterification reaction optimization, this study aims to validate its applicability in synthesizing aliphatic esters in SFS mediated by immobilized lipases. We applied this mathematic tool for lipase-catalyzed esterification reactions previously described in the literature and using experimental data obtained in this study (39 different examples).

2. Results

2.1. SER Validation from Literature and Experimental Data

Table 1 shows the SER outcomes calculated utilizing available data in the selected publications and the correlation with the optimal range suggested by our previous work. We observe that high conversions (above 80%) were associated, in most cases, with intermediate positive values of SER, between 0 and 65, as observed by Sousa and co-workers (2020) [6] for the octyl octanoate synthesis. In addition, conversion higher than 90% were observed within this SER range for a variety of cases—92–93% on butyl formate and octyl formate with SER 35–36 [8]; 92–94% on isobutyl propionate with SER 20 [4] and SER 13 [65]; 92% on butyl octanoate with SER 0.5 [7]; 96–99% on octyl octanoate with SER 4–9 [6]; 95–98% on cetyl tetradecanoate, cetyl hexadecanoate, cetyl stearate, and cetyl oleate with SER 4 [66].

Table 1. Enzymatic syntheses studies of aliphatic esters in solvent-free systems with calculated SER and its respective conversion results.

Acid	Alcohol	Lipase	Immobilization Support	T (°C)	Conversion Response Reported (%)	SER Calculated Range	SER with the Highest Conversion	Ref.
Good level of agreement								
Metanoic Acid	*n*-Butanol	Novozym 435®	Lewatit VP OC 1600	40	55.0–93.0%	35 to 39	36 (93.0%)	[8]
Metanoic Acid	*n*-Octanol	Novozym 435®	Lewatit VP OC 1600	40	-	-	35 (92.0%)	[8]
Propionic Acid	Isobutanol	Fermase CALB 10000	Polyglycidemethaacrylate	60	71.8–94.2%	0 to 20	13 (94.2%) ***	[65]
Propionic Acid	Isobutanol	Novozym 435®	Lewatit VP OC 1600	40	63.8–92.5%	0 to 30	20 (92.5%)	[4]
Butanoic Acid	*n*-Butanol	Novozym 435®	Lewatit VP OC 1600	37	41.0–48.6%	−3 to −1	−1 (48.6%) *	[67]
Pentanoic Acid	Ethanol	Lipase from S. *simulans*	$CaCO_3$	37	29.0–51.0%	−20 to −4	−4 (51.0%) **	[68]
Pentanoic Acid	Ethanol	Novozym 435®	Lewatit VP OC 1600	50	40.0–69.0%	−50 to −21	−33 (69.0%) ***	[55]
Octanoic Acid	*n*-Butanol	Novozym 435®	Lewatit VP OC 1600	37	-	-	−4 (38.0%) *	[67]
Octanoic Acid	*n*-Butanol	Novozym 435®	Lewatit VP OC 1600	50	85.0–89.0%	−24 to 27	27 (89.0%)	Data not published
Octanoic Acid	*n*-Butanol	Lipozyme RM IM®	Duolite ES 562	50	68.2–89.0%	−12 to 14	14 (89.0%)	Data not published
Octanoic Acid	*n*-Butanol	Novozym 435®	Lewatit VP OC 1600	60	76.5–92.5%	−14 to 9	0.5 (92.5%)	[7]
Octanoic Acid	*n*-Octanol	Novozym 435®	Lewatit VP OC 1600	65	74.7–96.1%	−19 to 207	9 (96.1%)	[6]
Octanoic Acid	*n*-Octanol	Lipozyme RM IM®	Duolite ES 562	65	19.6–99.0%	−10 to 171	4 (99.0%)	Data not published
Decanoic Acid	*n*-Propanol	Fermase CALB 10,000	Polyglycidemethaacrylate	60	19.0–83.8%	−96 to 8	1 (83.8%)	[9]
Dodecanoic Acid	*n*-Butanol	Novozym 435®	Lewatit VP OC 1600	37	28.0–35.0%	−10 to −3	−10 (35.0%)	[67]
Dodecanoic Acid	Hexadecan-1-ol	Lipozyme RM IM®	Duolite ES 562	70	67.0–98.1%	4 to 32	4 (98.1%)	[66]
Tetradecanoic Acid	Isopropanol	Novozym 435®	Lewatit VP OC 1600	60	7.0–87.7%	7 to 60	15 (87.7%)	[5]
Tetradecanoic Acid	Hexadecan-1-ol	Lipozyme RM IM®	Duolite ES 562	70	65.9–97.3%	4 to 33	4 (97.3%)	[66]
Hexadecanoic Acid	Hexadecan-1-ol	Lipozyme RM IM®	Duolite ES 562	70	61.8–97.1%	4 to 33	4 (97.1%)	[66]
Octadecanoic Acid	Hexadecan-1-ol	Lipozyme RM IM®	Duolite ES 562	70	60.5–95.8%	4 to 33	4 (95.8%)	[66]
Octadec-9-enoic Acid	*n*-Octanol	Lipase from *R. miehei*	Poly(ehtylene)-g-co-hydroxyethyl methaacrylate	37	-	-	56 (82.0%)	[69]
Intermediate level of agreement								
Propionic Acid	*n*-Butanol	Novozym 435®	Lewatit VP OC 1600	45	81.2–92.7%	100 to 600	100 (92.7%)	[70]
Propionic Acid	*n*-Butanol	Novozym 435®	Lewatit VP OC 1600	60	35.0–92.0%	39 to 160	85 (92.0%) ***	[56]
Butanoic Acid	*n*-Butanol	Novozym 435®	Lewatit VP OC 1600	53.9	-	-	91 (99.6%)	[64]
Decanoic Acid	*n*-Octanol	Novozym 435®	Lewatit VP OC 1600	50	92.0–96.0%	−1 to 0	−0.4 (96.0%)	[71]
DecanoicAcid	*n*-Octanol	Lipozyme RM IM®	Duolite ES 562	50	88.8–95.1%	−1 to 0	−0.2 (95.1%)	[71]
Dodecanoic Acid	*n*-Octanol	Novozym 435®	Lewatit VP OC 1600	50	93.9–96.3%	−10 to −1.5	−1.5 (96.3%)	[71]
Dodecanoic Acid	*n*-Octanol	Lipozyme RM IM®	Duolite ES 562	50	86.7–97.7%	−10 to −1.5	−4 (97.7%)	[71]
Octadec-9-enoic Acid	*n*-Butanol	Lipase from *R. oryzae*	$CaCO_3$	37	18.0–81.0%	−54 to −5	−5 (81.0%)	[72]
Low level of agreement								
Etanoic Acid	*n*-Butanol	Lipase from *R. oryzae*	Celite 545	37	12.0–61.0%	−7.5 to 9	1 (61.0%) **	[73]
Etanoic Acid	Isopentanol	Lipase from S. *simulans*	$CaCO_3$	37	2.0–64.0%	−26 to 35	−7 (64.0%) **	[74]
Etanoic Acid	Isopentanol	Novozym 435®	Lewatit VP OC 1600	30	46.7–68.4%	3 to 10	3 (68.4%)	[60]
Etanoic Acid	*n*-Hexanol	Lipase from S. *simulans*	$CaCO_3$	37	21.0–43.0%	3 to 14	3 (43.0%) **	[68]
Dodecanoic Acid	Ethanol	Fermase CALB 10,000	Polyglycidemethaacrylate	60	67.0–92.4%	−31 to −2	−15 (92.4%)	[75]
TetradecanoicAcid	Isopentanol	Novozym 435®	Lewatit VP OC 1600	60	82.0–97.0%	−197 to 17	−99 (97.0%) ***	[76]
Hexadecanoic Acid	Isopropanol	Novozym 435®	Lewatit VP OC 1600	75	33.5–88.0%	−35 to −10	−10 (88.0%)	[57]
Octadecanoic Acid	Ethanol	Novozym 435®	Lewatit VP OC 1600	60	66.0–92.0%	−65 to 27	−19 (92.0%)	[77]
Octadecanoic Acid	*n*-Butanol	Novozym 435®	Lewatit VP OC 1600	60	61.0–92.0%	−74 to 104	−48 (92.0%)	[77]
Octadec-9-enoic Acid	Ethanol	Lipozyme®	Duolite A568	40	72.0–99.0%	−41 to −4	−41 (99.0%)	[1]

* Included the use of molecular sieves or vacuum pressure to remove the water. ** With the addition of water. *** Included activation by microwave or ultrasound.

