

NITROGEN SOURCE: AN EFFECTIVE COMPONENT FOR THE GROWTH, AND VIABILITY OF *LACTOBACILLUS DELBRUECKII* SUBSP. *BULGARICUS*

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MATERIALS AND METHODS

Lactobacilli MRS Agar Medium

MRS agar medium was prepared by completely dissolving 55g of MRS (Neogen Co, Michigan, USA) and 0.5g of L-Cysteine in 1 L of deionized water (DW). Difco agar powder (15g) was added, and the agar medium was sterilized at 121°C for 15 min and then cooled in a water bath. All freshly-prepared media in the study were poured into sterile petri dishes and stored at 4 °C until further needed.

Modified Reinforced Clostridial Medium-Pyruvate (mRCM-PYR)

A reinforced clostridial medium according to Oyeniran *et al.* (2020) was optimized for selectivity and accurate enumeration of *Lb. bulgaricus* by dissolving 10g peptone #3, 10g beef extract, 5g yeast extract, 5g sodium chloride, 3g sodium acetate, 2g K₂HPO₄, 0.1g uracil, 0.25g calcium chloride, 5g dextrose, 5g fructose, 10g maltose, 2g sodium pyruvate, 0.2% Tween 80 and 0.5g L-Cysteine in 1 L DW. The final pH (6.0 ± 0.2) of the solution was obtained using 6N HCl before the addition of 0.008% aniline blue and 15g agar. The medium was autoclaved at 121°C for 15 min, cooled in a water bath, and poured into sterile petri dishes. All freshly-prepared media in sterile petri dishes were stored at 4°C until needed.

Validation of Nitrogen Sources as Growth Medium

Five yeast samples (X-Seed Nucleo Max, X-Seed KAT, X-Seed Peptone, X-Seed Carbo-Peptone, and X-Seed Carbo-Max) provided by Ohly GmbH, Germany were used and tested in different blends and proportions during the preliminary study. X-Seed Carbo-Peptone, and X-Seed Carbo-Max were products under development by Ohly GmbH. The preliminary work thus confirmed X-

Seed Nucleo Max, X-Seed KAT, and X-Seed Carbo-Max yeast samples as superior alternative complex nitrogen sources for use in the study. The Supplementary Table S2 highlights the total percentage of protein and the total percentage of nitrogen in X-Seed Nucleo Max, X-Seed KAT, and X-Seed Carbo Max, respectively.

Optimized Lactobacilli Growth Medium

The growth medium was prepared similarly to the composition of standard deMan Rogosa Sharpe (MRS) medium as follows: completely dissolving 20g dextrose, 3g potassium phosphate dibasic, 3g ammonium citrate, 0.2g magnesium sulphate, 3g sodium acetate, 3g sodium pyruvate, 0.1g manganese sulphate, 0.2% Tween 80, 0.1g Uracil, 0.35g calcium chloride, 0.5g L-Cysteine, 2g X-Seed Nucleo Max, optimized proportions of blends of X-Seed KAT, and X-Seed Carbo Max (5g and 10g) in 1 L of deionized water (DW). This medium was adjusted to a final pH of 6.4 ± 0.2 , using 6N HCl. Ten ml of the resultant solution was then dispensed into test tubes, autoclaved at 121°C for 15 min, and then cooled at room temperature.

Culture Propagation in Growth Medium with Different Nitrogen Blends

After 16 hours of growth in MRS and reaching an optical density (OD_{610nm}) between 0.7 and 0.9, the strains (S9 and LB6) were individually centrifuged at 5000 rpm for 20 minutes at 4°C (Thermo Scientific Sorvall ST 16R). The resultant supernatant was decanted, and cells were washed in a 0.1M phosphate-buffered saline (PBS) solution. The washed cells were then serially diluted ten-fold in peptone water. From the third-fold dilution, 250µl was inoculated in duplicates into each batch of test tubes as follows: MRS broth (control), basal growth medium with a nitrogen source (2g/L of X-Seed Nucleo Max: N-Max), basal medium with 2g/L of Nucleo Max supplemented with 5g/L and 10g/L Carbo Max respectively (C-Max 5% and C-Max 10%), basal medium with 2g/L of Nucleo Max supplemented with 5g/L and 10g/L KAT respectively (KAT 5% and KAT 10%), basal medium with 2g/L of Nucleo Max supplemented with 5g/L Carbo Max and 5g/L KAT respectively (KCMaX 5/5), basal medium with 2g/L of Nucleo Max supplemented with 5g/L Carbo Max and 10g/L KAT respectively (KCMaX 5/10), basal medium with 2g/L of Nucleo Max supplemented with 10g/L Carbo Max and 5g/L KAT respectively (KCMaX 10/5), and basal

medium with 2g/L of Nucleo Max supplemented with 10g/L Carbo Max and 10g/L KAT respectively (KCMax 10/10). The complex nitrogen source blends used in the present study are shown in Supplementary Table S3. All test tubes with the different batches of nitrogen sources were vortexed and anaerobically incubated at 42°C for 12 hours, and the growth of cultures was measured at time intervals of 0, 6, and 12 hours at OD_{610nm}. The initial bacterial population was determined using the bacterial enumeration method.

Determination of pH values for the different growth media

The pH values of each test tube sample were measured in duplicates at the start (0 h) and end (12 h) of the fermentation period using a pH meter (Accumet AB 150, Fisher Scientific, Pittsburgh, PA). The pH readings were taken after calibration with standard pH buffers 4.0 and 7.0. The pH electrode was rinsed thoroughly with deionized water between the different samples.

Determination of buffering capacity

The initial pH values of all the nitrogen-supplemented media; N-Max, C-Max 5%, C-Max 10%, KAT 5%, KAT 10%, KCMax (5/5), KCMax (5/10), KCMax (10/5), KCMax (10/10), and MRS (control) were stabilized and adjusted to pH 6.5. A standard 0.1N of hydrochloric acid (HCl) was prepared for sample titration. Two aliquots (50ml) of each sample were placed in a 100ml beaker, and 1 mL of HCl acid was slowly titrated into each sample in order to reach a pH of 4.5.

Bacterial Enumeration

The bacterial growth of the two strains (LB6, and S9) was monitored via spectrophotometric measurement of the strains' optical densities (OD_{610nm}) at different time intervals (0, 6, and 12 hours) using a Thermo Fisher Scientific Evolution 201 UV-visible spectrophotometer (Thermo Fisher Scientific, Inc. Waltham, MA, USA). The uncultured media were used as blanks during the spectrophotometric measurement, and the initial and final bacterial populations were determined. At the end of the fermentation period (12 h), a 1 mL sample was drawn from each inoculated sample and serially diluted in 0.9% peptone water. Appropriate dilutions were then surface plated (100 µl) onto duplicate MRS and mRCM-PYR agar plates. All plates were anaerobically incubated for 48 h at 42°C, and plates with 25 – 250 colonies were counted. The bacterial populations were then expressed in Log CFU/mL.

Determination of buffering capacity with L-Arginine and L-Histidine supplementation

The different nitrogen supplemented media were then prepared with and without the addition of 4g/L of L-Arginine and L-Histidine respectively and adjusted to pH 6.5. A standard 0.1N of hydrochloric acid (HCl) was prepared for sample titration. Two aliquots (50ml) of each sample were placed in a 100ml beaker, and 1 mL of 0.1N HCl acid was slowly titrated into each sample. The pH reading was recorded for each until the pH of the solution reached 4.5. The buffering capacity was then determined by adding the total amount of HCL acid consumed in order to reach the endpoint of pH 4.5.

Bacterial enumeration in growth media supplemented with and without L-Histidine

Bacterial growth and population counts were determined in the growth media (KCMax (5/10), KCMax (10/5), and KCMax (10/10) with and without L-histidine supplementation. A high and a low concentration (4g/L and 0.5g/L) of L-histidine were supplemented with the selected media. The control samples were MRS, and the standard growth media of KCMax (5/10), KCMax (10/5), and KCMax (10/10) had no L-histidine supplementation. The S9 strain of *Lb. bulgaricus* was used for this study and anaerobically fermented in the various growth media at 42°C for 12 hours. At the end of the fermentation period (12 h), a 1 mL sample was drawn from each inoculated sample and serially diluted in 0.9% peptone water. Appropriate dilutions were then surface plated (100 µl) onto duplicate MRS and mRCM-PYR agar plates. All plates were anaerobically incubated for 48 h at 42°C and plates with 25 – 250 colonies were counted. The bacterial populations were then expressed in Log CFU/mL.

Impact of calcium on the growth of *Lb. bulgaricus* in modified growth medium

Fifty (50) ml duplicate samples of the modified growth medium KCMax (10/10) were prepared with each containing varying concentrations of calcium; 4g/L (0.4%), 8g/L (0.8%) and 12g/L (1.2%), respectively. The control samples were prepared similarly but without calcium supplementation. The different calcium supplemented media and the control media were then inoculated with 1ml each of the S9 *Lb. bulgaricus* strain and were anaerobically fermented at 42°C for 12 hours. The pH measurements were taken, and population counts were determined by the enumeration method at the end of the fermentation period. Bacterial cells were then harvested by

centrifugation at 5000rpm for 15minutes at 4°C. One set was washed with 50ml sterile deionized water, centrifuged a second time, suspended in sterile deionized water, and vortexed in order to obtain a uniform mixture. The other set was treated similarly but without washing with deionized water. Both samples were sonicated with a Branson Sonifier 250 unit for 4 minutes in order to disrupt the cells and the samples were rapidly cooled with ice. Samples were then filter sterilized through a filter with a pore size of 0.2µm in order to obtain a clear filtrate. Calcium content was finally determined in the clear filtrates by the Inductively Coupled Plasma Mass Spectroscopy (ICP-MS) method using an Optima 800 ICP-OES unit (Perkin Elmer Company, MA, USA).

