Thieme

Statistical Experimental Designs for cLTB-Syn Vaccine Production Using *Daucus carota* Cell Suspension Cultures

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ABSTRACT

The carrot-made LTB-Syn antigen (cLTB-Syn) is a vaccine candidate against synucleinopathies based on carrot cells expressing the target antigen LTB and syn epitopes. Therefore, the development of an efficient production process is required with media culture optimization to increase the production yields as the main goal. In this study, the effect of two nitrogen sources (urea and glutamate) on callus cultures producing cLTB-Syn was studied, observing that the addition of 17 mM urea to MS medium favored the biomass yield. To optimize the MS media composition, the influence of seven medium components on biomass and cLTB-Syn production was first evaluated by a Plackett–Burman design (PBD). Then, three factors were further analyzed using a central composite design (CCD) and response surface methodology (RSM). The results showed a 1.2-fold improvement in biomass, and a 4.5-fold improvement in cLTB-Syn production was achieved at the shake-flask scale. At the bioreactor scale, there was a 1.5-fold increase in biomass and a 2.8-fold increase in cLTB-Syn yield compared with the standard MS medium. Moreover, the cLTB-Syn vaccine induced humoral responses in BALB/c mice subjected to either oral or subcutaneous immunization. Therefore, cLTB-Syn is a promising vaccine candidate that will aid in developing immunotherapeutic strategies to combat PD and other neurodegenerative diseases without the need for cold storage, making it a financially viable option for massive immunization.

ABBREVIATIONS

μ	Specific growth rate
2,4-D	2,4-Dichlorophenoxyacetic acid
B5	Gamborg medium
cLTB-Syn	Carrot-made LTB-Syn
CNS	Central nervous system
DW	Cell dry weight
FW	Cell fresh weight
GALT	Gut-associated lymphoid tissue
IgA	Immunoglobulin A
IgG	Immunoglobulin G
KIN	Kinetin
k_La	Gas-liquid volumetric mass transfer coefficient
MS	Murashige and Skoog medium
p. o.	*per os* (oral immunization)
PBD	Plackett–Burman design
RSM	Response surface methodology
s. c.	Subcutaneous immunization
td	Doubling time
vvm	Volume of air per liquid volume per min

Introduction

Synucleinopathies are a group of neurodegenerative diseases characterized by the manifestation of protein bodies rich in alpha synuclein (α-Syn) in the CNS. The constant damage represented by α-Syn aggregates in different regions and cell types and results in pathologies such as Parkinson's disease, multiple system atrophy, and dementia with Lewy bodies [1]. The rise in the incidence and prevalence of synucleinopathies (~ 1.5 million people only in the US) would be the result of the increase in life expectancy [2]. More than 35/100 000 new cases per year have been registered for Parkinson's disease, making it the second most important neurodegenerative disease of the world [3]. Multiple factors are implicated in synucleinopathies to manifest aging, environmental factors, oxidative stress, mitochondrial dysfunction, and genetic factors [4]. The α-Syn (140 amino-acid protein) has important functions related to the correct fusion of dopamine-loaded vesicles to the presynaptic membrane [5]. Most current treatments are palliative, and a specific biomarker has not been achieved to predict or diagnose precisely; however, contributions to the pathophysiological area have made it possible to design promising therapeutic strategies. Innovative approaches include reduction in α-Syn accumulation and cell-to-cell transfer through active immunization [6]. Immunization induced the production of antibodies with the ability to recognize α-Syn in histological sections of the brain with Parkinson's disease without evidence of cytotoxic effects [7]. The possibility of finding new protein production platforms with more significative advantages, such as suspension plant cells for producing recombinant proteins, was opened [8]. In addition to being feasible for oral administration, plant-based vaccines offer low production costs, high biosynthetic capacity, greater safety, easy scale-up, stability, and the ability to produce N-glycosylated proteins [9].

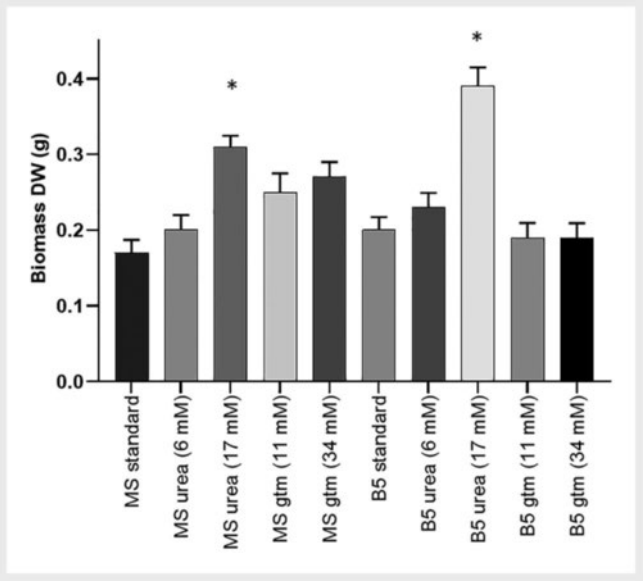
Carrot (*Daucus carota* L.) is a member of the Apiaceae family, and it is classified as a root vegetable. The edible taproot of carrot is widely consumed due to its dietary value in carotenoids, flavonoids, anthocyanins, vitamins, and minerals. Transformation in carrot cells has proven to be an attractive bioreactor for obtaining different secondary metabolites of industrial interest [10–12]. Furthermore, transgenic carrot cells have produced other therapeutic proteins and pharmaceuticals [13, 14]. The optimization media composition has become an attractive approach for increasing the productivity of plant-made recombinant proteins [15]. The 'one-factor-at-a-time' is a time-consuming and laborious approach; thereby, these limitations can be overcome by optimizing all influencing factors together by applying factorial design and response surface methodology (RSM) [16]. Factorial designs are employed when it is necessary to investigate the impact of multiple factors on a response simultaneously. RSM is the statistical method and analytical strategy widely applied in optimization studies by exploring the influence of experimental variables and their interactions with a few experimental trials [17]. The use of optimization statistical approaches has been investigated with plant cell suspension cultures for secondary metabolites production [18] or for increased antibody production in tobacco BY-2 cells [19].

In the present study, we used PBD to screen the essential factors and RSM to evaluate the interactions among the influential factors and select optimum conditions for the optimization of MS media for transformed *Daucus carota* cell suspension cultures, aiming to enhance the growth and cLTB-syn yields at the shake-flask and bioreactor scales. Moreover, the immunogenic properties of cLTB-Syn in terms of inducing systemic and mucosal humoral responses in mice were confirmed.

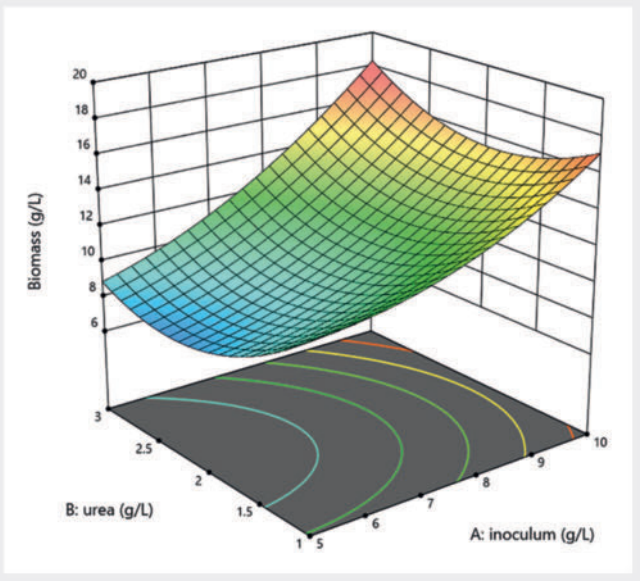
Results

The maximum dry weight biomass of Z4 calluses was achieved with 17 mM of urea in the B5 (0.41 g DW) and MS media (0.31 g DW), representing two- and threefold increases in comparison to standard salt B5 and MS media, respectively (▶ **Fig. 1**). However, it was observed that the biomass obtained with glutamate was lower (0.19 g DW in B5 and 0.22 g DW in MS media), and no significative difference ($p < 0.05$) was found by statistical analysis (Tukey test) in comparison to the standard B5 and MS media.