SER optimum range seems to be applicable for different carboxylic acid and alcohols of different chain lengths, as showed by the first section of Table 1, but some important aspects should be highlighted. For short-chain acids and alcohols, as studied by Aljawish et al. (2019) [8] and Kuperkar et al. (2014) [4], optimum SER was found in the middle of the

proposed range, between 20 and 36, due to the use of significant molar excess of alcohol for reducing the strong inhibition potential of short-chain acids. As short-chain alcohols also promote inhibition of lipases and may cause damages in their hydration layers [1,57,72,76], the formed water in the system helps to attenuate this problem, resulting in high conversions. Jaiswal & Rathod (2017) [65] did not use the same level of a stoichiometric excess of isobutanol, which resulted in SER equals 13, but the reaction was assisted by microwave. Similarly, the formed water remained in the system. Contrarily, for long-chain acid and alcohols, as reported by Arnaldos et al. (2018) [66], optimum SER was found to be close to 0, as shown by Table 1. Since long-chain acids or alcohols have less potential to affect the biocatalysts' hydration layer, it is essential to remove the formed water due to the possible water accumulation in lipase vicinity in such a hydrophobic environment, forming a diffusional barrier for the substrate and lipase active sites [2,37,78]. In these cases, it is possible to adopt biocatalyst loadings lower than those used for esterification with short-chain reagents, which is coherent with the reduced probability of substrate enzyme inhibition when using long-chain reagents [1,79,80]. The same rationale can be extended for mid-chain acids and alcohols, as suggested by the SER in our previous study [6] and the experimental data collected in this work, as shown in the first section of Table 1. Studies in which water was not removed, include own data, showed conversions slightly lower [5]. Data from Ghamgui et al. (2004) [72] and Sousa et al. (2021) [71] show that mid and long-chain acids and alcohols seem to have optimum SER close to 0, although within a broader range of values including slightly negative outcomes. These studies showed optimum conditions using discrete stoichiometric excess of alcohol, associated with low biocatalyst loadings, which resulted in a low SER number.

It is plausible to compare different studies of solvent-free enzymatic esterification with immobilized lipases because (i) chemical equilibrium in an esterification reaction, using monofunctional acids and alcohols, is not dependent on the (bio)catalyst adopted [31,81,82]; (ii) the theoretical equilibrium constant should be the same for the same type of reaction independently of the reagents, even though slight variations may occur due to effects of the solvation of different reagents and products and the ionization of the different carboxylic acids, as observed in experimental data [31,82]. Furthermore, external mass transfer limitations are generally neglected in immobilized lipase-catalyzed esterification reactions at a lab-scale [1,5,10,83,84].

Considering the many different aspects that define the kinetics and yields of enzymatic esterifications in SFS using immobilized lipases, it is counterintuitive to think that a simple mathematical equation is effective in predicting the behavior of the reaction with different reagents. The numerator of SER represents, in terms of mass, the quantity of exceeding reagent. Indirectly, the analysis of its nature coupled with its quantity may give an idea if the solvation of the second reagent by the exceeding reagent will be favored or not. Solvation of reagents and products are associated with their respective thermodynamic activities, which govern the equilibrium equation of the reaction [31,81,85–88]. The denominator of SER is the mass of biocatalyst that provides information about the reaction rate: in the absence of harmful interferences, increasing biocatalyst loadings in the reactor will linearly increase the reaction rate. The stoichiometric excess of one of the reagents (the numerator of SER) and the biocatalyst loading (denominator of SER) are linked by the ability of the exceeding reagent to affect the enzyme properties, causing modifications in the essential hydration layer of the enzyme or generating enzyme inhibition. An intermediate outcome is obtained by a relation between a proper biocatalyst loading and an excess of reagent that potentially leads the system to shift the equilibrium towards synthesis without noticeable inhibition or inactivation of enzymes. If a highly hydrophilic reagent is present in the system, a sizeable molar excess of the second reagent or adopting high biocatalyst loading is required to counterbalance its deleterious effect using to compensate for the possible reaction rate reduction, as observed in the study of Vadgama and co-workers (2015) [5]. This condition is associated with an intermediate SER outcome, depending on the nature of the reagents and biocatalysts involved. The proposition of a different range of values that

correspond to high conversions is a way to deal with the variability of reaction conditions that include the nature of reagents and biocatalysts and different methods of activation and reactional strategies. SER should be understood as a general tool, not a mathematical simulator of the reaction.

The data of short-chain esterifications lead us to hypothesize that the optimum range established for mid-chain esterifications should be shifted for higher SER values. The synthesis of butyl propionate carried out by Dai and co-workers (2014) [70] is an interesting case because only conditions with high SER were evaluated. Although the lowest SER number tested—the closest to the previously defined optimum range—resulted in the highest conversion, this result was obtained using a similar strategy than Aljawish et al. (2019) [8] and Kuperkar et al. (2014) [4] maintaining the formed water in the media. The reduction in the reaction rate seems clear in this case due to the long reaction times (30 h) compared to the synthesis of isobutyl propionate carried out by Kuperkar and co-workers (10 h) [4]. The study of Rahman and co-workers (2017) [64] reinforces the relevance of water maintenance in the esterification of short-chain acids and alcohols, and although only one condition was tested, the optimum SER value in this study (91) is above the supposed optimum range.

The studies of short-chain esterifications classified as low level of agreement with our previous work also reinforce this hypothesis, because conversions below 80.0% with SER close to 0 (supposedly inside the proposed optimum range) were obtained [68,73,74,89]. However, three studies of the same research group [68,73,74] adopted specific conditions in the reactions related to the addition of a considerable quantity of water (10% or more) to the system. As potent inhibition caused by highly polar acids (as etanoic or pentanoic acid) and short-chain alcohols are expected in these cases, the authors tried to avoid this using stoichiometric excess of some of the reagents. Thus, the addition of the water (acting as a co-solvent) reduced the acid concentration and may have restored the hydration layer of the enzymes; however, as water is a product of the reaction, thermodynamic equilibrium was shifted to hydrolysis, as discussed by the authors. Moreover, the syntheses studied by Karra-Châabouni et al. (2006) [68] were carried out only in equimolar substrate conditions at different biocatalyst loadings, indicating why the SER trend is not followed in this case.