Impact of calcium on the freeze-stability and viability of *Lb. bulgaricus*

Cells from the S9 strain of *Lb. bulgaricus* were harvested from overnight cultures, washed with phosphate-buffered saline (PBS) solution, serially diluted and appropriate dilutions were then inoculated into 50ml of MRS media and KCMMax (10/10) respectively. All samples were performed in triplicates and anaerobically incubated at 42°C for 12 hours. The samples were then centrifuged at 5000 rpm for 15 minutes at 4°C following the fermentation period, in order to harvest the cell pellets. Bacterial cells from both the MRS and KCMMax (10/10) media were then washed with and without 0.4% calcium chloride solution. Cells washed without the calcium chloride solution were assigned as the control and were only washed with sterile deionized water. Bacterial cells from both treatments were thinly spread in petri-dishes and pre-frozen overnight at -80°C before freeze-drying. Pre-frozen cells were freeze-dried in a Labconco unit for 48 hours. The freeze-dried samples were resuspended into 3ml of skimmed milk supplemented with 0.1ml yeast extract and 10µl Tween 80 in order to enhance the recovery of stressed bacterial cells. The mix was then incubated at 42°C for 6 hours, serially diluted with peptone solution, and 100µl of appropriate dilutions were subsequently plated in duplicates by the enumeration method. Plates were anaerobically incubated at 42°C for 48 hours, and colonies were counted.

RESULTS AND DISCUSSION

*Preliminary Study (validation of the nitrogen source on the growth of *Lb. bulgaricus* strains)*

The different nitrogen sources, X-Seed Nucleo Max (N-Max), X-Seed KAT (KAT), X-Seed Peptone (Pep), X-Seed Carbo Peptone (C-Pep), and X-Seed Carbo Max (C-Max) were validated and tested in different blends and formulations (Supplementary Table S3). Consequently, X-Seed Nucleo Max, X-Seed KAT, and X-Seed Carbo Max were considered as superior complex nitrogen sources and were blended as the optimized growth media. The viability of all the strains is shown in Supplementary Figure S1 and is linked to the first formulated media (Supplementary Table S3). Formulations two, and four did not yield promising growth results; however, formulation three (Supplementary Figure S2) included a basal medium with 2g/L of Nucleo-Max (N-Max) in conjunction with various blends of KAT (K) and Carbo-Max (C-Max) yielded exceptional results.

Several trials were done with various strains of *Lb. bulgaricus* until a final optimized growth media formulation with comparable results to MRS was obtained. The first formulation had a blend of 1% (w/v) of the different nitrogen sources, and five (5) strains (E22, S9, LB6, RDA, and ATCC 11842) were used. MRS was also used as a control medium in all of the preliminary formulations. Bacterial growth was monitored for eight (8) hours at an incubation temperature of 42°C. Although the optical density (OD_{610nm}) results of the strains in the first formulated growth media were not promising, MRS (the standard control) had acceptable growth which confirmed the viability of the strains as shown in Supplementary Figure S1.

It was also observed that *Lb. bulgaricus* strains S9 and LB6 were fast-growing, especially in the MRS medium, hence their selection for the fermentation study. Formulations two, and four did not yield promising growth results; however, formulation three (Supplementary Figure S2) which included a basal medium with 2g/L of Nucleo-Max (N-Max) in conjunction with various blends of KAT (K) and Carbo-Max (C-Max) yielded exceptional results.

Based on the positive outcome of formulation three, formulation five (5) was developed and consequently chosen as the final optimized recipe for the study. The fifth and final growth media formulation was based on 5% (5g/L) and 10% (10g/L) of the validated nitrogen sources (KAT and C-Max, respectively), and a blend of both KAT and C-Max (KCMaX) in a supplemented

basal medium containing 2g/L of Nucleo-Max (N-Max). Thus, the various formulations employed were C-Max 5%, C-Max 10%, KAT 5%, KAT 10%, KCMMax (5/5), KCMMax (5/10), KCMMax (10/5), and KCMMax (10/10), all of which were supplemented with a basal medium (2g/L of N-Max). MRS was also used as the control medium in all cases for the fermentation study. The nitrogen source blends KCMMax (5/5) represented 5g/L of KAT and 5g/L of C-Max, and KCMMax (5/10) was a combination of 5g/L of KAT and 10g/L of C-Max. The blend KCMMax (10/5) constituted 10g/L of KAT and 5g/L of C-Max and the final blend, KCMMax (10/10) was a combination of 10g/L of KAT and 10g/L of C-Max respectively.

References

Oyeniran A, Ibrahim SA, Gyawali R, Tahergorabi R, Zimmerman T and Krastanov A (2020). A modified reinforced clostridial medium for the isolation and enumeration of *Lactobacillus delbrueckii* ssp. *bulgaricus* in a mixed culture. *Journal of Dairy Science* **103**, 5030 – 5042. 10.3168/jds.2019-17894.

Table legends:

Supplementary Table S1:

Probiotic strains used in the preliminary study

Supplementary Table S2:

Total Protein (%) and Nitrogen (%) of validated yeast samples for the study

Supplementary Table S3

Nitrogen sources used for the growth media formulation

Supplementary Table S4:

Composition per liter of deMan Rogosa Sharpe medium (MRS) and the optimized growth medium

Supplementary Table S1

No	Product Code	Sample	Source	Bacterial Composition as labeled ¹
1.	S9	Pure Industrial Strain	Bulgaria	<i>Lb. bulgaricus</i>
2.	LB6	Pure Industrial Strain	Bulgaria	<i>Lb. bulgaricus</i> ,
3.	THT	Pure Industrial Strain	Belgium	<i>Lb. bulgaricus</i>

4.	ATCC 11842	Pure Industrial Strain	ATCC	<i>Lb. bulgaricus</i>
5.	DAW	Yogurt	USA	<i>Lb. bulgaricus</i> , other live culture
6.	S8	Pure Industrial Strain	Bulgaria	<i>Lb. bulgaricus</i>
7.	E22	Yogurt	USA	<i>Lb. bulgaricus</i> , other live culture
8.	S5	Pure Industrial Strain	Bulgaria	<i>Lb. bulgaricus</i>
9.	S19	Pure Industrial Strain	Bulgaria	<i>Lb. bulgaricus</i>
10.	RDA	Probiotic Supplement	Bulgaria	<i>Lb. bulgaricus</i>

¹*Lb.* = *Lactobacillus*
Supplementary Table S2

Yeast Samples	Protein (%)	Total Nitrogen (%)
X-Seed Nucleo Max	71	11.4
X-Seed KAT	75	12
X-Seed Carbo Max	26 - 30	4.2 – 4.8

Supplementary Table S3

Growth Media Formulation	Nitrogen Sources (X-Seed)	Ratio / Blend	Standard
1	Nucleo Max, KAT, Carbo Max, Carbo Peptone, and Peptone	1% w/v of each nitrogen source	MRS
2	Nucleo Max, KAT, Carbo Max, Carbo Peptone, and Peptone	0g/L, 5g/L, 10g/L of each nitrogen source	MRS
3	Nucleo Max, KAT, Carbo Max, Carbo Peptone, and Peptone	0g/L, 2.5g/L, 5g/L, 7.5g/L and 10g/L of KAT, Carbo Max, Carbo Peptone and Peptone respectively <i>in a basal medium</i> (2g/L Nucleo Max)	MRS
4	Nucleo Max, KAT, and Peptone	1% w/v blend of KAT/Nucleo Max, 1% w/v blend of KAT/Peptone/Nucleo Max and 1% w/v blend of Nucleo Max/Peptone	MRS

5	Nucleo Max, KAT, and Carbo Max,	5g/L, and 10g/L of blends of KAT, and Carbo Max, respectively <i>in a basal medium</i> (2g/L Nucleo Max)	MRS
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Supplementary Table S4

1

MRS (Neogen) (g/L)	Optimized Growth Medium (g/L)
20g Glucose	20g Dextrose
5g Sodium Acetate	3g Sodium Acetate
0.2g Magnesium Sulfate	0.2g Magnesium Sulfate
0.05g Manganese Sulfate	0.1g Manganese Sulfate
1.08g Tween 80	2ml Tween 80
2g Ammonium Citrate	3g Ammonium Citrate
*10g Peptone	3g Ammonium Phosphate Dibasic
*5g Yeast	3g Sodium Pyruvate
*10g Meat Extract	0.35g Calcium Chloride
	0.5g L - Cysteine
	0.1g Uracil
	*2g X- Seed Nucleo Max
	*5g / 10g X- Seed KAT
	*5g / 10g X- Seed Carbo Max

Ingredients marked * indicate a nitrogen source

Figure legends:

Supplementary Figure S1:

Growth of *Lb. bulgaricus* strains in the first formulated growth media (1% w/v of the different nitrogen sources) after 8 hours of incubation at 42°C.

Supplementary Figure S2:

Growth of *Lb. bulgaricus* strains (S9 and LB6) in the third formulated growth media after 14 hours of incubation at 42°C. Nitrogen source legends: KAT, Carbo Max (C-Max), Peptone (PEP), Carbo Peptone (C-Pep), 2.5% = 2.5g/L, 5% = 5g/L, 7.5% = 7.5g/L, and 10% = 10g/L.

Supplementary Figure S3a, S3b, and S3c:

The different buffering capacities of the N-Max medium with and without L-Arginine against the MRS control medium

Supplementary Figure S4a, S4b, S4c, and S4d:

The varying buffering capacities of the C-Max (5% and 10%) medium supplemented with and without L-Arginine.

Supplementary Figure S5a, S5b, S5c, and S5d:

The different buffering capacities of the KAT (5% and 10%) medium supplemented with and without L-Arginine.

Supplementary Figure S6a, S6b, S6c, and S6d:

The varying buffering capacities of the KCMa_x (5/5) and KCMa_x (5/10) medium supplemented with and without L-Arginine.

Supplementary Figure S7a, S7b, S7c, and S7d:

The buffering capacities of the KCMa_x (10/5) and KCMa_x (10/10) medium supplemented with and without L-Arginine.

Supplementary Figure S8a, S8b, and S8c:

The different buffering capacities of the N-Max medium supplemented with and without L-Histidine against the buffering capacity of the control medium (MRS).