The PDB model was used to describe the interaction among factors and to screen and evaluate the important factors that influence the response. Experimental design and results for the PBD experiments are shown in ▶ **Table 1**. According to the PBD analysis, there were four treatments with higher biomass production of up to 18.9 g/L DW and three treatments with higher cLTB-Syn production of up to 0.23 µg/g DW. Student's *t*-test for ANOVA was used to screen the variables exhibiting significant effects (**Table 1S**, Supporting Information), and the effects of each factor are graphically represented in a Pareto chart, which independently shows the principal factors that have a positive effect on the response variable (**Fig. 1S**, Supporting Information). In addition, the model F value of 5.75 indicates the significance of the model used, and the values of $p < 0.05$ indicate that model terms are significant to inoculum size, pH, and urea factors. The statistical sig-



▶ **Fig. 1** Biomass yields (dry weight) of *D. carota* Z4 line calluses producing cLTB-Syn. Biomass production was evaluated in different media and analyzed 30 days after inoculation. The asterisks indicate significant differences within each treatment ($P < 0.05$) versus the standard MS and B5 media culture. Data are expressed as mean ± SD of three independent experiments.



▶ **Fig. 2** Surface response plot for biomass production in *D. carota* cell suspension culture showing interaction between urea and inoculum size when pH variable is held at zero level.

nificance of the model was also confirmed by the coefficient of determination, R^2, which was found to be 0.9875, indicating the model could explain 98% of the variability in the response. The predicted R^2 value of 0.9154 was in reasonable agreement with the adjusted R^2 of 0.9273.

Based on PBD and Pareto chart analysis, only three independent variables (inoculum size, urea, and pH) exhibited positive *t*-values, and therefore, high impact on growth and cLTB-Syn accumulation were selected. The light, sucrose, and phytoregulators factors were maintained at the same levels according to the standard MS medium.

For optimizing, the CCD model was used to obtain the combination of values that optimizes the three input variables, leading to a minimal number of experiments to identify the optimal conditions for the desired response. The experimental design and results for CCD responses in optimal conditions are given in ▶ **Table 2**. The analysis of variance (ANOVA) representing the results of the quadratic response surface model (**Table 2S**, Supporting Information) revealed that the values of the adjusted determination coefficient (adj. R^2) and determination coefficient (R^2) were 0.9276 and 0.9741, respectively, which indicated the significance of the model to describe the experimental observations. The lack of a fit value of 19.95 was observed, suggesting that the model was highly significant and showed the feasibility of the model to predict the variations accurately.

A polynomial mathematical model incorporating the different interactions of low and high concentrations of different variables was proposed concerning growth production:

$$\text{Biomass} = -130.087 + 1.07286 \times F + 1.69048 \times B + 48.06463 \times E + 0.38000 \times F \times B - 0.628571 \times F \times E - 2.57143 \times B \times E + 0.202667 \times F^2 + 2.21667 \times B^2 - 3.53741 \times E^2$$

where F, B, and E are inoculum size, urea, and pH, respectively.

The results suggest that interactions between (F) and (B) were significant ($p < 0.05$) for biomass production. The interactions between (F), (B), and (E) were not significant. Regarding the quadratic terms, (F^2), and (B^2) showed significant effects ($p < 0.05$) in contrast to the nonsignificant effect of (E^2), based on interactions between factors. These results indicate that the concentrations of urea and inoculum size in the cultivation media have substantial influence on the biomass of *D. carota*.

The model Equation (1) representing biomass production was solved for different sets of nutrient concentrations to develop contour plots and study the response of nutrient interactions. Thus, three-dimensional response surface plots were generated for the interactions between two variables by keeping the other variables at their central levels for biomass production. The effect of interaction between the inoculum size and urea had a positive effect on growth when the optimal values were kept to those closest to its level (+ 1) (▶ **Fig. 2**).

The maximum biomass was obtained when the inoculum size was around 10 g/L and urea was 3 g/L; then, biomass declined with decreasing urea and inoculum. Because of that, it was decided to keep the inoculum size and urea concentrations at higher levels. Similar trends were observed in the interactions effect between inoculum size and pH (**Fig. 2S**, Supporting Information). The inoculum size presents a positive trend effect on the response, while the pH generates a decrease in the biomass values, so the optimal pH values would be found at the level (− 1). In the last interaction, the effect between pH and urea when the inocu-

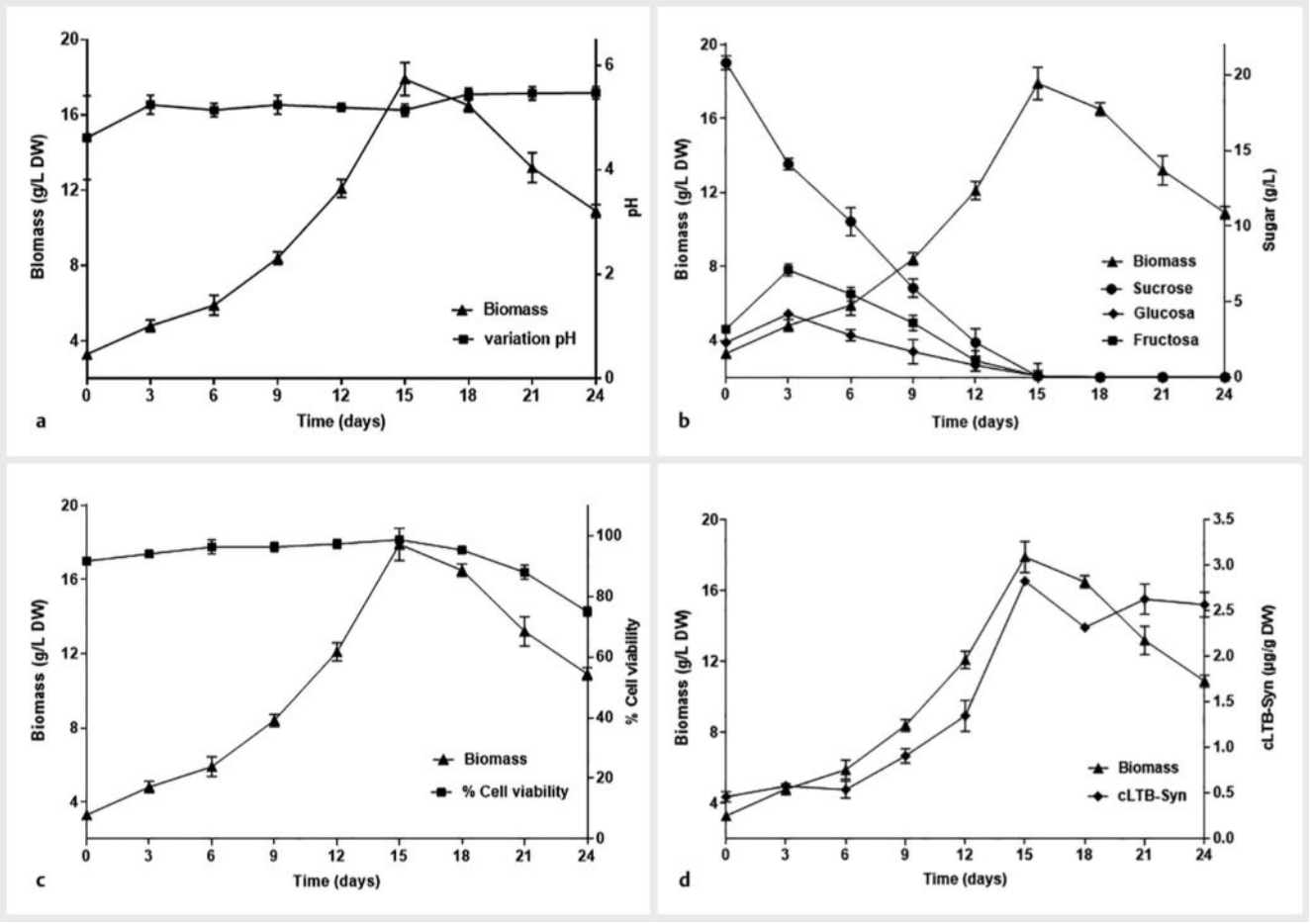
▶ **Table 1** Design of the trial experiments and response by Plackett–Burman design evaluated for cLTB-Syn production. Data are expressed as the mean ± SD derived from experiments performed in triplicate.