The use of microwave irradiation can be included in the same set of specific reaction conditions. Bhavsar & Yadav (2018) [55,56] studied the syntheses of *n*-butyl propionate and ethyl valerate—two pairs of short-chain substrates—assisted by microwave. The first study shows SER value equals 85 as the optimum; the lowest conversions observed were associated with SER value 39 and 170 (respectively, 52.0% and 35.0%), indicating a range of optimum reaction conditions with different values than the initially suggested SER trend. However, when the condition corresponding to SER 85 was evaluated under conventional heating (60 °C), the reaction presented a 72.0% of conversion after 8 h, not so far from the expected trend. The second study evaluates reaction conditions corresponding to negative SER outcomes (between −50 and −21). Besides the use of microwave—which affects the reaction media behavior and immobilized lipase performance differently than conventional heating—the reaction time was too short (40 min), suggesting that the equilibrium may not have been achieved. Comparing the results obtained in optimum reaction conditions under microwave and conventional heating indicates that the reaction presented a low conversion (53.1%) with SER −33. Then, the adoption of microwave in SFS brings additional difficulties for analyzing the data. However, utilizing the data obtained using conventional heating in the same studies, we observe an acceptable level of agreement with the SER trend.

The most remarkable exceptions are the studies involving long-chain acids (above C12) and short-chain alcohols (below C7) [1,57,76,77]. High conversions were obtained with negative SER results far from 0, within a range of −10 and −48. The reason is that the SER equation, as firstly established, does not consider the molar masses of the different involved reagents. When a long-chain acid reacts with short-chain alcohol, the subtraction of utilized masses of alcohol (even using an excess of alcohol) and acid generates a negative SER number due to the accentuated differences in molar masses. For instance, the highest

conversion obtained by Pereira and co-authors [77] for butyl stearate synthesis (92.0%) was obtained with acid:alcohol molar ratio 1:2, which corresponded to the SER value of −48. Yadav and Thorat (2012) [76] obtained a high yield on isopentyl tetradecanoate with a very negative SER value, −99. The authors indicated the equimolar ratio of reagents with a low biocatalyst loading of Novozym 435® (0.38% *wt*/*wt*) as the optimum condition for this synthesis, but the system was submitted to microwave. The same reaction conditions under conventional heating resulted in a low 56.0% of conversion. Considering these cases, we have elements to hypothesize that, for esterifications between long-chain acids and short-chain alcohols, SER negative outcomes also follow the same trends as the positive ones, keeping the same rationale about extreme values.

Summarizing: (i) SER optimum range (0 to 65) is also observed for other mid-chain esters syntheses besides octyl octanoate mediated by Novozym 435®; (ii) SER optimum range seems to be shifted for higher values (20 to 100) in short-chain esters syntheses and lower values in long-chain esters syntheses (−65 to 0); (iii) the trend is not followed either when the reaction is assisted by microwave or ultrasound, nor when water is added to the media.

Few studies evaluated a wide range of SER values at a constant temperature [6,9,76,77]. The highest conversion observed in these studies were achieved not only by the manipulation of molar ratio and biocatalyst loadings at a constant temperature, but with the application of some additional strategy to obtain the maximum yield, as the continuous removal of the water from the media [6,7,9,66], or different methods of activation, as microwave irradiation [55,56,76]. Therefore, we believe that SER will only indicate a range of conditions in which thermodynamics and kinetics aspects have a convergence towards high conversions but not predicting the exact condition that will result in the maximum conversion. The effective use of SER to optimize molar ratios and biocatalyst loadings implies that temperature and stirring rate are enough to promote a proper reaction performance.

The work of Santos and co-authors (2007) [67] illustrates well the limitations caused by stirring and temperature. The authors adopted stoichiometric excesses of acid for esterifications of *n*-butanol with butanoic, octanoic, or dodecanoic acid, with high biocatalyst loadings (5, 10, and 15% *wt*/*wt*) of Novozym 435®, at 37 °C and 150 rpm of stirring rate. Besides the unfavorable relation between these variables using SER as a parameter, 37 °C seems inadequate in the esterification of dodecanoic acid for a proper mixture of reagents since the dodecanoic acid melting point is 43 °C; its use in stoichiometric excess may limit the diffusion of *n*-butanol inside the biocatalyst particle. Moreover, the stirring rate (150 rpm) could not be enough to properly mix biocatalyst and substrates, considering that most cited studies used 200 rpm or higher stirring rates in these reactions [4,7–9,72–74,77]. Moreover, high biocatalyst loadings were adopted, and the poor conversions after 72 h (below 50.0%) of reaction may be associated with biocatalyst particle aggregation, increasing the diffusional limitations on the system. Partial inactivation of the immobilized lipase may also be suggested in the esterification of butanoic acid by reducing pH in the enzyme environment. To reinforce the hypothesis of diffusional limitations caused by high biocatalyst loadings in that stirring rate, we obtained 73.0% of conversion in butyl octanoate synthesis using 2.0% Novozym 435® and an acid:alcohol molar ratio of 1:2 (SER equals to 2), at 30 °C and 150 rpm, in just 3 h. Similar issues may be suggested for the results obtained by Güvenç and co-workers (2007) [89] in the esterification of etanoic acid and *n*-pentanol, where high biocatalyst loadings (6% and 10%, which corresponds to SER 3 and 10, respectively), temperatures of 35 °C, and 150 rpm of stirring rate were employed.

As we can see in Table 1, most of studies used Novozym 435® as biocatalyst. Its immobilization support material is a moderately hydrophobic resin [20]. The same feature can be observed in Lipozyme RM IM®, whose support is Duolite ES 562® [18]. By adopting proper reaction conditions, reactions mediated by these biocatalysts achieve high conversions. Thus, it is possible to observe that moderately hydrophobic materials are enough to promote a proper partitioning of the water in the media, keeping the hydration layer of enzymes and simultaneously avoiding the occurrence of hydrolysis of formed esters.

2.2. Thermodynamics and Kinetics Aspects

SER contains a term indirectly correlated with thermodynamics (difference between substrates masses) and another term indirectly correlated to kinetics (biocatalyst mass in the system). An exploration of these topics is required to check how SER is coherent with the phenomena involved in enzymatic esterification. Chemical equilibrium is established by the relation of products and reagents concentrations, or more accurately, its thermodynamics activities (in non-ideal systems) [31–34]. Substrates thermodynamic activities should increase to favor the esterification, and this increase will not happen if the reagents are very well solvated (by the solvent or by the exceeding reagent in SFS); contrarily, the thermodynamic activities of the products should decrease to avoid hydrolysis [1,31,36,90]. The quantity and the nature of the exceeding reagent can provide simple predictions about solvation and thermodynamic activities, helping obtain a good overview of the reaction. For instance, in a reaction between short-chain alcohol (C3) and a long-chain acid (C14) with a molar ratio acid:alcohol 1:3, the mutual solubility of the substrates will not be favored—highly polar alcohol with a non-polar acid. Due to the differences in polarity, the alcohol will not solvate the acid completely, although some degree of solvation may affect the final conversion due to its excess. In the case of a reduction in alcohol quantity that corresponds to a discrete stoichiometric excess, it is expected that the alcohol will be unable to solvate a more significant percentage of the acid, which means that the acid conversion will be favored. The opposite situation—the increase of alcohol concentration in the system—may increase the probability of damages in the hydration layer of lipases or inhibition [1,4,72,76,91]. Not only surplus reagent can cause these damages, but its probability is higher than that of the limiting reagent. SER helps visualize these situations by a simple way. A discrete stoichiometric excess of the mentioned alcohol that is unable to solvate the acid is associated with low number in the SER numerator; similarly, discrete stoichiometric excess of alcohol that reduces the probability of damages on immobilized lipases or inhibition is also associated with low numbers.