Supplementary Figure S9a, S9b, S9c and S9d:

The different buffering capacities of the C-Max (5% and 10%) medium supplemented with and without L-Histidine.

Supplementary Figure S10a, S10b, S10c, and S10d:

The varying buffering capacities of the KAT (5% and 10%) medium supplemented with and without L-Histidine.

Supplementary Figure S11a, S11b, S11c, and S11d:

The different buffering capacities of the KCMa_x (5/5) and KCMa_x (5/10) medium supplemented with and without L-Histidine.

Supplementary Figure S12a, S12b, S12c, and S12d:

The varying buffering capacities of the KCMa_x (10/5) and KCMa_x (10/10) medium supplemented with and without L-Histidine.

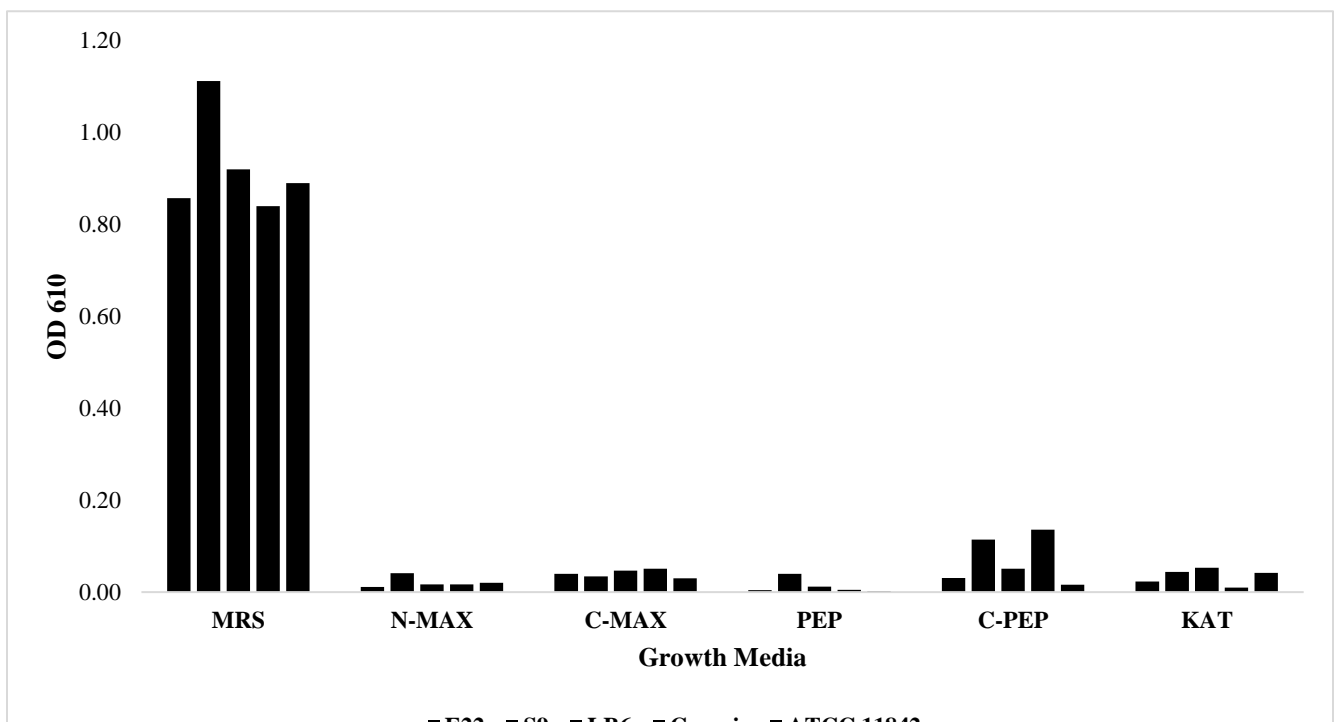
Supplementary Figure S13:

Calcium concentrations detected in KCMa_x (10/10) medium after fermentation with S9 *Lb. bulgaricus* strain with different treatments of cell wash. Error bars indicate SD for an experiment performed in triplicate.

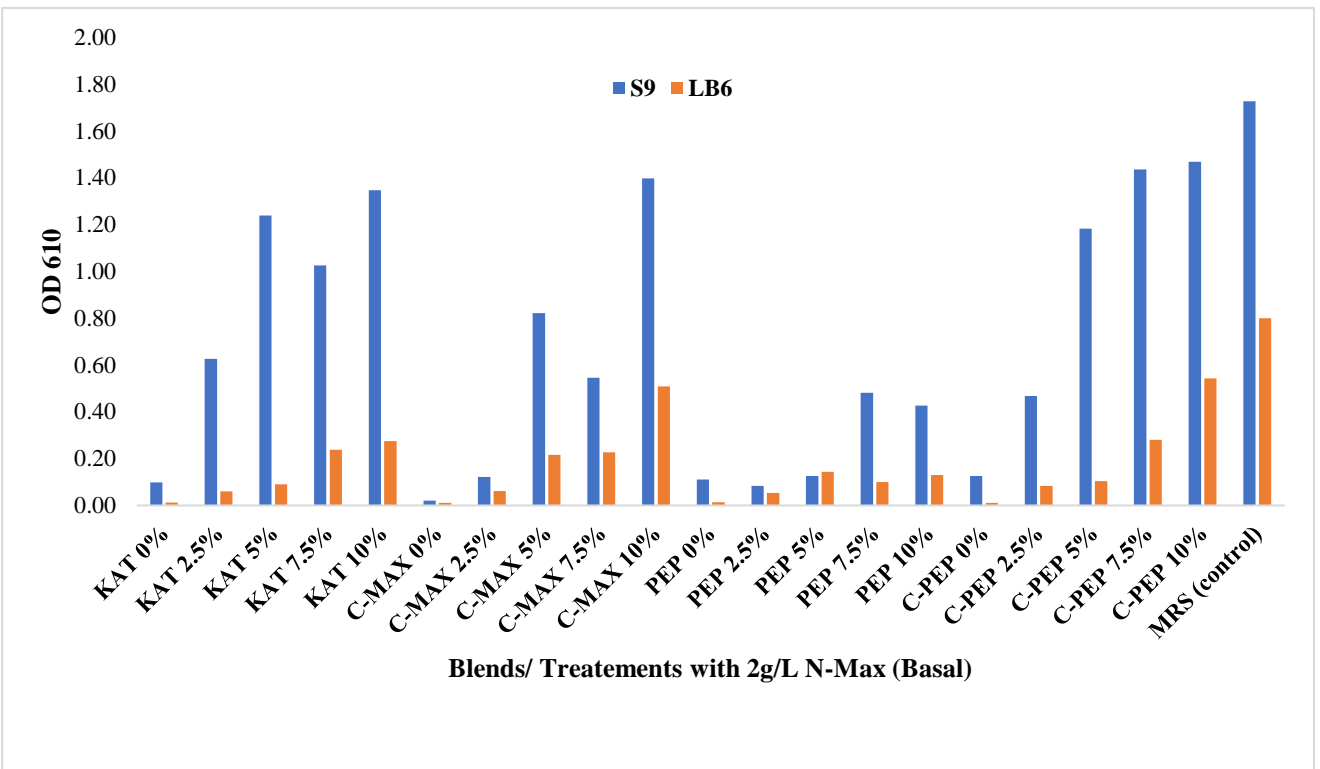
Supplementary Figure S14:

The bacterial count of S9 *Lb. bulgaricus* strain after calcium supplementation in the fermentation medium KCMAX (10/10) with two different treatments of cell wash. Error bars indicate SD for an experiment performed in duplicate.

Supplementary Figure S1:

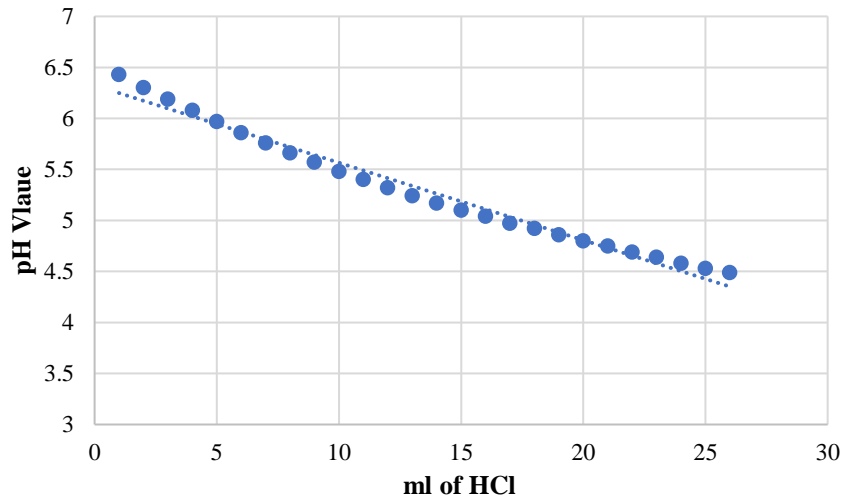


Supplementary Figure S2:

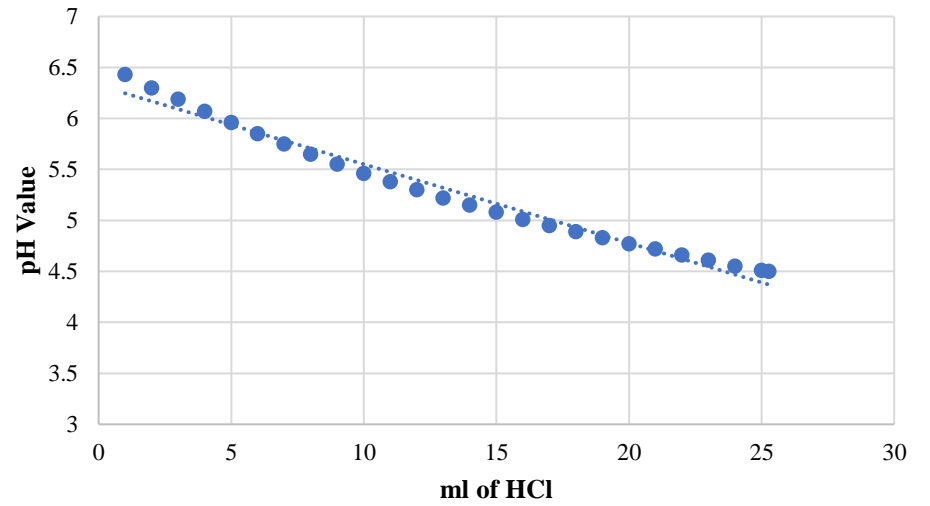


Supplementary Figures S3a, S3b and S3c (Arg: Arginine):