Run	F1	F2	F3	F4	F5	F6	F7	Biomass (g/L DW)	cLTB-Syn (µg/g DW)
1	1	1	1	−1	−1	−1	1	6.9 ± 0.81	0.10 ± 0.01
2	1	1	−1	−1	−1	1	1	18.8 ± 0.82	0.23 ± 0.05
3	1	−1	1	1	−1	1	1	15.8 ± 0.99	0.20 ± 0.01
4	−1	−1	−1	−1	−1	−1	−1	7.4 ± 0.26	0.11 ± 0.01
5	−1	−1	−1	1	−1	1	1	14.6 ± 0.65	0.13 ± 0.01
6	−1	1	1	1	−1	−1	−1	12.8 ± 0.62	0.08 ± 0.02
7	1	−1	1	1	1	−1	−1	9.0 ± 0.82	0.17 ± 0.09
8	−1	1	1	−1	1	1	1	18.7 ± 0.50	0.09 ± 0.07
9	1	1	−1	1	1	1	−1	17.6 ± 0.80	0.21 ± 0.01
10	−1	−1	1	−1	1	1	−1	18.9 ± 0.68	0.14 ± 0.05
11	1	−1	−1	−1	1	−1	1	17.1 ± 0.65	0.13 ± 0.09
12	−1	1	−1	1	1	−1	1	11.9 ± 0.28	0.12 ± 0.08
13	1	1	−1	1	1	1	1	16.1 ± 0.55	0.15 ± 0.01
14	−1	1	−1	1	1	1	1	16.5 ± 0.85	0.13 ± 0.03

F1: sucrose (g/L); F2: urea (mM); F3: glutamate (mM); F4: Light (Dark/Photoperiod); F5: pH (5.0–5.7); F6: Inoculum (g/L); F7: Phytoregulators (2,4-D, Kin)

▶ **Table 2** Experimental set-up of CCD and responses in the different runs for the production of cLTB-Syn. Data are expressed as the mean ± SD derived from experiments performed in triplicate.

Run	Inoculum size	Urea	pH	Biomass (g/L DW)	cLTB-Syn (g/L DW)
1	0	0	0	10.7 ± 3.04	0.075 ± 0.026
2	0	−1	1	13.7 ± 1.49	0.116 ± 0.042
3	1	0	1	15.2 ± 2.04	0.134 ± 0.208
4	−1	0	−1	6.32 ± 0.15	0.075 ± 0.094
5	0	1	−1	12.6 ± 1.91	0.102 ± 0.027
6	0	0	0	10.2 ± 1.47	0.064 ± 0.055
7	−1	0	1	7.41 ± 1.79	0.071 ± 0.072
8	1	−1	0	16.5 ± 1.02	0.185 ± 0.071
9	0	0	0	10.5 ± 2.21	0.056 ± 0.065
10	0	−1	−1	11.4 ± 3.23	0.122 ± 0.027
11	1	1	0	17.6 ± 2.92	0.179 ± 0.051
12	0	1	1	11.3 ± 0.52	0.107 ± 0.063
13	−1	−1	0	12.2 ± 0.80	0.114 ± 0.016
14	1	0	−1	16.3 ± 0.10	0.133 ± 0.085
15	−1	1	0	9.5 ± 0.351	0.119 ± 0.081

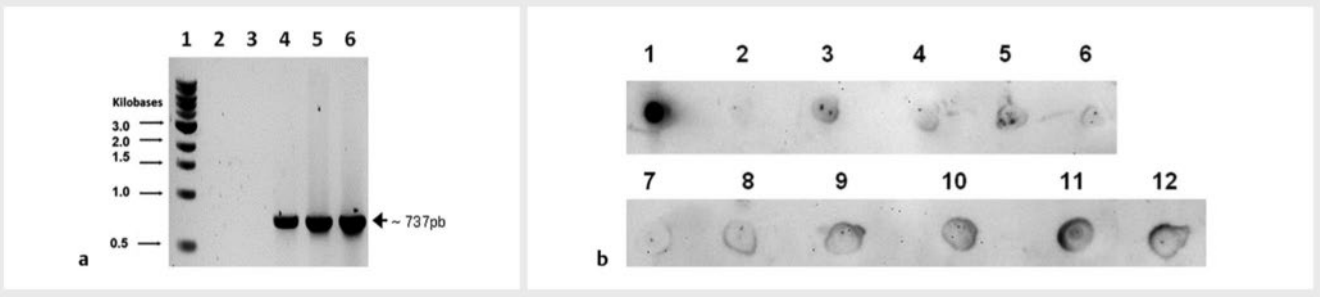


▶ **Fig. 3** Growth profile kinetics of *D. carota* MSRZ4 cell suspension line producing cLTB-Syn in shake flasks for 24 days: **a** medium pH variation; **b** carbohydrates consumption; **c** % cell viability; **d** cLTB-Syn production. Data are expressed as the mean ± SD derived from experiments performed in triplicate.