Adopting a significant stoichiometric excess of alcohol in this hypothetical case may cause inhibition, with a consequential reduction in the reaction rate. Then, an increase in the biocatalyst loading may be adopted to compensate for this reduction. Mathematically, an increase in the biocatalyst loading means an increase in the SER denominator, reducing the outcome of SER. From a thermodynamic perspective, a discrete stoichiometric excess of alcohol seems adequate to shift the chemical equilibrium, avoiding excessive solvation of acid in this case and with a reduced probability of inhibition on lipases. These effects will depend on the amounts and the nature of the surplus reagent in the media. The combination of this condition, expressed by SER numerator, associated with low biocatalyst loading, expressed by SER denominator as the mass of biocatalyst, will result in a low SER outcome. As shown in Table 1, this observation is coherent with high conversions observed in the literature (and own data) for the esterification of long-chain acids and short-chain alcohols [1,72].

Water plays an essential role in enzymatic esterification, affecting both thermodynamic and kinetics aspects. For example, in a C14 acid and C3 alcohol esterification, the formed water is expected to be solubilized mainly in the alcohol. However, some water may remain in the vicinity of the enzyme if the immobilization support of the enzyme is moderately hydrophobic [92–94]. The ester formed is always more apolar than both substrates, and its concentration will be growing along with the reaction time. Partitioning of the water between the reaction media and the catalytic phase may occur, driving to favor the lipases hydrolytic activity. The effects of an excess of the alcohol (indicated by an increase in SER numerator) may cause a complete strip-off of the hydration layer of the enzyme with probable enzyme inhibition/inactivation. As the remaining quantity of alcohol will decrease along with the reaction, the essential hydration layer of the immobilized enzyme may be less affected at the end than at the beginning of the reaction, depending on the biocatalyst loading and the excess of alcohol in the system (indicated by SER denominator and numerator, respectively). Most of the water formed may be bounded to the biocatalyst,

forming a water layer around the enzymes, affecting enzyme activity and stability. Also, the water layer may produce a partition of the ester from the active site of the enzyme, considering the ester hydrophobicity; then, diffusional limitation caused by the water layer may avoid the access of the ester into the active site of the enzyme, preventing likely inhibitions by the reaction product.

Further information of the reaction media may be predicted in qualitative terms, and SER can be used to a quantitative understanding of these phenomena. Overall considerations about the behavior of the reaction from a chemical equilibrium and kinetics perspective are summarizing in Table 2.

Table 2. Predictions of the behavior of enzymatic esterifications in different conditions of molar ratio using stoichiometric excess of reagents.

Reaction Condition	Initial Condition of the Media	Effect on Biocatalyst	Condition on Equilibrium	Possible Optimization Path
Large stoichiometric excess of a highly polar reagent	Possible strip-off of essential water on enzymes pH acid (if exceeding reagent is the acid) Low log P; low viscosity	Possible reduction in the enzymatic activity	Accumulation of the water on the organic phase pH slightly acid Low log P; increased viscosity	Adoption of high biocatalyst loadings Control of the water activity Fractioned additions of the exceeding reagent
Discrete excess of a highly polar reagent	pH slightly acid (if the surplus reagent is the acid) Limited solubility/poor solvation (if limiting reagent is non-polar) Intermediate log P; intermediate viscosity (if limiting reagent have a long-chain)	-	pH neutral (or slightly acid) Intermediate log P; considerable viscosity	Possibility of adopting reduced biocatalyst loadings
Discrete excess of a non-polar reagent	pH slightly acid (if the surplus reagent is the acid) Limited solubility/poor solvation (if limiting reagent is highly-polar) Intermediate log P; intermediate viscosity (if limiting reagent have a long-chain)	-	pH neutral (or slightly acid) Intermediate log P; considerable viscosity	Possibility of adopting reduced biocatalyst loadings
Large stoichiometric excess of a non-polar reagent	pH acid (or slightly acid) High log P; high viscosity (if surplus reagent have a long-chain)	Possible reduction in the enzymatic activity	Accumulation of the water on catalytic phase pH neutral (or slightly acid) High log P; high viscosity	Adoption of high biocatalyst loadings Control of the water activity Increase the temperature

For different reasons, we can observe that large stoichiometric excesses of one of the reagents seem to be unfavorable to enzymatic esterifications since it increases the solvation of the limiting reagent requires the use of high biocatalyst loading or other strategies to avoid inhibition of lipases. Nevertheless, some high conversion results showed in Table 1 were attained with a large stoichiometric excess of one of the reagents [4,8,64], diminishing the inhibition potential of the utilized acid by increasing its solvation. Vadgama et al. (2015) [5] did not observe deleterious effects on Novozym 435® above 4% biocatalyst loading using a substrate molar ratio acid:alcohol of 1:15. This stoichiometric alcohol excess was applied to dissolve the tetradecanoic acid, increasing its solvation. These authors, however, did not explore different molar ratios. Besides the thermodynamic and kinetics issues, this excessive amount of alcohol must be removed after the reaction completion, bringing additional difficulties for the downstream processes.

When varied molar ratios and biocatalyst loadings in short-term evaluations (limited reaction time), the conversion reductions of the limiting reagent may be associated exclusively with a reaction rate reduction. In these cases, it is possible to observe which concentration of surplus reagent (correlated to the biocatalyst loading in the system) is not favorable for the enzymatic activity, indicating some potential inhibition. For this reason, SER may be helpful to indicate in which conditions (or a range of conditions) inhibition by exceeding reagent may arise. Another essential aspect to emphasize is the possibility of aggregation of biocatalyst particles when high biocatalyst loadings are adopted. The aggregation will reduce the reaction rates due to increased substrate diffusion limitations [83,95]. SER values close to zero (positive or negative) indicate, in some cases, the employment of high biocatalyst loadings in the system, increasing the possibility of aggregation of the biocatalysts. This phenomenon is favored when immobilization support has affinity by

water or when high water content is present in the system [91]. For example, Novozym 435®, the most widely studied lipase in these studies, is immobilized in Lewatit VP OC 1600, an organic carrier composed of poly(methyl methacrylate) and divinylbenzene. This support material is moderately hydrophobic and susceptible to absorb some water [20]. Observations like this should be considered, mainly when highly hydrophilic reagents are present and high biocatalyst loadings are adopted.

To check the accuracy of these kinds of predictions with SER, a more detailed analysis of the reaction condition of a selected study—Parikh et al. (2019) [9]—was carried out, as showed in Table 3.

Table 3. SER and conversions results of the reaction conditions studied by Parikh et al. (2019) in the synthesis of propyl decanoate mediated by Fermase at 60 °C, in the solvent-free system, 300 rpm, and 10 h. The biocatalyst loading is showed based both on *wt*/*wt* of the total mass of reagents and based on *wt*/*wt* of acid.