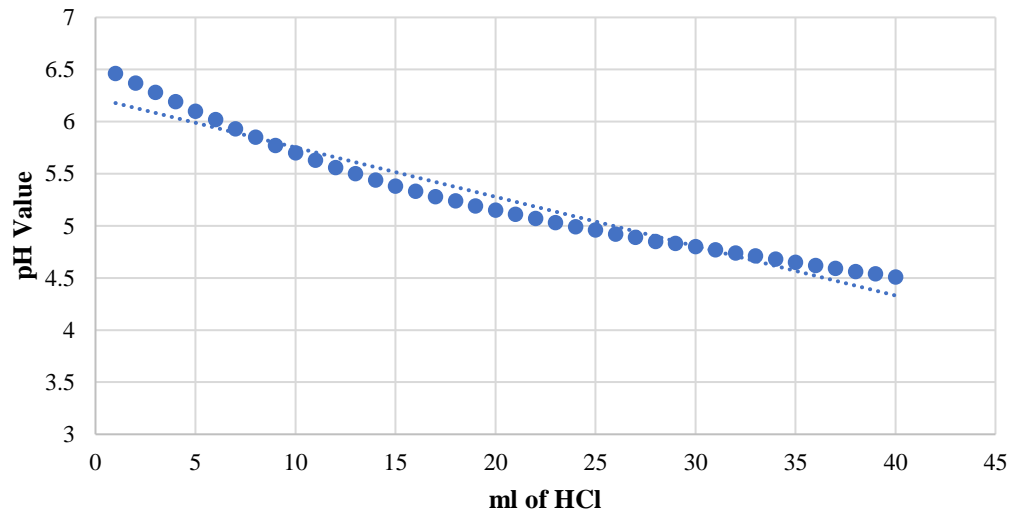
(3a) Buffer Capacity of N-Max only



(3b) Buffer Capacity of N-Max-Arg only

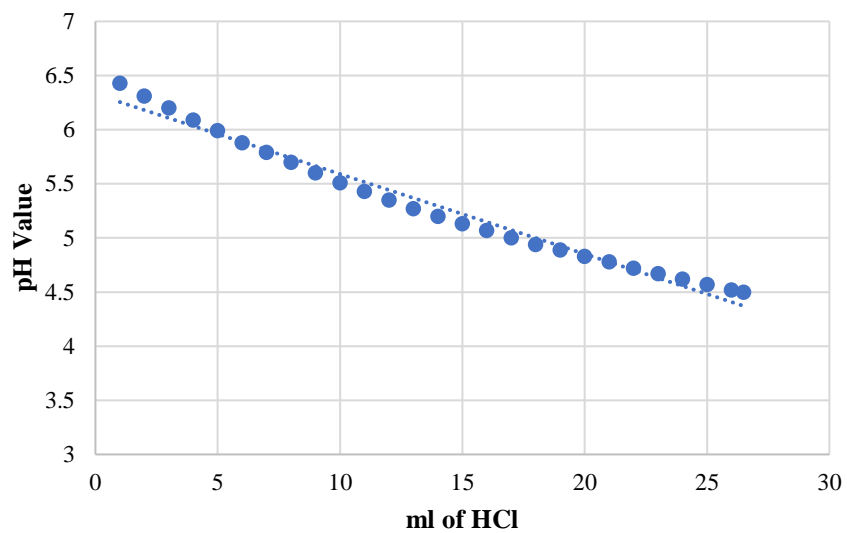


(3c) Buffer Capacity of MRS

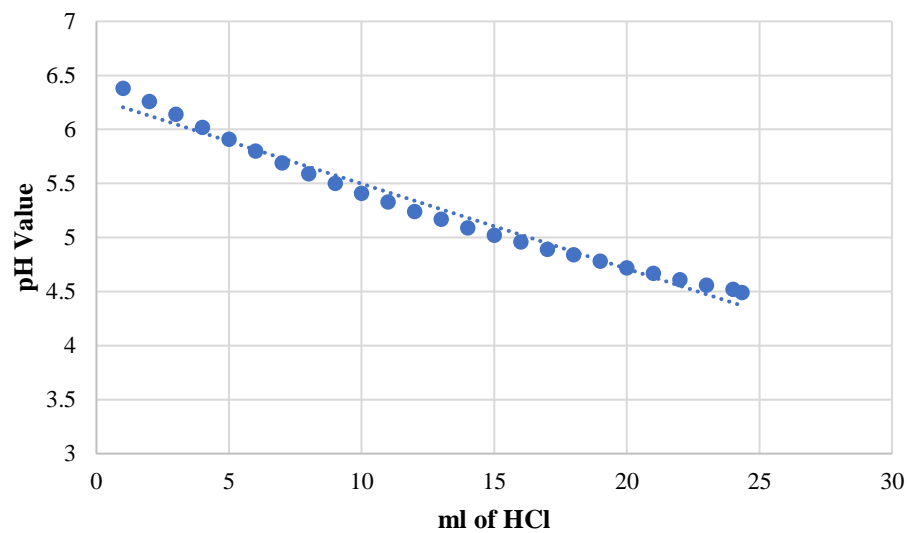


Supplementary Figures S4a, S4b, S4c and S4d (Arg: Arginine):