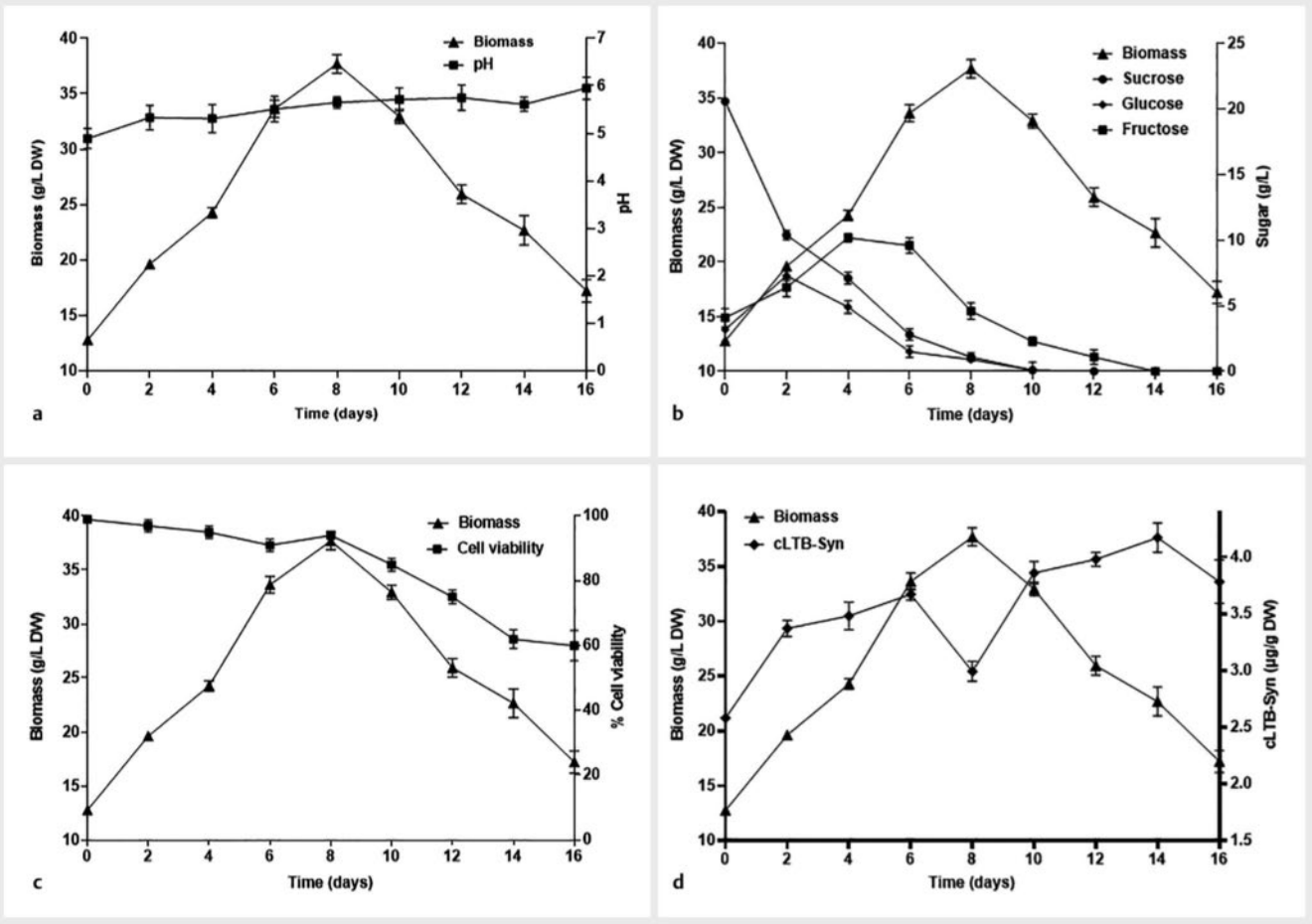
lum size remains at the central level was assessed (**Fig. 3S**, Supporting Information). In this case, both urea and pH, by increasing their quadratic effect, generate a slight increase in growth values, so the optimal values of both factors would be at the level of (+ 1 and − 1). The observed surface graphs, together with their respective contour graphs, presented elliptical shapes, which indicates the simultaneous or interaction effects between the factors on the response variable. Desirability charts of the RSM optimization are given in **Fig. 4S** (Supporting Information). The maximum desirability was achieved within an inoculum size range of 9.98 g/L to 2.9 g/L of urea. Desirability was zero below 5 g/L of inoculum and below 1.9 g/L of urea (**Fig. 4S**, Supporting Information).

Based on the different response curves generated, the optimum concentrations for the three components as obtained from the maximum point of the model were calculated as follows: 30 g/L of sucrose; 3 g/L (17 mM) of urea; 9.97 g/L of inoculum size (FW), and pH: 5.0. The model predicted 19.2 g/L biomass production at these optimum nutrient concentrations.

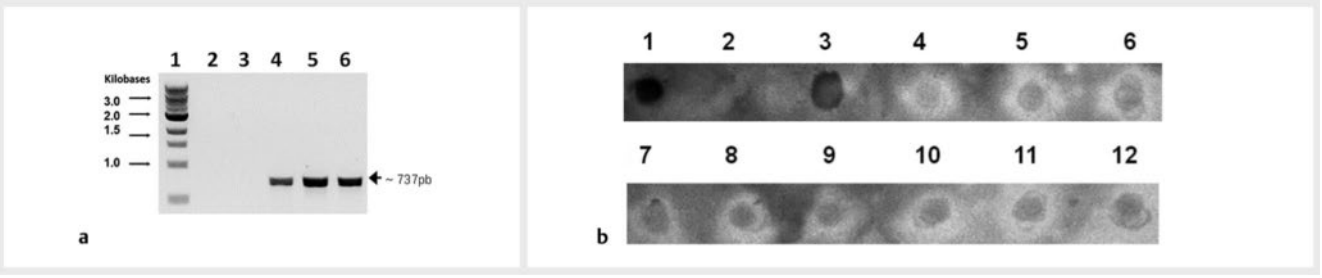
The growth profile of suspension cells from the MSRZ4 (▶ **Fig. 3**) line in a shake flask containing 100 mL of MSR media showed a lag phase (3 days), log/exponential phase (9 days), stationary phase (3 days), and death phase (6 days) (▶ **Fig. 3a**). The maximum biomass was 17.9 g/L DW after 15 days of culture, with a growth rate of µ= 0.111/d, a td of 6.2 days, and biomass yield ($Y_{X/S}$) of 0.53. Variation in pH is also shown in ▶ **Fig. 3a**. Initially, the pH drops to 4.6 within the first 4 days, then stabilizes between 5.4 and 5.5 until the end of the kinetics. The consumption of sugars started in the first three days of culture (lag phase). Initially, sucrose is converted into glucose and fructose due to cytoplasmic invertase; then, the cells first begin to consume glucose and then fructose until they are entirely depleted at day 15 (▶ **Fig. 3b**). During the kinetics, it was observed that the cell viability decays slowly after day 21 with up to 85% viability (▶ **Fig. 3c**). The cLTB-Syn production increased (on 6 day) until reaching maximum expression during exponential phase at 15 days (2.83 µg/g DW) and was maintained until the end of the growth kinetics (▶ **Fig. 3d**). The maximum protein yield ($Y_{P/X}$) reached was 0.16 µg cLTB-Syn/g cell with a qp of 0.14 µg cLTB-Syn/g cell/d. On the other hand, PCR analysis confirmed the presence of the LTB-Syn gene in the cell suspension MSRZ4 line cultured in shake flasks (▶ **Fig. 4a**). Moreover, the presence of the LTB-Syn protein in the soluble extracts during the shake-flask kinetics was confirmed by dot blot (▶ **Fig. 4b**).



▶ **Fig. 4** Characterization of MSRZ4 line expressing cLTB-Syn in flask samples. **a** Detection of the LTB-Syn transgene by PCR. The analysis was conducted using genomic DNA extracted from transgenic or wild-type samples, where the presence of 737 bp amplicons indicates specific amplification. Lanes: 1, molecular weight marker (1 kb ladder, New England Biolabs); 2, negative control (water); 3, wild-type cell line; 4, positive cell line control (Z4+); 5 and 6, shake-flask samples at 0- and 24-day cultures, respectively. **b** Dot-blot analysis. Total protein extracts were analyzed to detect the cLTB-Syn in native conditions using anti-CT hyperimmune serum. Dots: 1, CT as positive control (100 ng); 2, protein extract from wild-type cell line; 3, protein extract from positive cell line control (Z4+); 4–12, protein extracts from shake-flask cultures at different timepoints.