Acid:Alcohol Molar Ratio	Biocatalyst Loading (% *wt*/*wt* Acid)	Biocatalyst Loading (% *wt*/*wt* Total)	SER	Conversion (%)
1:1	0.7	0.5	−97	19%
1:1	1.3	1.0	−48	47%
1:1	2.7	2.0	−24	59%
1:1	4.0	3.0	−16	62%
1:2	3.4	2.0	−9	71%
1:3	4.1	2.0	1	83%
1:4	4.8	2.0	8	78%
2:1	2.3	2.0	−35	36%
3:1	2.2	2.0	−40	24%

Table 3 shows that the intermediate SER values (−9 to 8) resulted in the highest conversions observed in the conditions tested. The equimolar substrate condition and biocatalyst loadings below 2.0% (*wt*/*wt* total mass) were unfavorable for the synthesis. Above 2.0% biocatalysts loading at equimolar condition, it is possible to observe that the 60% conversion was reached without shifting the equilibrium due to the lack of surplus reagent. The molar ratios 1:2 to 1:4 improved the yields and showed that the excess of *n*-propanol shifted the equilibrium, promoting the low acid solvation without generating an unfavorable partitioning of the water. Although this information is insufficient to indicate the most proper conditions for this synthesis, it is possible to discard the evaluation of molar ratios superior to 4, as well as the conditions with an excess of acid (SER negative) due to the increase of the viscosity and the decrease of pH. Considering the molar mass differences between decanoic acid and *n*-propanol, SER optimum condition seems to be close to 0 (positive or slightly negative values), implying that the evaluation of molar ratio conditions between 1:2 and 1:3 with biocatalyst loadings close to 2.0%. Another important observation is that slight differences in SER values seem to be associated with slight variations in conversion (the difference between SER 1 and 8, resulting in conversions of 83% and 78%), as observed initially by Sousa et al. (2020) [6]. This trend may be used to optimize the conditions, simultaneously reducing the molar ratio (avoiding the waste of alcohol) and the biocatalyst loading (reducing the costs of the process).

In this sense, important consequences can be extracted: (i) the interdependence of substrate molar ratio and biocatalyst loading; (ii) the possibility of using reduced biocatalyst loadings in the reactor in some cases. We emphasize that this kind of analysis is not trivial, or even feasible, to be made using equilibrium constant or kinetics parameters (Vmax, Km, Kcat, Ki), whose determinations are laborious, reinforcing the potential utility of SER as a tool for predicting enzymatic esterification behavior. Figure 1 shows, in a simplified way, the convergence between molar ratio (expressed as a mass ratio) and biocatalyst loading that may lead to high conversions in esterification reactions in SFS. This convergence may be numerically translated by SER, which can be used as a tool for designing experiments and optimizing reaction conditions.

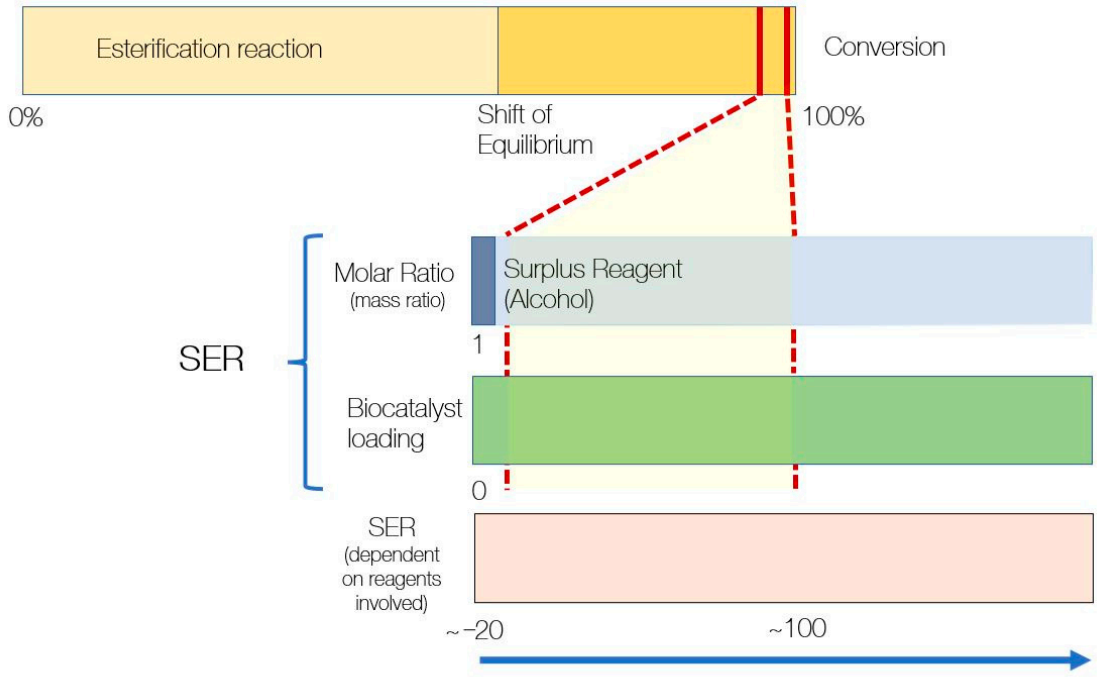

Figure 1. Graphical representation of the optimal reaction condition (molar ratio and biocatalyst loading) range for optimizing enzymatic esterification and its mathematical expression using SER.

2.3. Handling SER for Design Experiments

We discussed up to now the use of SER to analyze reaction conditions already established. However, the real interest is the use of SER to simplify the design of the process, thus predicting an optimum range of conditions and discarding exhaustive assays in extreme conditions.

When designing an optimization study, some additional information should be known to allow a reasonable prediction based on SER. The first one is the enzymatic activity of the biocatalyst used, which will establish a "starting point" of the biocatalyst loading studied and the reaction time. Enzymatic activity expresses the rate of the conversion of a given acid per time (usually per minute) per mass of biocatalyst (usually per gram); if we extrapolate this data for the acid used, we can obtain an estimation of the minimum quantity of biocatalyst is required to consume the acid within a specific time of the reaction. The second one is temperature, which should promote activation of the reaction without denaturation of the enzyme, taking into account the maximum temperature tolerated by the enzyme and the melting point/boiling point of the reagents. Moreover, we should establish the mass of the acid used and some bounds in which we want to find the biocatalyst loadings (considering its cost and the reaction time). Then, we should establish some SER values within the optimum estimated range (considering the nature of the reagents) and the respective mass of alcohol. The general procedure to design experiments with SER is given in Figure 2, and hypothetic examples of the application shown in Table 4.

The enzymatic activity of Novozym 435® is expressed as PLU (propyl dodecanoate units), which means that this data refers to the conversion of dodecanoic acid to esters in the conditions tested. It is not possible to extrapolate this data to the conversion of any other acid, and in this sense, we adopt an estimation that 260 PLU will be sufficient. This biocatalyst content is sufficient to convert 0.025 mols of dodecanoic acid to ester in 1.5 h. We can suggest that this biocatalyst loading will convert these different acids in esters in less than 3 h, considering the broad substrate specificity shown by Novozym 435® [20]. As the moles of hexadecanoic acid are much lower than 0.025, we suggested using minimum biocatalyst content (135 PLU). The temperature selection considers favoring the reaction kinetics, the melting of the hexadecanoic acid, and the fact that Novozym 435® is stable at

high temperatures [20]. The selection of SER values was empiric, considering the analysis of the previous data carried out in this study, but we adopt the premise to make a coupled increase of molar ratio and enzyme loading. We already observed that the conditions that conciliate large stoichiometric excesses of reagents with low biocatalyst loadings resulted in a poor conversion due to the increased probability of enzyme inhibition. Moreover, we did not consider adopting an excess of acid to avoid potential issues related to the pH media.