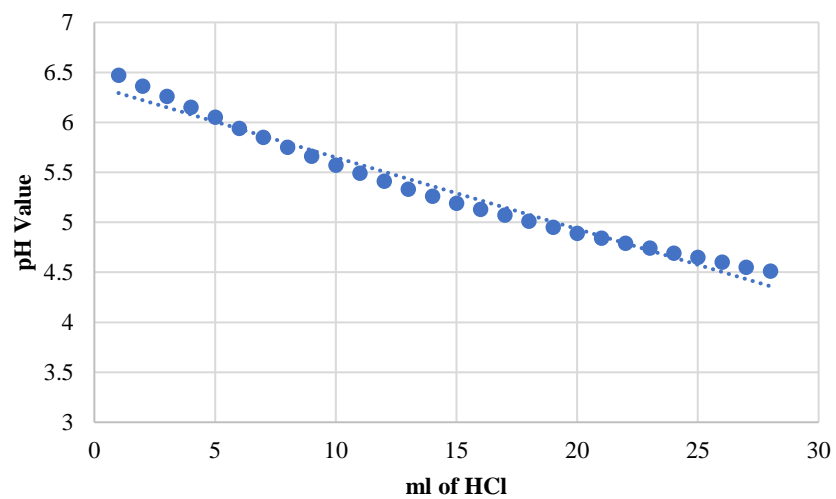
(S4a) Buffer Capacity of C-Max 5%



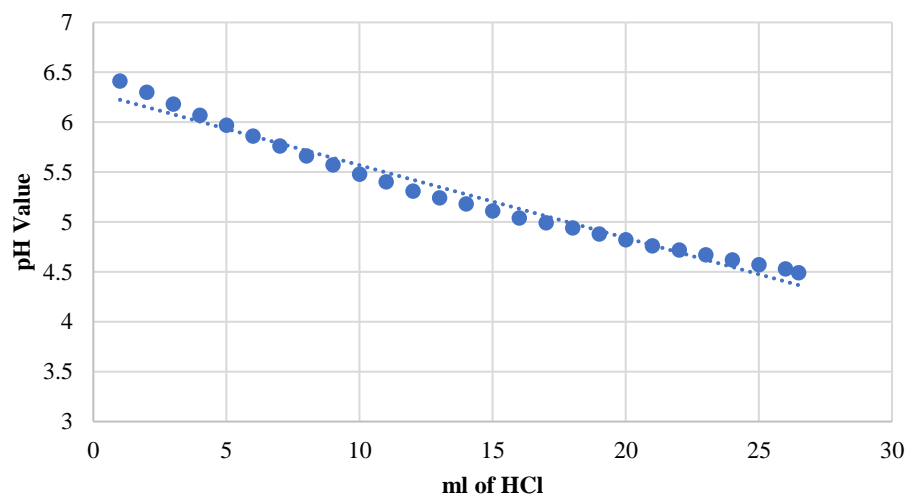
(S4b) Buffer Capacity of C-Max 5% - Arg



(S4c) Buffer Capacity of C-Max -10%

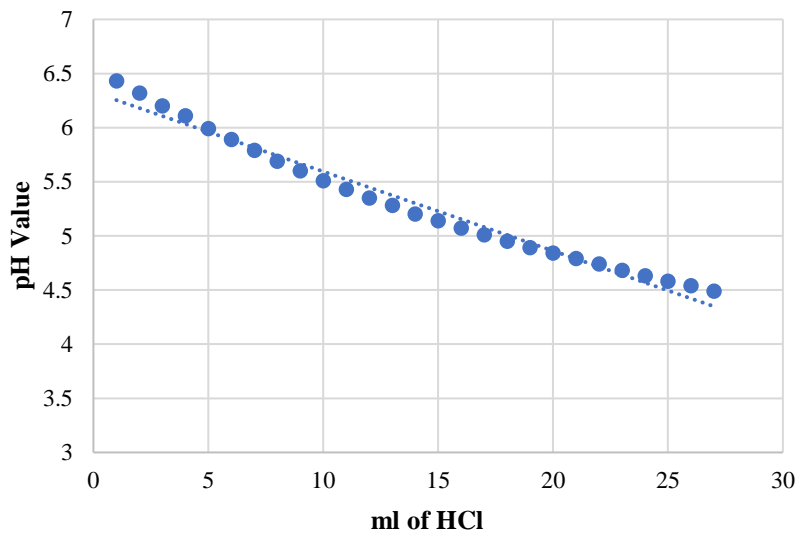


(S4d) Buffer Capacity of C-Max 10% - Arg

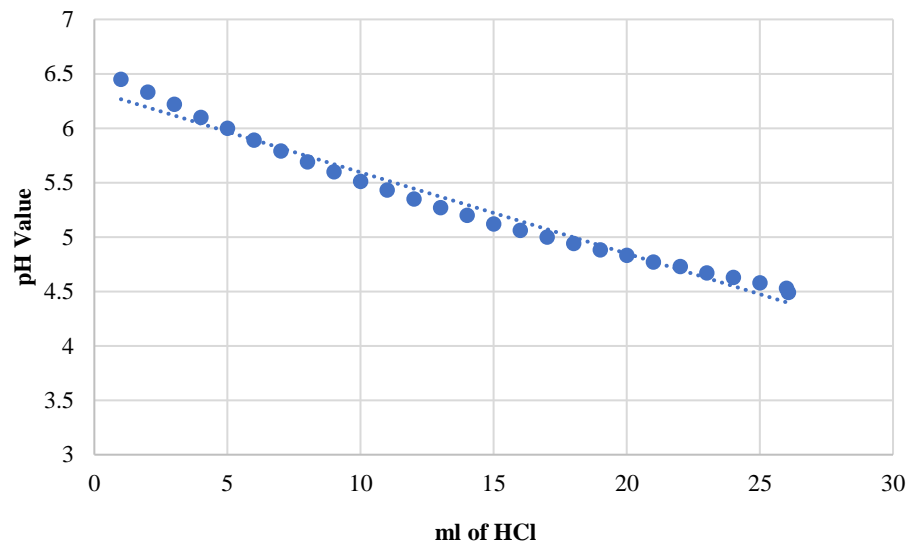


Supplementary Figures S5a, S5b, S5c and S5d (Arg: Arginine):

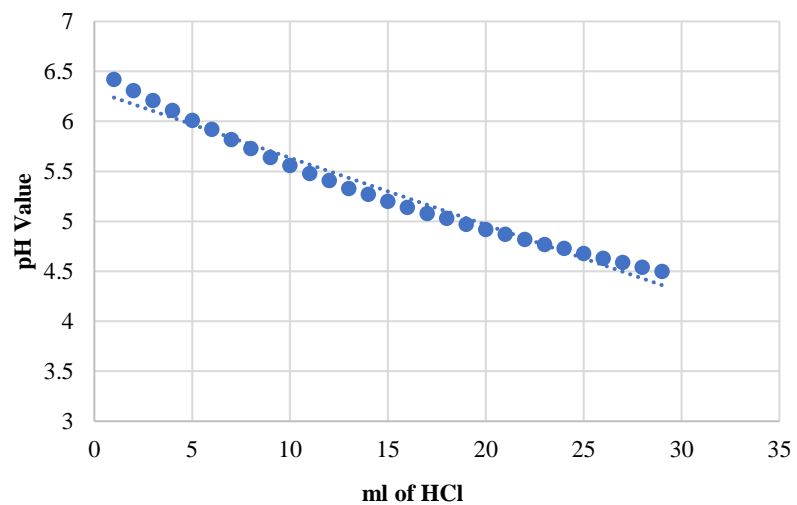
(S5a) Buffer Capacity of KAT 5% only



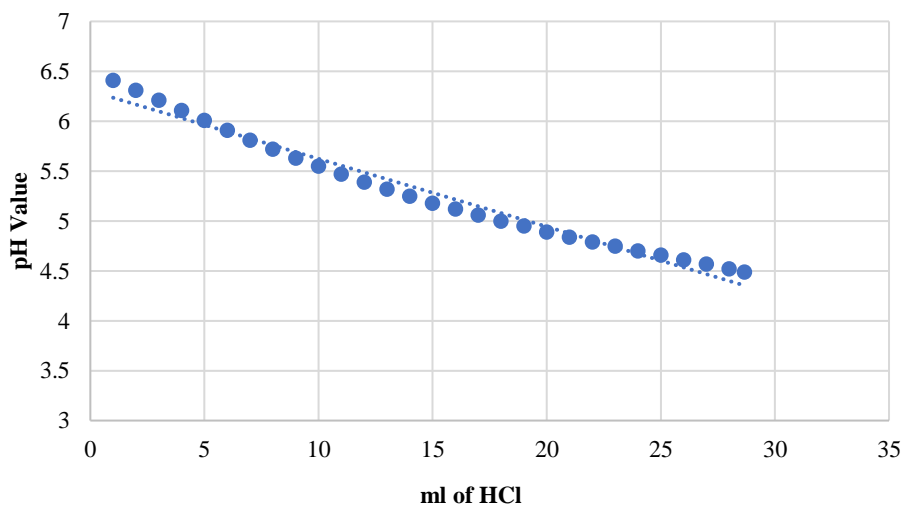
(S5b) Buffer Capacity of KAT 5% - Arg



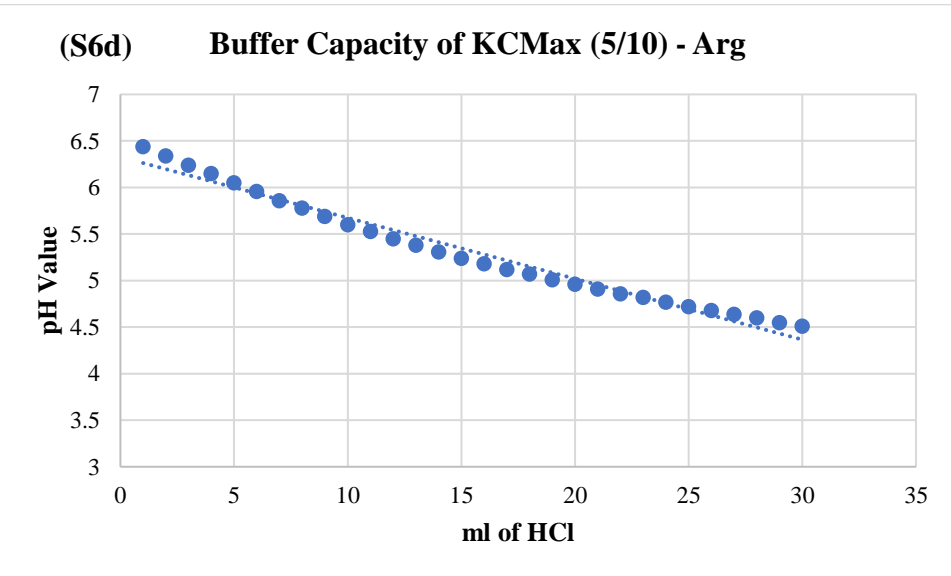
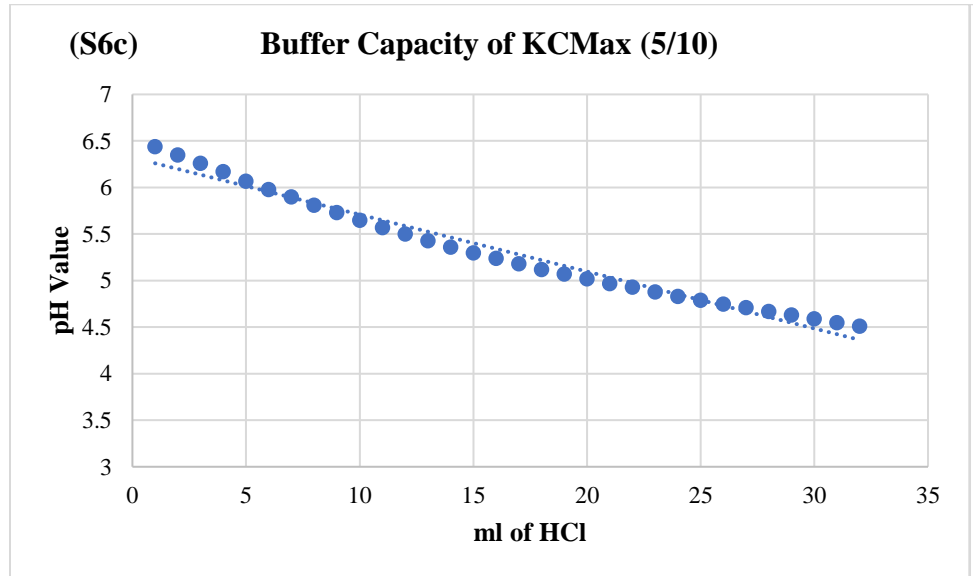
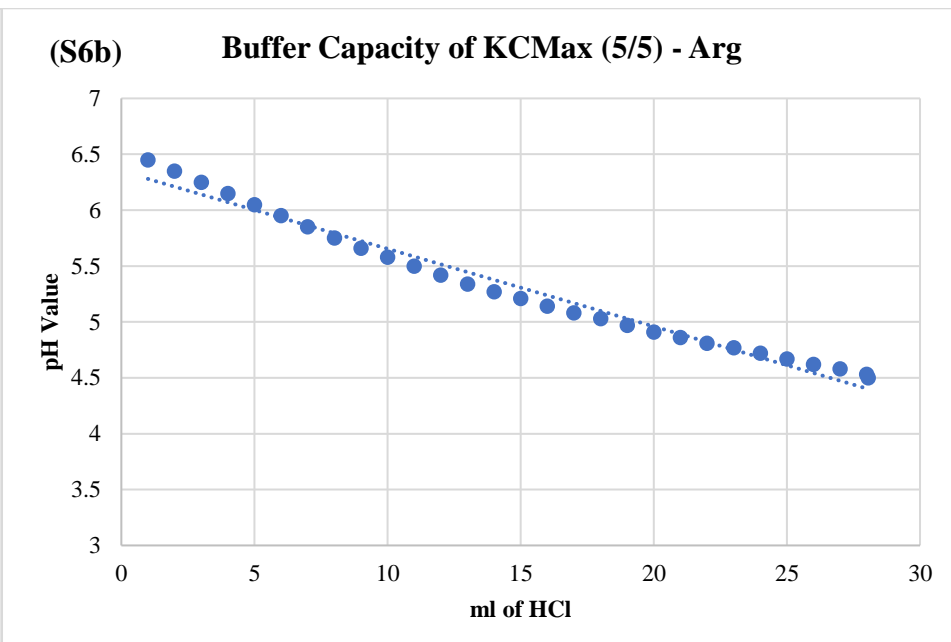
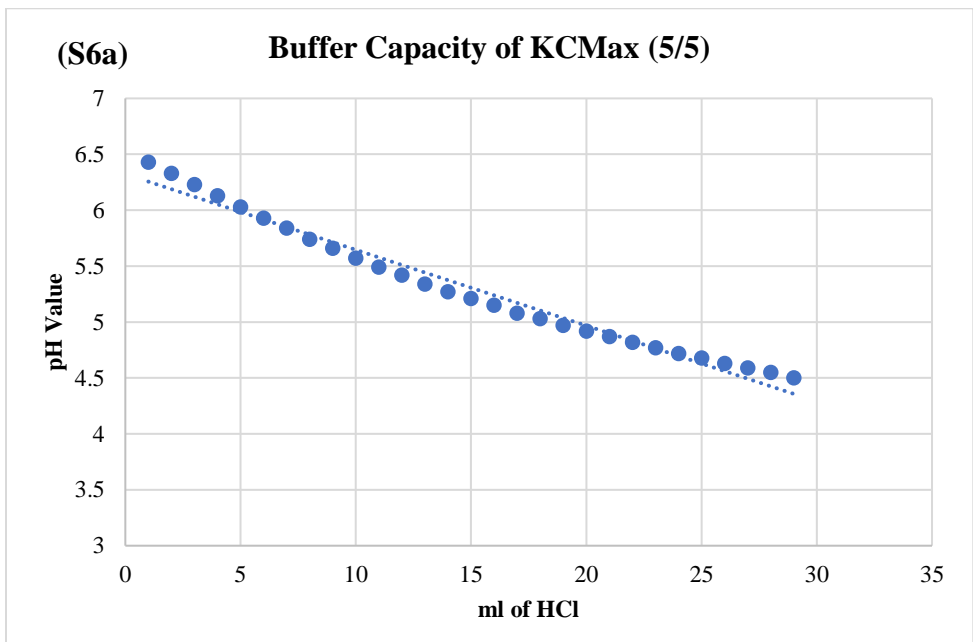
(S5c) Buffer Capacity of KAT 10%