▶ **Fig. 5** Growth profile kinetics of *D. carota* MSRZ4 cell suspension line producing cLTB-Syn in an airlift bioreactor for 16 days: **a** medium pH variation; **b** carbohydrates consumption; **c** % cell viability; **d** cLTB-Syn production. Data are expressed as the mean ± SD derived from experiments performed in triplicate.



▶ **Fig. 6** Characterization of MSRZ4 line expressing cLTB-Syn in bioreactor airlift samples. **a** Detection of the LTB-Syn transgene by PCR. The analysis was conducted using genomic DNA extracted from transgenic or wild-type samples, where the presence of 737 bp amplicons indicates specific amplification. Lane: 1, molecular weight marker (1 kb ladder, New England Biolabs); 2, negative control (water); 3, wild-type cell line; 4, positive cell line control (Z4+); 5 and 6, airlift samples at 0- and 16-days culture, respectively. **b** Dot-blot analysis. Total protein extracts were analyzed to detect the cLTB-Syn in native conditions using anti-CT hyperimmune sera. Dots: 1, CT positive control (100 ng); 2, wild-type cell line; 3, positive cell line control (Z4+); 4–12, total protein extracts protein extracts from airlift bioreactor cultures at different timepoints.

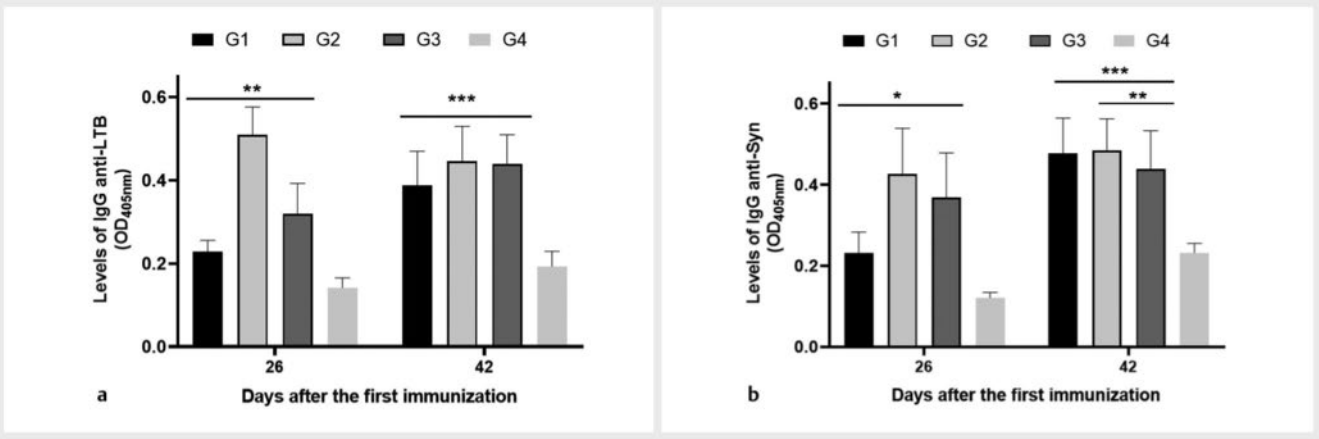
After obtaining the optimal medium by statistical design, we tested its feasibility in a 2 L airlift bioreactor with an aeration rate of 1.5 vvm, a temperature of 25 °C, and a DO level of 80%. The growth profile of the suspension cell MSRZ4 line in a 2 L bioreactor under optimal culture conditions is shown in ▶ **Fig. 5 a**. After the first 2 days, the cells grew exponentially with a μ=0.121/d, a td of 6 days, and a biomass yield ($Y_{X/S}$) of 0.93. The maximal cell dry mass reached was 37 g/L in 8 days. During the growth kinetics, the variance in pH value was similar to that observed for shake flask; initially, the pH dropped from 5.1 to 4.8 after, and then it remained stable and relatively high at the end of the cultivation period (from 5.3 to 6.1) (▶ **Fig. 5 b**). The kinetic data for the cell growth and C-source consumption curves during growth in the airlift bioreactor are shown in ▶ **Fig. 5 c**. During the exponential phase, glucose was completely consumed after 10 days. However, the carbon sources were not completely consumed at this time, observing that fructose remained in the culture medium on day 10 with a subsequent total consumption 4 days later (about 14 days at the end of the cultivation period). The cLTB-Syn production profiles for the optimal culture conditions are shown in ▶ **Fig. 5 d.** The curve for cLTB-Syn production increased gradually during the exponential growth phase (in 2 days) and then increased sharply with a maximal production of 4.3 µg cLTB-Syn/g DW in 14 days and a qp of 0.18 µg cLTB-Syn/g cell/d. During the growth phase, the DO decreased gradually and reached a minimal value of 25% saturation after 8 days and then increased gradually, reaching about 75% saturation by the end of the cultivation process.

PCR analysis confirmed the presence of an LTB-Syn transgene in the growth kinetics of MSRZ4 line at the beginning and end in the airlift bioreactor culture (at day 0 and day 16) (▶ **Fig. 6 a**). Moreover, the detection of cLTB-Syn in the soluble extracts during the airlift bioreactor kinetics was confirmed by dot blot (▶ **Fig. 6 b**).

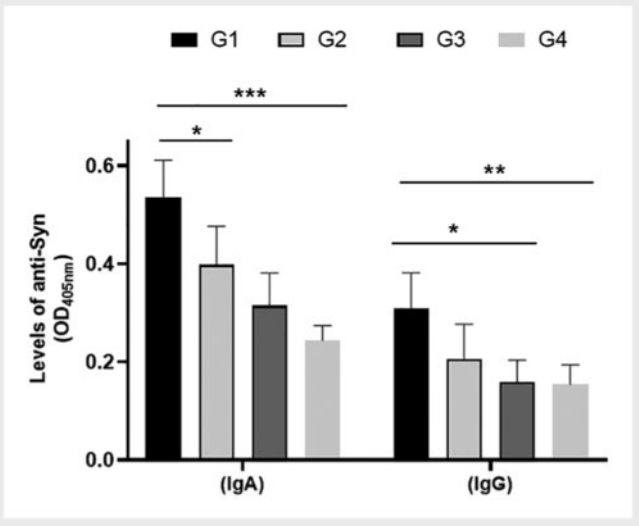
The immunogenicity of the cLTB-Syn was tested in mice using biomass from the MSRZ4 cell line produced in the bioreactor due to its higher antigen content and consistent growth kinetics. The immunization schedules comprised either oral or s. c. immunization or two s. c. immunizations followed by an oral boost. Antibody measurements performed by ELISA to measure IgG against LTB or Syn peptides revealed low humoral responses on day 27, with a notable increase on day 42 after the last boost (▶ **Fig. 7 a**). Oral immunization with the MSRZ4 line led to significant anti-LTB and anti-Syn peptides IgG responses, which are similar in magnitude to the response induced by the scheme based on the s. c. immunization with Syn peptides (▶ **Fig. 7 b**). An oral boost in mice s. c.-immunized with Syn peptides did not improve the systemic IgG response. This result was seen for serum IgG and intestinal IgA responses to either LTB or Syn epitopes. Regarding mucosal humoral responses, significant anti-Syn IgA levels were detected on day 42 in feces from mice subjected to s. c., immunization followed by oral boosting with MSRZ4, whereas the group immunized orally only with MSRZ4 did not induce a significant IgA response (▶ **Fig. 8**). None of the experimental groups showed significant IgG responses at the mucosal level. Overall, these data suggest that oral boosting after systemic immunization led to a better mucosal immune response.