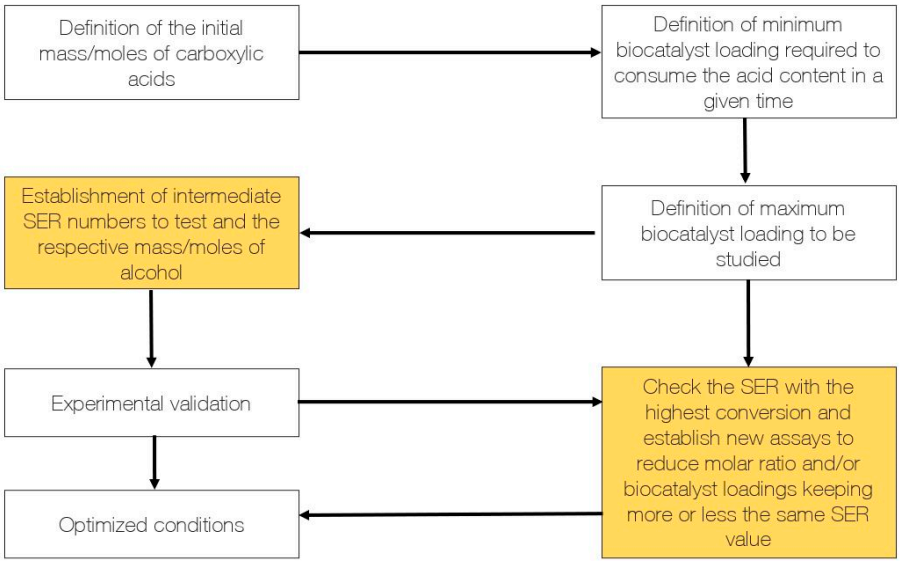

Figure 2. Fluxogram of steps required to design a set of experiments using SER.

Table 4. Hypothetical experimental design for enzymatic esterification using SER.

Synthesis (Product)	Pentyl Propionate	Ethyl Hexadecanoate	Dodecyl Hexanoate
Enzyme (commercial lipase)	Novozym 435® (7000 PLU)	Novozym 435® (7000 PLU)	Novozym 435® (7000 PLU)
Temperature	60 °C	70 °C	60 °C
Initial quantity of acid	1.85 g (0.025 mol)	1.85 g (0.0072 mol)	1.85 g (0.016 mol)
Minimum biocatalyst loading	0.037 g (260 PLU)	0.019 g (135 PLU)	0.037 g (260 PLU)
Maximum biocatalyst loading	0.065 g (455 PLU)	0.037 g (260 PLU)	0.065 g (455 PLU)
SER and the respective reaction conditions	SER 18 Molar ratio 1:1.1/Bioc. loading 2.0%	SER −43 Molar ratio 1:3.2/Bioc. loading 1.0%	SER 27 Molar ratio 1:0.9/Bioc. loading 2.0%
	SER 24 Molar ratio 1:1.5/Bioc. loading 3.5%	SER −30 Molar ratio 1:3.9/Bioc. loading 1.0%	SER 38 Molar ratio 1:1.1/Bioc. loading 2.0%
	SER 39 Molar ratio 1:2/Bioc. loading 2.0%	SER −18 Molar ratio 1:3.6/Bioc. loading 2.0%	SER 42 Molar ratio 1:1.4/Bioc. loading 3.5%
	SER 42 Molar ratio 1:1.5/Bioc. loading 2.0%	SER −1 Molar ratio 1:5.4/Bioc. loading 2.0%	SER 68 Molar ratio 1:1.7/Bioc. loading 3.5%
	SER 74 Molar ratio 1:3/Bioc. loading 3.5%	SER 4 Molar ratio 1:6/Bioc. loading 2.0%	SER 75 Molar ratio 1:2/Bioc. loading 3.5%

Bioc. loading = Biocatalyst loading, expressed as % *wt*/*wt* of the acid mass.

One may argue against using the mass of biocatalyst instead of using the enzymatic activity in SER calculation. Some reasons support our decision. The first one is that enzymatic activity data are expressed by many different methodologies, which may make the comparison between different studies unfeasible. Although enzymatic activity is indeed

a more precise measure of catalytic activity than the mass of biocatalyst (which considers a significant proportion of immobilization support mass), the enzymatic activity is measured in units per mass (as U g^{-1}), which means a direct correlation between mass and quantity of active enzymes in the media. In an industrial context, it is reasonable to think that frequent enzymatic activity measures must be carried out as part of quality control of raw materials, giving the relation between the mass of biocatalyst and enzymatic activity. The second and most important reason is the influence of the immobilization support on the partition of the water in the system, which impacts the equilibrium position depending on the reaction media [1,10,90,96] and the influence of the immobilization support on kinetics aspects [42,92,97,98]. The third reason is that the measure of the mass is practical; indirectly, the mass of the biocatalyst in the system may bring valuable information about the operational costs, productivity, design of the reaction system, stirring rate, downstream processes, among others.

It is noteworthy that if the enzymatic esterification study aims to understand the mutual interaction between reaction variables, an extensive set of assays is required to generate accurate data that support the results. On the other hand, if the aim is to obtain optimized conditions for a specific synthesis, SER helps find the optimum molar ratio of reagents and enzyme loading. However, the reaction yield can still be increased by using different methods for shifting the reaction equilibrium, activating the reaction, and designing the reaction system [19,42,99–102].

Some experiments were carried out with different SER in various syntheses to obtain additional pieces of evidence of the relation between molar ratio and biocatalyst loading, as shown in Table 5. As a result, we can observe a general trend—variations in molar ratio and biocatalyst loading that generate discrete differences in SER resulted in similar conversions. In these cases, we increase simultaneously molar ratio (the stoichiometric excess of alcohol) and biocatalyst loading.

Table 5. Enzymatic syntheses of ethyl, butyl, and octyl dodecanoate, butyl octanoate, and butyl decanoate in different reaction conditions, respective SER, and conversion results.

Ethyl Dodecanoate		Butyl Dodecanoate		Octyl Dodecanoate		Butyl Octanoate		Butyl Decanoate	
SER	**Conversion**	**SER**	**Conversion**	**SER**	**Conversion**	**SER**	**Conversion**	**SER**	**Conversion**
13		8		10		1		7	
(1:5.2)	89.0%	(1:3)	88.9%	(1:1.7)	90.7%	(1:1.9)	87.4%	(1:2.6)	89.5%
(1.5%)		(1.5%)		(1.5%)		(1.5%)		(1.5%)	
19		27		16		13		20	
(1:6.4)	85.2%	(1:3.7)	92.1%	(1:2.1)	90.9%	(1:2.7)	90.3%	(1:3.5)	91.9%
(2.5%)		(2.5%)		(2.5%)		(2.5%)		(2.5%)	

The molar ratio acid:alcohol and biocatalyst loading (*wt*/*wt* mass of acid) used in each synthesis are described below the SER value. All syntheses were mediated by Novozym 435®, in closed stoppered flasks for 24 h, under 50 °C, 200 rpm, in a solvent-free system.

It is possible to obtain similar results when we carried out an equivalent reduction in keeping the same (or similar) resultant SER, which means the possibility of attaining advantageous conditions of molar ratio and biocatalyst loading. The variation of the conversion result with SER was also checked with esterification of decanoic acid and *n*-butanol mediated by Novozym 435® at 65 °C, using the SER equals 20 in two different conditions. The first one was equivalent to molar ratio acid:alcohol 1:3 and biocatalyst loading 1.5%, and the second one was molar ratio acid:alcohol 1:3.5 and biocatalyst loading 2.5%. Very similar conversions were observed: 93.5% and 93.6% after 24 h. Although both conditions did not represent a significant change in the system, we emphasize that this kind of analysis is helpful to reduce the biocatalyst loading. As the biocatalyst cost represents the highest cost item in a biocatalytic process, reduced biocatalyst loadings in the reactor are a way to overcome the main obstacle for a broader application of biocatalysis in the production of commodities and chemical specialties [49,50,52,83]. We also observed a discrete increase in the conversion of decanoic acid by increasing the temperature from 50 °C (Table 5) to 65 °C.