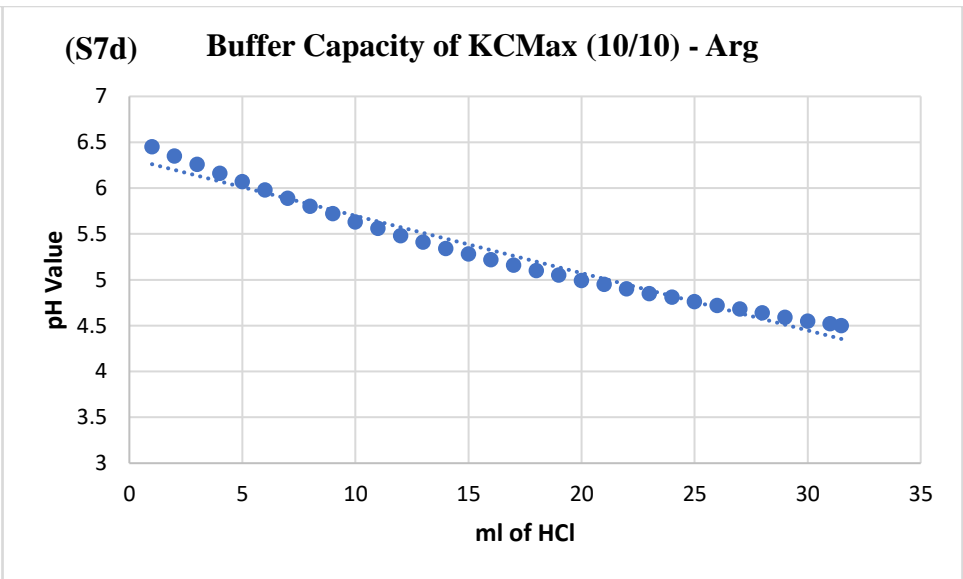
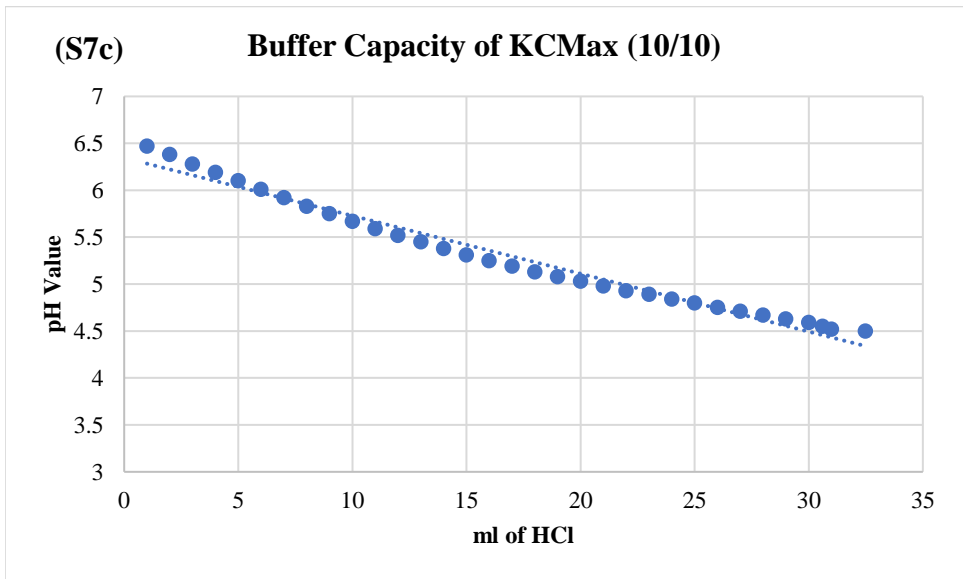
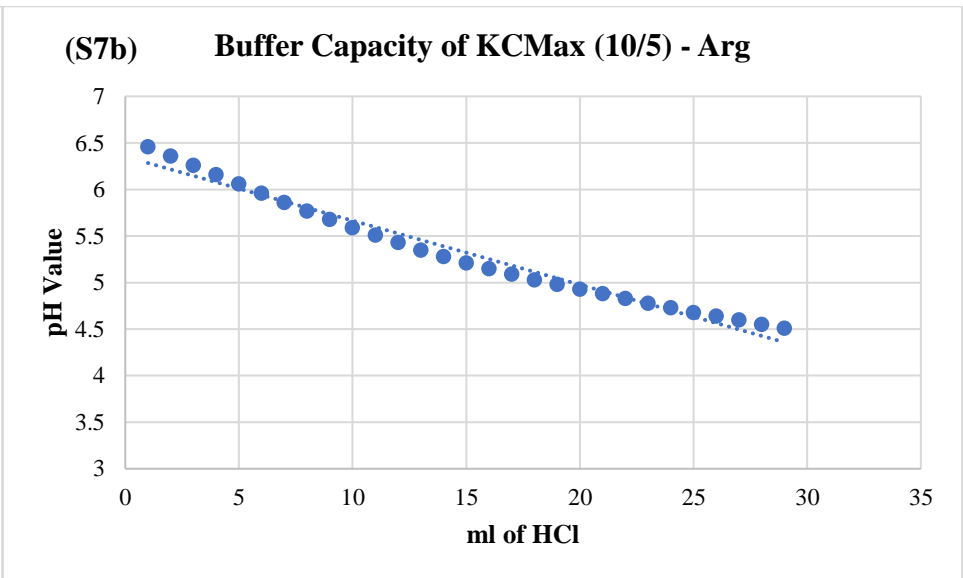
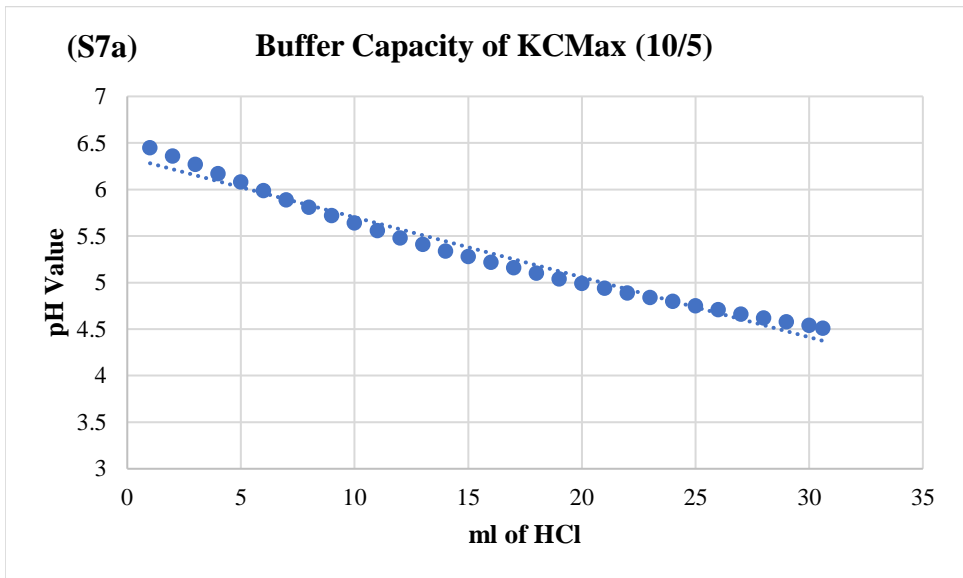
(S5d) Buffer Capacity of KAT 10% - Arg