Discussion

Nitrogen has a fundamental role in the growth of plant cells and is directly related to the biosynthesis of amino acids and proteins. Here, we observed that the urea (17 mM) present in MS and B5 media had a better response over the growth of calluses. This strongly suggests that an additional nitrogen source did not affect the metabolism and was necessary for stable biomass production in the carrot Z4 line. This could be because urea provides a readily available nitrogen source, implying that the formation is necessary for the carbon skeleton or the reduction of nitrate to ammonium. Moreover, it has been demonstrated that plants possess sufficient urease activity to process urea even at higher external concentrations [20]. However, when cell plant culture grows solely with urea, there are higher levels of amino acid concentrations (in particular glutamine and asparagine) that could cause growth depression, possibly due to these amino acids leading to the sensing of a high nitrogen abundance, resulting in the restriction of further nitrogen uptake and distribution [21]. Interestingly, in our study, growth deficits were not observed when urea was sup-



▶ **Fig. 7** Assessment of the immunogenicity of the cLTB-Syn vaccine in BALB/c mice. Systemic humoral responses induced under the following treatments: s. c., administration of Syn1-3 peptides on days 0 and 14, followed by an oral boosting with MSRZ4 biomass on day 28 (G1); s. c., administration of Syn1-3 peptides (G2) on days 0, 14, and 28; p. o., administration of MSRZ4 biomass on days 0, 14, and 28 (G3); p. o. administration of wild-type carrot biomass on days 0, 14, and 28 (G4). **a** The anti-LTB antibodies or (**b**) anti-Syn antibodies were determined by ELISA using the corresponding targets (LTB and Syn1-3 synthetic peptides, respectively). A 1 : 50 dilution of sera samples was used. Data are expressed as the mean ± SD derived from experiments performed in triplicate. The asterisks denote statistical difference between days versus the group treated with WT plant (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).



▶ **Fig. 8** Assessment of the immunogenicity of the cLTB-Syn vaccine in BALB/c mice. Mucosal humoral responses induced in feces under the following treatments: s. c., administration of Syn1-3 peptides on days 0 and 14, followed by an oral boosting with MSRZ4 biomass on day 28 (G1); s. c., administration of Syn1-3 peptides (G2) on days 0, 14, and 28; p. o., administration of MSRZ4 biomass on days 0, 14, and 28 (G3); p. o. administration of wild-type carrot biomass on days 0, 14, and 28 (G4). The antibodies levels were determined by ELISA using the Syn1-3 synthetic peptides as target. Data are expressed as the mean ± SD derived from experiments performed in triplicate. The asterisks denote statistical difference versus the group treated with WT plant (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

plied together with nitrates present in MS and B5 media. Similar findings have been reported for nitrate boosts, urea uptake, and urea nitrogen assimilation in *Arabidopsis* and wheat cultures [22, 23]. Glutamate added in MS and B5 media was not efficient as organic nitrogen sources, having an inhibition effect on the growth of calli cultures compared to the media added with urea. This effect could be due to the specific inhibition of one or more of the steps of nitrate assimilation leading to nitrogen starvation. Some reports showed that using amino acid sources inhibits the growth of plant cell suspension cultures [24, 25] due to the inhibition of nitrate reductase activity affecting the assimilation of intracellular ammonium [26].

Based on the results described above, we decided to explore the optimal operational conditions and environmental parameters for carrot Z4 cell line cultures in order to have a favorable impact on cLTB-Syn vaccine yields, which is a candidate for the treatment of synucleinopathies. Previously, we reported cLTB-Syn production with *D. carota* using standard MS medium (an approach without changing the medium composition) and represented a viable platform to optimize culture conditions and achieve short doubling times in shake-flask cultures, as well as to enhance the productivity of cLTB-Syn in an airlift bioreactor [27]. Therefore, we initiated the optimization of culture conditions based on multifactorial experimental designs with PBD and RSM. The PBD considered three parameters, pH, urea, and inoculum size, as significant for achieving optimum biomass yield. These parameters were further optimized by central composite designs (CCD) under RSM, and after validating the model, the kinetic study was carried out. Here, a 1.2-fold enhancement of biomass yield and a 4.5-fold enhancement of LTB-Syn yield was achieved in the shake-flask scale. Statistical experimental designs have frequently been adopted to optimize microbial systems; however, fractional factorial designs were applied to tobacco BY-2 cells to

maximize yields of antibody production using a combination of KNO_3, NH_4NO_3, $CaCl_2$, 2,4-D, and BAP in MS media [28]. Another process of optimization consisted of testing the combinatorial effect of different cultivation parameters such as temperature, incubation time, inoculum density, and lighting on biomass production in pear cell suspension cultures [29]. Other studies determined favorable conditions using RMS designs to improve metabolite production using different inoculum densities, inoculum age, and carbon source concentrations in cell suspension of *T. officinale* [30]. In summary, these results show that multifactorial designs are as important as cell line and vector development for increasing plant cell culture productivity. In this study, to enhance productivity, the cLTB-Syn production cultivation in a 2 L airlift bioreactor under the operating mode described in methodology was implemented. The value used in the bioreactor was k_La for the 1.5 vvm with an 80% saturation level of the dissolved oxygen (DO) parameter settings. Here, k_La aids in determining the capacity of the bioreactor to sustain optimum cell growth, and it is also one of the most essential scale-up factors [31]. With these conditions, it was possible to increase the biomass yield by 1.5 times and cLTB-Syn yield by 2.8 times compared to that obtained previously [27]. The enhancement of these yields in airlift systems may be due to better mixing between the air/liquid phase and increased oxygen availability, which is important for continuous nutrient supply in the cultured cells [32]. In addition, the k_La value use increased proportionally along with the aeration rate (1.5 vvm) and favored the growth of carrot cells, which could be due to a greater amount of oxygen provided by the increased air flow rate. However, an increase in the aeration rate also demands more energy, which needs to be considered in the overall cost analysis.

▶ **Table 3** compares all kinetic parameters obtained in shake flasks and the airlift bioreactor operated at the above-reported optimal conditions. By comparison of these two cultivation strategies, cell viability, change in pH medium, and sugar consumption were similar in both cases despite the changes in the variables evaluated. The μ values and td in the airlift bioreactor culture were similar in the shake-flask culture. Nevertheless, in the airlift bioreactor, the $Y_{X/S}$ was 1.75 greater, $Y_{P/X}$ was 0.5 greater, and qp was 1.2 times greater than in the shake-flask scale. Moreover, the cLTB-Syn expression was higher since day 6 in the bioreactor, which represents 9 days less than the previously obtained data in the shake flask and bioreactor with standard MS medium [27]. We hypothesized that this increase in cLTB-Syn expression was due to active cell division provided by the optimal conditions in the bioreactor. Therefore, we demonstrated that cLTB-Syn expression in the bioreactor could be used for constant and continuous production within a short period of time. Similar observations were obtained with recombinant miraculin in carrot cell cultures using an airlift bioreactor [33]. It is worth noting that the aeration volume (1.5 vvm) used in the bioreactor ensured sufficient nutrient supply to the cultured cells for enhanced biomass and cLTB-Syn production. Moreover, aeration volume provides important gases such as oxygen, carbon dioxide, and possibly others, such as ethylene, to the culture cells [34]. Selection of suitable aeration volume in airlift yielded optimal results for the production of ginsenosides [35], protopanaxadiol [36], and diterpenoids [37]. cLTB-Syn production can be further improved by employing advanced culture optimization strategies such as fed-batch culture, perfusion culture, or semi-continuous culture in airlift bioreactors.