To illustrate how changes in biocatalyst loading impact SER, we can take as an example the synthesis of medium-chain alcohol (C8) and acid (C6). If we use molar ratio acid:alcohol 1:2 and biocatalyst loading (*wt*/*wt* of acid mass) of 5%, resultant SER will be 25; if we use the same molar ratio with biocatalyst loading 10%, a not considerable variation will occur—SER value becomes 12. In practical terms, this discrete difference can be associated with a common observation present in enzymatic esterification studies—an increase in biocatalyst loading after a certain level did not increase the conversion in a specific time [4,5,8,67,100]. Although the biocatalyst does not affect the equilibrium position (i.e., the yield of the reaction), this observation refers to the conversion obtained after a short interval of time, which turns evident the alterations in the reaction rate. As already mentioned, discrete differences in SER (inside the range of intermediate values) seem to be associated with marginal differences in the reaction conversion. Both conditions show high biocatalyst loadings, and it seems evident that there is an unnecessary excess of biocatalyst in the system—as biocatalysts are high-cost incomes, it is necessary to recycle them for obtaining a cost-effective biocatalytic process. However, if we adopt a biocatalyst loading of 1%, the resultant SER will be 124, which is an extreme value. In this case, it is possible to evaluate a coupled reduction of the enzyme loading and the stoichiometric excess generating a more favorable condition. SER will remain equals to 25 if we use biocatalyst loading 2% and a molar ratio 1:1.3. We reduce the potential inhibitor effect of the reagent (keeping their effect on the shift of equilibrium), coupling these effects with a proportional reduction of biocatalyst loading using SER as a guide. However, the reduction of biocatalyst loading is limited by the minimum quantity of enzyme required to process the reagents within a given time. This kind of rationale using SER as a guide shows how interesting this tool could be for economic purposes, considering the strict correlation between raw materials quantities (reagents and biocatalyst) or, in other words, between different operational costs terms.

2.4. Limitations of SER

The first explicit limitation of SER is related to the temperature of the reaction. It is well-known that temperature influences the equilibrium constant, but Flores and co-workers (2000) [81] found that the equilibrium position variation of a reaction between octadec-9-enoic acid acid and *n*-butanol was negligible between 35 and 50 °C. As already pointed out, we are dealing with reaction conditions (molar ratio and biocatalyst loading) at a constant temperature in a given synthesis to reduce the complexity of our analysis. The temperature probably produces some SER response variation due to the thermodynamics and kinetics temperature effects that should be further investigated. In here, we considered that the temperature was enough to promote a proper activation of the reaction without causing the enzyme denaturation.

The SER formulae, as proposed, did not consider the mass molar between reagents. The lack of this parameter in SER calculation may lead to inaccurate conclusions when a reaction between reagents with substantial differences in chain length (for example, a long-chain acid and short-chain alcohol) is evaluated. For example, using 5% of biocatalyst loading, an SER equals 20 is equivalent to the molar ratio acid:alcohol 1:10 in a reaction between tetradecanoic acid (C14) and ethanol (C2), but the same SER equals 20 is equivalent to the molar ratio acid:alcohol 1:1.1 in a reaction between butanoic acid (C4) and decanol (C10). We can manipulate the SER formulae to consider the molar mass of reagents keeping the outcome as a dimensionless number. Moreover, this alteration (proposed in Equation (2)) will avoid the outcome equals to zero, which was considered non-applicable when SER was first described [6], which is another explicit limitation of the equation. However, this alteration will produce specific ranges for every combination of reagents and, although noticeable gain in precision of the analyses may be obtained, general trends and predictions may be offset.

$$\mathrm{SER} = \frac{((\mathrm{m}/\mathrm{M})\ \text{alcohol} - (\mathrm{m}/\mathrm{M})\ \text{acid})(\mathrm{M}\ \text{alcohol})}{\mathrm{m}\ \text{biocatalyst}} \quad (2)$$

Another expected limitation of the SER equation is that the same outcome can be associated with more than one experimental condition (molar ratio and biocatalyst loading), even though both variables proportionality is maintained. However, there is no guarantee that this trend will be observed in reactions between short-chain acids and long-chain alcohols.

As the SER was described for synthesizing an aliphatic ester in SFS, this study focuses on applying the SER for other aliphatic molecules in the same condition. We can suppose that applying a mathematic relation like SER (as it is, or with modifications) could help predict enzymatic esterifications with alicyclic reagents, polyfunctional reagents (like ethylene glycol, glycerol, acrylic acid, for example), using free lipases, or in the presence of solvents.

Table 6 summarizes which information can be extracted from SER and its limitations, showing the advantages and the disadvantages of its use. Further studies should be performed to address all these issues, considering the practical applicability of SER in predicting different kinds of esterification reactions in SFS.

Table 6. Advantages and disadvantages of SER.

Advantages
Predictability of reaction' s behavior
Easy to handle
Reduced number of experiments to achieve high yields
Offer a range of reaction conditions that achieve high yields
Possibility to reduce the biocatalyst loading keeping high yields
Disadvantages
One SER number may be associated with more than one reaction condition
Optimum range may be shifted depending on the nature of the reagents
Temperature effects are not considered
Differences in molar ratio of reagents are not considered
Differences in esterification activity of lipases are not considered

3. Materials and Methods

3.1. Data Obtained from the Literature

A bibliographic survey was carried out to collect relevant enzymatic esterification studies in literature in which SER could be appropriately calculated and compared with our previous work [6]. To obtain a direct comparison and to facilitate the SER validation, only monofunctional and aliphatic reagents were considered. The following criteria were adopted for their selection: (i) ester syntheses should involve monofunctional carboxylic acids and alcohols in SFS using immobilized lipases; (ii) the selected studies should provide a detailed description of the quantities of reagents and biocatalysts (enzyme + support) used in the assays, allowing SER calculation. Studies that simultaneously fulfilled these criteria were considered for further analysis. However, we did not differentiate studies that applied a one-by-one variable optimization approach or RSM. In addition, studies that synthesize aliphatic esters from mixtures of different fatty acids or alcohols were not considered to facilitate the validation of SER.

As SER does not include the temperature effects, the data utilized for SER calculation were studied only at a constant temperature. For example—if a given study evaluated three different molar ratios and three different enzyme loadings at two different temperatures, only the set of data in which higher conversions were reported was used for SER application. In RSM studies, the maximum amount of data at a constant temperature was selected. The same rationale was applied when different stirring rates were evaluated. We considered that the selected temperature and stirring rate were high enough to favor the reaction but not so high to generate deleterious effects on immobilized lipases. Figure 3 shows the schematic procedure used to collect and calculate the data in both cases.