Supplementary Figures S6a, S6b, S6c, and S6d (Arg: Arginine):

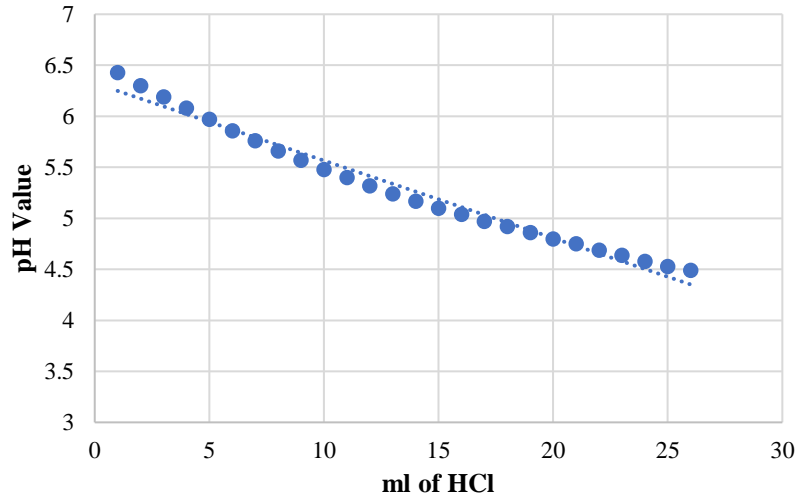


Supplementary Figures S7a, S7b, S7c, and S7d (Arg: Arginine):

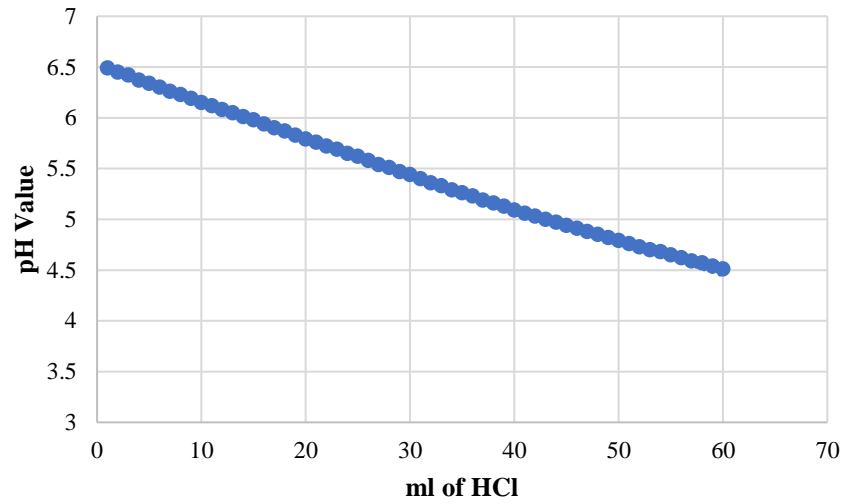


Supplementary Figures S8a, S8b, and S8c (His: Histidine):

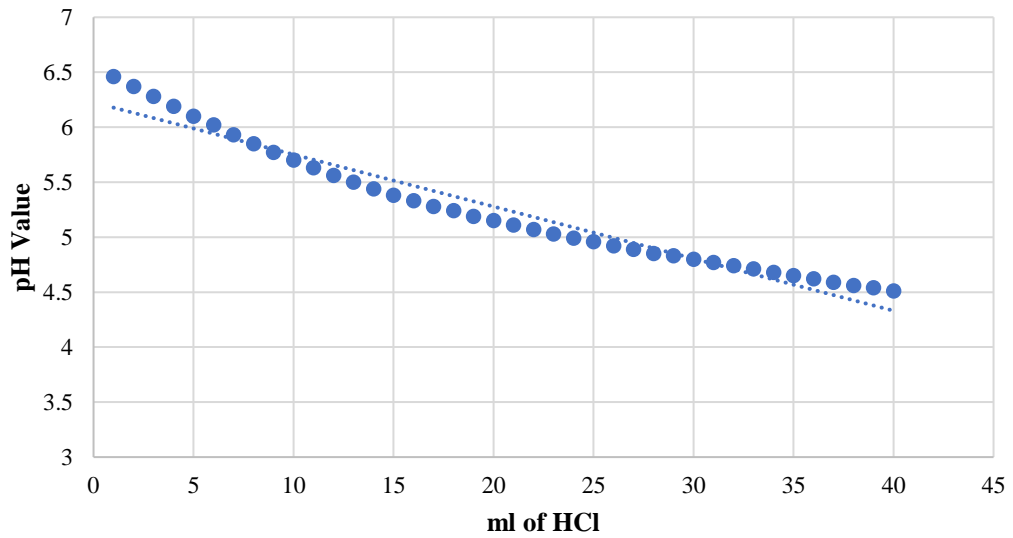
(S8a) Buffer Capacity of N-Max only



(S8b) Buffer Capacity of N-Max-His only

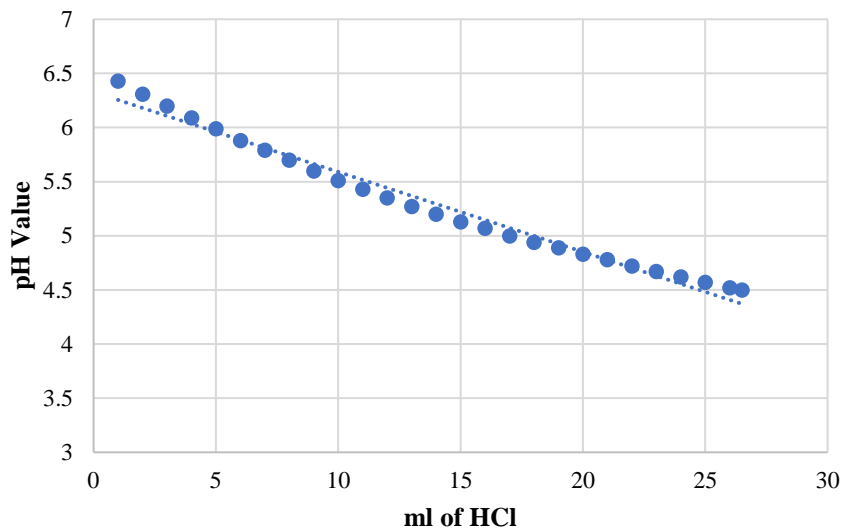


(S8c) Buffer Capacity of MRS

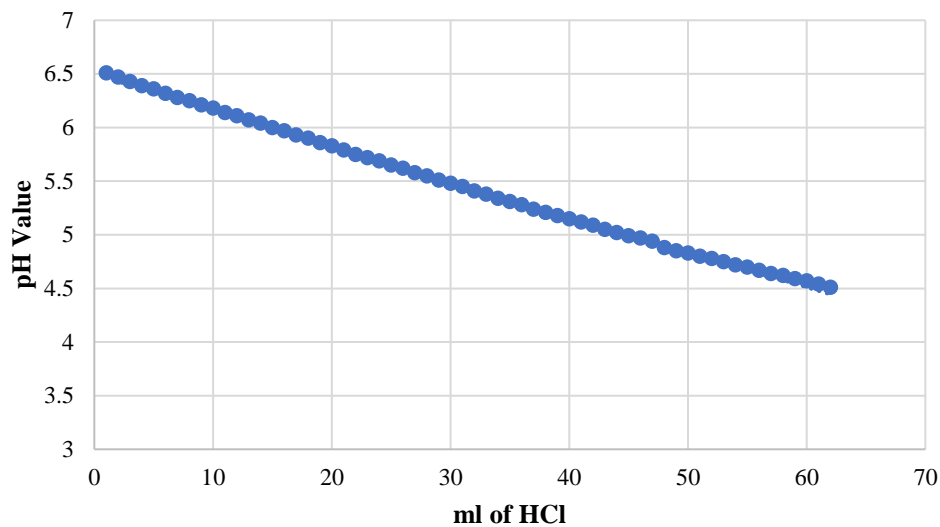


Supplementary Figures S9a, S9b, S9c, and S9d (His: Histidine):