▶ **Table 3** Kinetic parameters of MSRZ4 line of *Daucus carota* in shake flask and airlift bioreactor under optimal culture conditions for cLTB-Syn production.

Parameters	Type of cultivation vessel	
	Shake flask (250 mL)	Bioreactor (2 L)
X_{max} (g/L)	17.9	37.7
μ (1/h)	0.111	0.114
td (days)	6.2	6.0
P_{max} (μg/g DW)	2.8	4.3
$Y_{X/S}$ (g/g)	0.53	0.93
$Y_{P/X}$ (μg/g)	0.16	0.05
qp (μg/g/d)	0.14	0.18

Abbreviations: X_{max}, maximal cell dry weight; μ, specific cell growth rate; td; duplication time; P_{max}, maximal cLTB-Syn production; qp, specific production rate. yield coefficients: $Y_{P/X}$, [μ] cLTB-Syn produced/[g] cell dry weight; $Y_{X/S}$, [g] biomass/[g] sucrose

Second, we explored different immunization schemes in which the administration route was selected as the main variable to determine if mucosal immunization is a feasible approach to optimize the immune response induced by cLTB-Syn. Here, we demonstrated that oral boosting with LTB-Syn bioencapsulated in carrot cells is as effective as the s. c. formulation based on synthetic peptides and a strong adjuvant. In addition, we observed that oral boosting using the chimeric LTB-Syn antigen from carrot material without adjuvant led to an increase in the systemic IgG and the induction of intestinal IgA responses in mice previously s. c.-immunized with the Syn1-3 peptides compared with the group treated with WT carrot cells, which confirms the oral immunogenicity of the antigen delivered to GALT by the carrot cells regardless of the presence of exogenous adjuvants. As a result of these benefits, adjuvants may not be necessary for oral vaccine formulations to initiate an immune response, while injectable vaccines are usually needed to strengthen the immune response. In line with these findings, an oral vaccine candidate against HBV based on maize plants expressing the HBsAg antigen showed the ability to induce IgG responses in the absence of adjuvants and also intestinal IgA responses detected in feces [38]. In other reports, freeze-dried lettuce leaves expressing HBsAg served as oral boosters inducing robust humoral responses in mice previously primed with a commercial HBV vaccine [39]. Thus far, several antigens produced in *D. carota* have shown a promising immunogenicity when used in prime/boosting schemes including mucosal administration, which include candidates against viral [40], bacterial [41], and noncommunicable diseases [42].

In our study, we observed the highest IgA levels in mice-primed s. c. boosted orally, suggesting that the plant-made antigen delivered by the plant cell achieves the activation of GALT,

which may contribute to attaining an optimal immune profile, that is, protection at both the systemic and mucosal levels. Considering the earliest evidence that Parkinson's disease may arise in the gut, hypothetical α-Syn aggregates progress through the vagus nerve to the brain in a prion-like fashion [43]. Oral vaccines against synucleinopathies aimed to block or prevent the α-Syn aggregate propagation at these sites are a potential approach to fight it. Thus far, several approaches have focused on this aspect, such as modulation of the gut microbiota [44], the use of probiotics and prebiotics [45], and lately, the employ of levodopa-carbidopa intestinal gel infusion (LCIG) [46]. Considering that the most expensive part of biopharmaceuticals production is the downstream processing [47], our oral carrot-based vaccine candidate, which requires minimal processing, stands as a promising and accessible candidate having the ability to induce both systemic and mucosal immune responses for integral protection against synucleinopathies onset and treatment. Our study represents a step forward in the development of oral vaccines against synucleinopathies. The path to continuing this development contemplates the evaluation of the vaccine in a disease model followed by toxicity and safety assessment to propose a phase I clinical assay. For the latter, the production of GMP batches will be greatly facilitated by the production process based on fermentation in airlift bioreactors.

In conclusion, the bioprocess to enhance the production of the cLTB-Syn vaccine was suitable for using an airlift-type 2 L bioreactor. In consequence, the airlift bioreactor is appropriate for scale-up in cLTB-Syn production. In addition, the cLTB-Syn vaccine is capable of inducing mucosal immune responses when orally administered, which supports its potential to be evaluated in a disease animal model and determine if cLTB-Syn could be translated into human clinical trials to determine its safety and efficacy against synucleinopathies.

Materials and Methods

Calluses and cell suspension culture conditions

The carrot cell line Z4 was originally obtained and transformed in a previous work [48]. The carrot Z4 cell line was maintained by our work group for more than three years and used in this work. Calluses of carrot Z4 cell lines were propagated on MS and B5 media solid (Phytotech Labs) + with 30 g/L of sucrose, 2 mg/L of 2,4-D, 2 mg/L of KIN, 2.5 g/L KNO_3 (only to B5 medium), and 1.5 g/L of phytagel. The urea and glutamate (added separately) were used as additional nitrogen sources at two different concentration levels (**Table 3S**, Supporting Information), maintaining the rest of the standard salts of the MS and B5 culture media. The calli cultures were incubated at 25 °C under 16/8 h light/dark photoperiod conditions. Six subcultures were performed at 20-day intervals by inoculating 1.0 ± 0.3 g, FW of biomass into fresh medium to obtain friable callus.

To establish the Z4 cell line suspension cultures, nine subcultures (4.5 months) were performed later by transferring 10% v/v inoculum into fresh medium for their optimization (named the MSRZ4 line). The cultures were maintained at 25 °C in a shaking incubator (120 rpm) under continuous light (50 µmol/m^2/s). The non-transformed cell line (WT) was used as the control for PCR, dot-blot, and immunization analyses.

Plackett–Burman design (PBD)

The PBD model was used to evaluate the relative importance of various nutrients on biomass and cLTB-Syn production. Based on preliminary experiments of this laboratory (data not shown), seven independent variables (sucrose, pH, phytoregulators, inoculum size, radiation, urea, and glutamate) were selected for PBD. Each variable was tested at two levels: the minimum (level − 1) and maximum (level + 1) ranges selected for the above variables are given in **Table 4S** (Supporting Information).

Twelve randomized experimental trials were conducted in triplicates, and the average value was measured to identify the components affecting cell mass and LTB-syn production of the Z4 cell line. After 15 days of incubation at 25 °C under continuous light and mixing at 120 rpm, the output of variable response was estimated. Variables exerting a significant effect on the reaction were selected using ANOVA analysis and used in RSM. The PBD was generated by the computer software Design-Expert (version 11).

Response surface methodology (RSM)

After the adequate nutrients were identified from the PBD, RSM was used to determine the optimum concentration level of the parameters and condition for optimization of biomass and cLTB-Syn production. A 2^3 factorial central composite design (CCD) model, with six axial points and three replicates at the center points leading to a total of 15 sets of experiments, was used to optimize the three selected variables (inoculum, urea, and pH) to give maximum biomass and cLTB-Syn (**Table 5S**, Supporting Information). The cultures were incubated in a shaking incubator at 120 rpm and maintained at 25 °C under continuous light. The flasks were harvested in triplicate after 15 days and analyzed for dry cell weight and cLTB-Syn production. The RSM design was developed using Design-Expert software (version 11). A second-order polynomial equation (ANOVA) was used to determine the interaction between the manipulated variables, and the response surface plot was obtained.