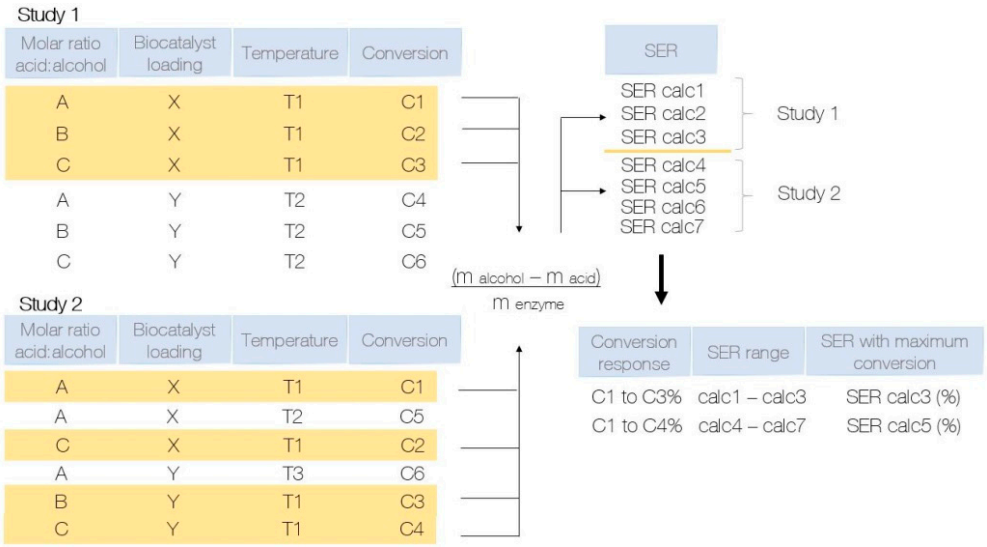

Figure 3. Schematic representation of collecting data from two hypothetical studies of enzymatic ester syntheses for SER calculation and compilation of obtained data.

The molar ratio and biocatalyst loading data extracted from the studies were compiled in a sheet, converted to masses (g) of alcohol, carboxylic acids, and biocatalysts when presented in mol or enzymatic activity per gram. The mass of biocatalyst considers the mass of the whole immobilized enzymes, accounting for protein and immobilization supports masses. The data collected were displayed in a Table, including the description of reagents (in IUPAC names) and immobilized lipase adopted. The respective SER outcomes were calculated as described in Equation (1) for each data of mass of reagents and biocatalyst. These SER outcomes were correlated to the conversion result obtained for each reaction condition. Thus, a range of SER numbers and conversion results were obtained. However, in some cases, only one condition of molar ratio and enzyme loading was available for calculations.

The range of SER numbers and the respective conversion results for the different ester syntheses were compared to the data reported by our group [6] to check if similar conclusions could be obtained—conversions above 80.0% but frequently around 90.0%, are associated with intermediate values of SER, i.e., between 0 and 65. Similarly, conversions below 80.0% could be associated with extreme values of SER, i.e., negative values or SER higher than 100. Therefore, we divided the data into three different sections: studied with (i) a good level of agreement, with optimum range between 0 and 65; (ii) an intermediate level of agreement, whose optimum SER are deviated from the estimated optimum range but still delimited between −10 and 100; (iii) and a low level of agreement, in which high conversions were observed outside the range or low conversions (using the maximum conversion observed as reference) were observed inside the range. In these cases, the likely reasons for the discrepancies were discussed. For the purpose of this text, in the discussion of the results the term "extreme values of SER" were considered as SER numbers largely outside the proposed optimum range (for example, ≥ -25 or ≤ 120); on other hand, the term "intermediate values of SER" were considered as SER within the proposed optimum range, or more specifically in a narrower range (for example, 5 or 30).

3.2. Data Obtained Experimentally

Part of the data used in this study were obtained experimentally by our group. Esterification reactions in a solvent-free system were carried out in 150 mL stoppered closed flasks under orbital stirring (200 rpm). Different molar ratios of carboxylic acid and alcohols were mixed, starting from 2.0 g of acid. For the syntheses of octyl decanoate and octyl dodecanoate, molar ratio acid:alcohol 1:1.3 were evaluated using different enzyme loadings of Novozym 435® and Lipozyme RM IM®, varying from 1.5% to 10.0% (*wt*/*wt* acid). To

synthesize butyl octanoate, molar ratios acid:alcohol varied from 1:1 to 1:3 with enzyme loading 2.0% *wt*/*wt* acid. The synthesis of octyl octanoate mediated by Lipozyme RM IM® evaluated molar ratios acid:alcohol varied from 1:1 to 1:3 and enzyme loadings from 2.5% to 4.0% *wt*/*wt* acid. Syntheses of dodecanoates—ethyl dodecanoate, butyl dodecanoate, and octyl dodecanoate—and butyl decanoate mediated by Novozym 435® were carried out in the reaction conditions described in Table 6, aiming to check slight variations in reaction condition using SER as a parameter. These systems were studied considering the previous experience of our group in medium-chain esters syntheses [6]. The immobilized lipases used, Novozym 435® and Lipozyme RM IM®, were provided by Novozymes Latin American (Araucária, Brazil). Reactions between octanoic acid and *n*-butanol or *n*-octanol were carried out at 65 °C for 3 h; reactions between decanoic acid and *n*-butanol or *n*-octanol, and dodecanoic acid with ethanol, *n*-butanol, and *n*-octanol were carried out at 50 °C for 24 h. All reagents were of analytical grade. The titration of the samples was used to monitor the progress of the reaction by the reduction of the acidity index. The samples were collected at the beginning and the end of the reactions. The conversions of the esterification reactions were calculated by the percentual reduction observed between the final and initial acidity index.

4. Conclusions

This study shows that SER is an empiric mathematical relation that presents a reasonable agreement with the literature results for solvent-free enzymatic syntheses of aliphatic esters. SER offers a simple way to conciliate the thermodynamics and kinetics aspects towards high conversions in enzymatic ester syntheses, providing a helpful overview of the reaction variables. In combination with other physicochemical parameters, a general prediction of the reaction's behavior can be obtained using SER. Its main advantage is to indicate a range of conditions in which the equilibrium shift towards synthesis may be attained without inhibition of immobilized lipases. By analyzing the application of the SER trend which indicated a range of reaction conditions correspondent to 0 from 65 as an optimum in a mid-chain ester synthesis, we observed an expanded range of −20 from 100 as potentially able to cover a variety of cases—short, mid, and long-chain esters syntheses—taken into account certain exceptions when esterifications are carried under ultrasound or microwaves, or with addition of water. A fast and simple methodology to design experiments using SER was proposed, by defining some boundaries of temperature, reaction time, and maximum biocatalyst loading, and after the determination of molar ratio using arbitrary SER values inside the estimated optimum range for the esterification reaction, considering the nature of the reagents involved. Although some limitations of the SER application are evident, its use brings attractive features—the possibility of achieving high yields with a reduced number of experiments, the possibility of understanding the practical implications of thermodynamics and kinetics on the syntheses, and the obtaining of more realistic reaction conditions for scaling-up (reduced biocatalyst loadings). Specific studies are required to evaluate how robust this mathematical tool is in various cases and an accurate definition of the optimum SER range and extreme values for each case. SER was applied successfully for the synthesis of octyl octanoate and its application can be extended to the optimization of other similar syntheses, considering the analyses carried out in this study with an extensive set of data.

Author Contributions: This article was performed as collaborative research between R.R.d.S. and A.S.d.S. For R.R.d.S., the work included: conceptualization, methodology, formal analysis, data curation, visualization, and writing—original, draft preparation; A.S.d.S. performed conceptualization, methodology, review, and corrections; V.S.F.-L. and R.F.-L., performed review, corrections, main supervision, project administration, and funding acquisition. All authors have read and agreed to the published version of the manuscript.

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