Kinetic growth conditions in shake flask and bioreactor

The Monod model was used in growth kinetics to describe the specific growth rate and the decay of biomass based on the concentration of the limiting substrate in the culture medium. For shake-flask kinetic studies, the Z4 cell suspension line was inoculated at 10% v/v (10 ± 0.2 g FW) ratio into 250 mL Erlenmeyer flasks with 100 mL of MSR medium (supplemented with 30 g/L of sucrose, 2 mg/L of 2,4-D + 2 mg/L of KIN, 17 mM of urea, and pH 5.0). The shake-flask cultivations were incubated at 25 °C under continuous light (50 µmol/m^2/s) in a gyratory shaker (120 rpm) for 24 days. The bioreactor cultivations were carried out in a 2 L concentric draft-tube internal-loop airlift operated previously [27]. The k_La that describes the efficiency with which oxygen can be delivered to a bioreactor was determined by the gassing-out method using MSR medium at 25 °C depending on the aeration volume (0.5, 1.0, 1.5, and 2.0 vvm) [49]. The airlift bioreactor was sterilized for kinetic experiments and filled with a

working volume of 80 %. The bioreactor was inoculated with 10 % v/v (160 g FW) and was grown at 25 °C under continuous light (50 µmol/m^2/s) for 16 days. The bioreactor was operated at an aeration rate of 1.5 vvm. The pH and the DO were not controlled, but they were monitored continuously. Any foam formation was governed by the addition of a silicone antifoaming agent (Drogueria Cosmopolita Inc.), and the DO concentrations were analyzed using a polarographic electrode sensor (Cole-Palmer).

Statistics and reproducibility

All singular experiment results where statistical calculations were applied used three independent replicates. All data are presented as the mean ± the standard deviation. In the case of bioreactor cultures, two independent experiments were conducted.

Growth kinetic sampling

Three randomly chosen shake flasks were harvested every three days for kinetic parameters (fresh weight, dry weight, pH variation, cell viability, carbohydrate consumption, and protein production). In the airlift bioreactor, 20 mL of the culture medium samples was taken every two days for the kinetic parameters.

Specific growth rate and yields

The specific growth rate (µ) was calculated by the change in biomass concentration per unit of time (d^{-1}). All values were taken from the exponential phase of growth. From µ, the doubling time (td) was calculated as follows:

$$td = \ln 2/\mu$$

Cell yield ($Y_{X/S}$) was calculated as (g maximum biomass – g initial biomass)/(sucrose consumed). Product yield ($Y_{P/X}$) was calculated as (µg protein produced)/(g biomass), and specific productivity (qp) was calculated as (µg protein produced)/(biomass per day).

Analytical procedures

Biomass concentration determination

Fresh weight (FW) was measured by filtering a biomass sample into a pre-dried, pre-weighed, paper disk (4 µm retention) filter connected to a vacuum and then weighing the cells. Dry weight (DW) was determined after freeze-drying the retained cells at − 60 °C for 3 days until constant weight.

Sugar analysis

HPLC analysis to determine sucrose, fructose, and glucose concentrations were assayed by an IR detector. The column was of the NH_2 type, measuring 3.9 × 300 mm, with 125 Å pore size and 10 µm particle diameter (Waters). The predetermined conditions were a mobile phase 80 : 20 acetonitrile : water with an operational flow of 1.0 mL/min.

Transgene detection by PCR

The transgene *LTB-Syn* presence was determined by PCR. First, total DNA was isolated from 10 mg DW of carrot MSRZ4 cells and wild-type (WT) lines cultivated in flasks and the bioreactor [50]. PCR and cycling conditions were assessed as previously reported [51]. This procedure was performed in a MultiGene Mini Personal Thermal Cycler (Labnet). The amplicons were analyzed by electrophoresis using 1 % agarose gels.

ELISA and dot-blot assays

The LTB-Syn expression levels in 10 mg DW of the carrot MSRZ4 cell line was determined by the ELISA (GM1-ELISA) method according to a previously reported protocol [51]. Protein extract from WT carrot lines was used as a negative control. OD values were at 405 nm (Thermo Scientific, Waltham, MA). A standard curve was included in the assay using known amounts of unmodified pure CTB to quantify the content of the LTB-Syn protein in the carrot cell suspension. The detection of LTB-Syn protein was achieved with a dot-blot assay according to a protocol described formerly [52]. Antibody binding was detected by incubation with Super Signal West Dura solution following instructions from the manufacturer (Thermo Scientific, Waltham, MA). Chemiluminescence detection was performed on the iBright system FL1000 equipment.

Immunogenicity assessment

Animal handling was conducted according to the Guide for Care and Use of Laboratory Animals of the National Institute of Health (USA) and protocols approved by the Committee on Research Ethics of the Faculty of Chemistry/University of San Luis Potosi (CEID2015048, issued on January 26th, 2015). The immunogenicity of cLTB-Syn expressed in carrot cells was evaluated in 12-week-old BALB/c female mice (body weight = 25 ± 3 g). Experimental mice were randomly assigned to experimental groups (n = 5) that received one of the following treatments: s. c., administration of Syn1-3 peptides on days 0 and 14, followed by an oral boosting with MSRZ4 biomass on day 28 (G1); s. c., administration of Syn1-3 peptides (G2) on days 0, 14, and 28; p. o., administration of MSRZ4 biomass on days 0, 14, and 28 (G3); p. o. administration of wild-type carrot biomass on days 0, 14, and 28 (G4). s. c. formulations were prepared as follows: the doses of Syn1-3 peptides mixed in PBS were emulsified with one volume of complete Freund's adjuvant (CFA) for immunization on day 0, while for immunization on days 14 and 28, incomplete Freund's adjuvant was used (IFA). Oral formulations were prepared with dry cells from transgenic (MSRZ4) or wild-type carrot lines; 30 mg per dose were resuspended in 300 µL of PBS and administered intragastrically.

Determination of antibody titers

Blood samples were obtained on days 0, 28, and 42 and after clotting samples were centrifuged at 13 000 ×g for 10 min. Serum samples were stored at − 80 °C until antibody determination. Feces samples (100 mg) were resuspended in 500 µL of 5 % fat-free dry milk in PBS and 1 mM of PMSF (ice cold). Feces suspensions were centrifuged for 15 min at 16 000×g at 4 °C.

An ELISA assay was performed to determine anti-α-Syn and anti-LTB IgG and IgA antibodies in serum and fecal samples, respectively. ELISA plates were coated overnight at 4 °C with 100 ng/well of LTB or 50 ng/well of synthetic Syn1-3 peptides in carbonate buffer. The performed ELISA was based on a previously reported protocol [51]. The ELISA data of the antibody analysis of immunized mice are presented as mean OD values obtained per

group at different time points. Statistical differences in antibody levels among groups were determined by a one-way ANOVA using the GraphPad Prism 8 software ($p < 0.05$).

Supporting information

The tables and figures used in this study of the ANOVA analysis with Plackett–Burman and CCD designs and the response surface plots for optimization of cLTB-Syn are presented in the supporting information.

Contributors' Statement

Writing-original draft, interpretation of the data; statistical analysis: C. Carreño-Campos; Data collection and methodology: D. O. Govea-Alonso, A. Romero-Maldonado and M. Morales-Aguilar; Conceptualization and supervision: E. Villegas and ME Jimenez-Capdeville; Conceptualization, supervision, funding acquisition, writing-review and editing: S. Rosales-Mendoza, M. L. Villarreal and A. Ortiz Caltempa.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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