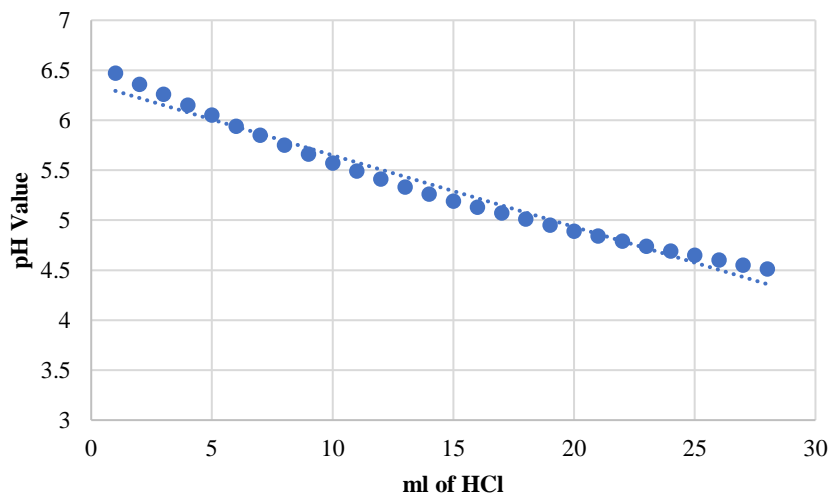
(S9a) Buffer Capacity of C-Max 5%



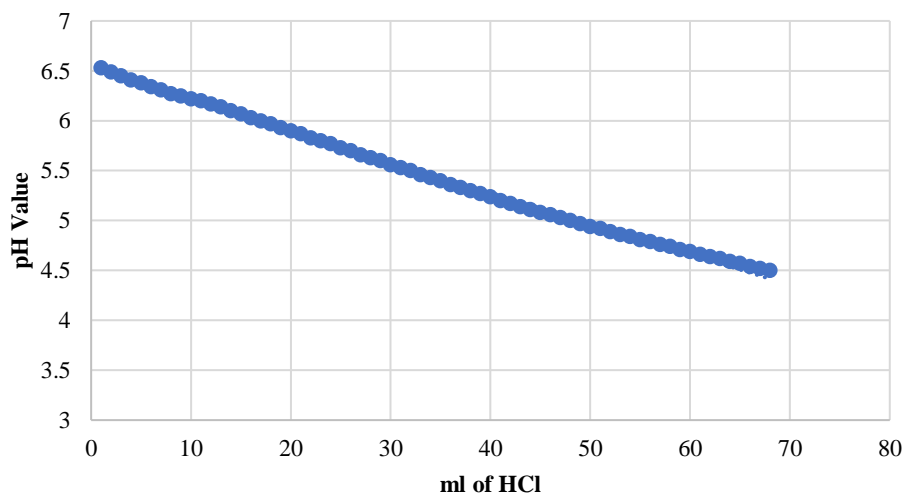
(S9b) Buffer Capacity of C-Max 5% - His



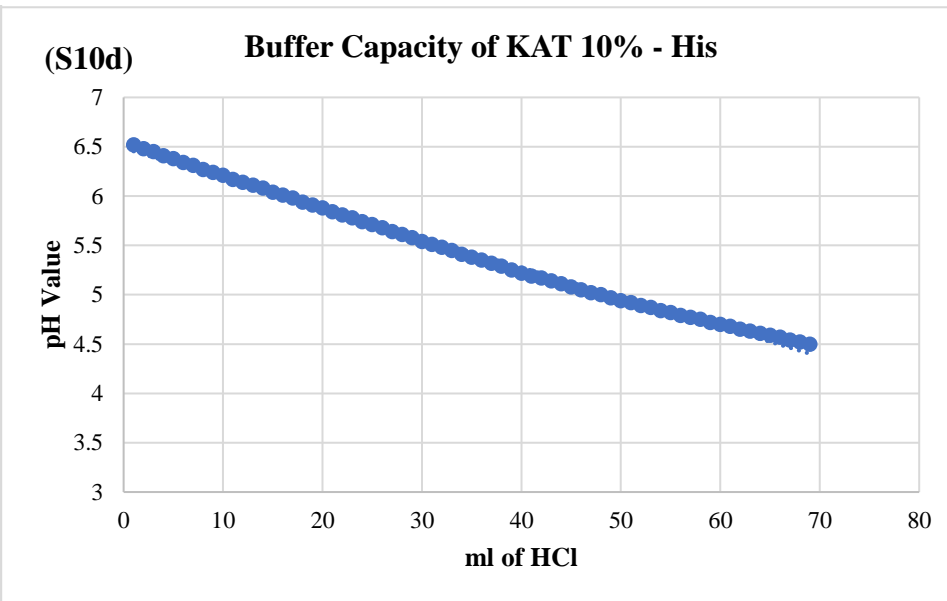
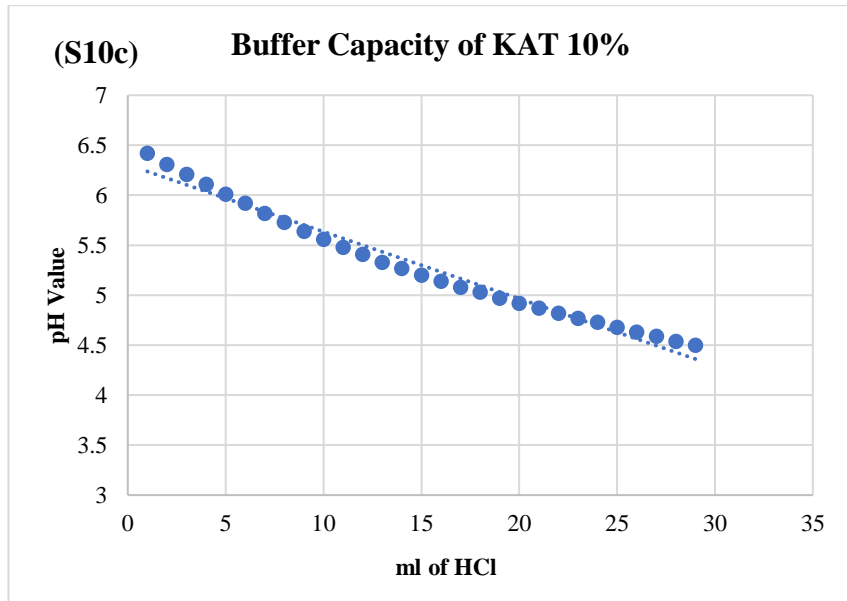
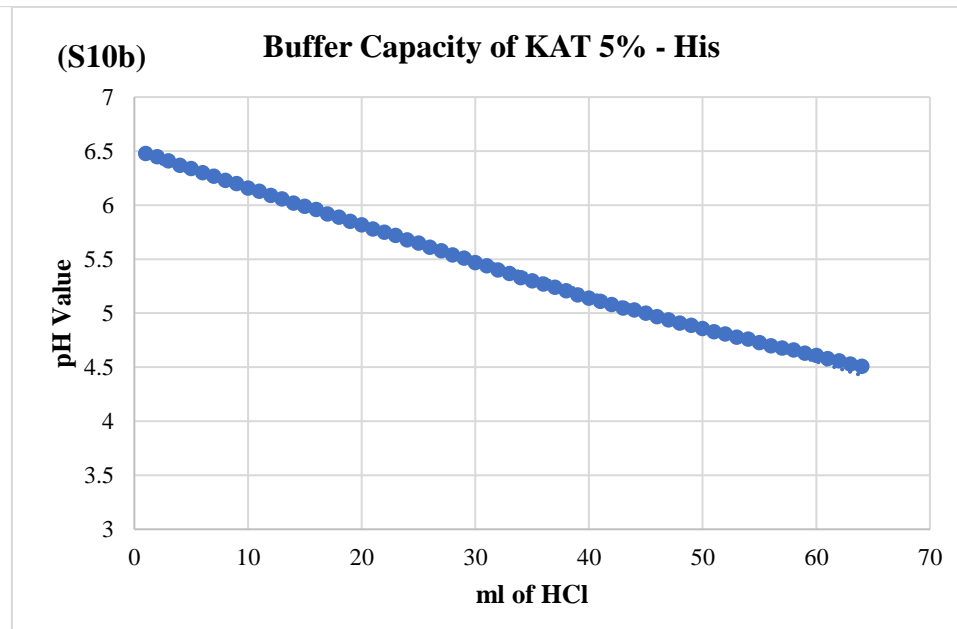
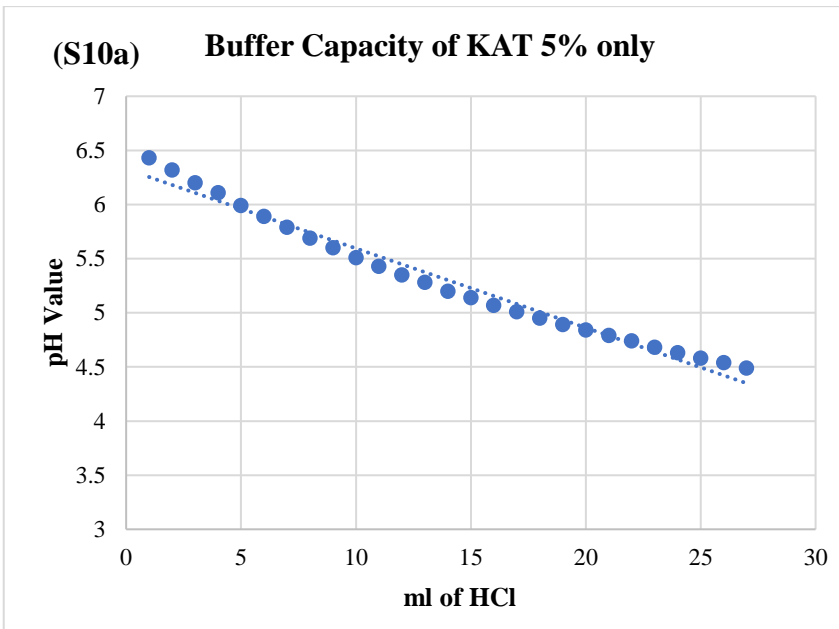
(S9c) Buffer Capacity of C-Max -10%



(S9d) Buffer Capacity of C-Max 10% - His

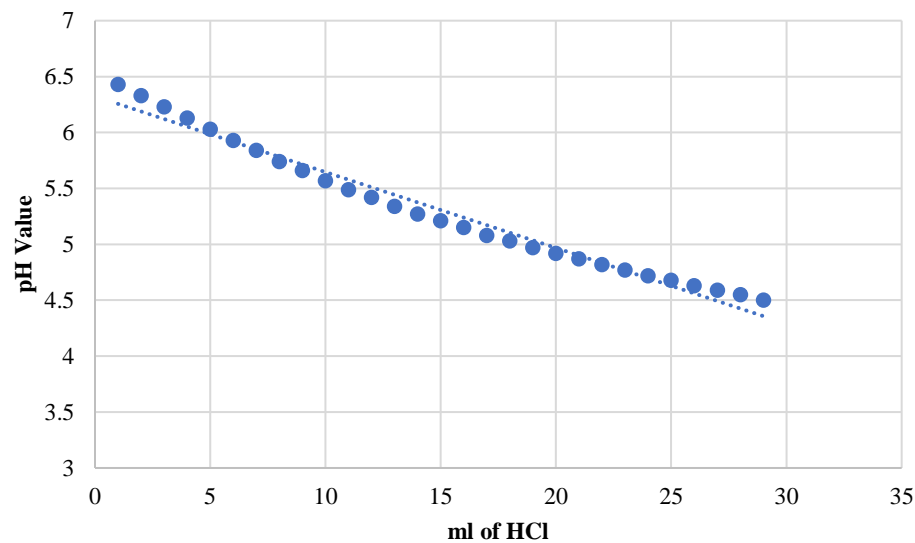


Supplementary Figures S10a, S10b, S10c, and S10d (His: Histidine):

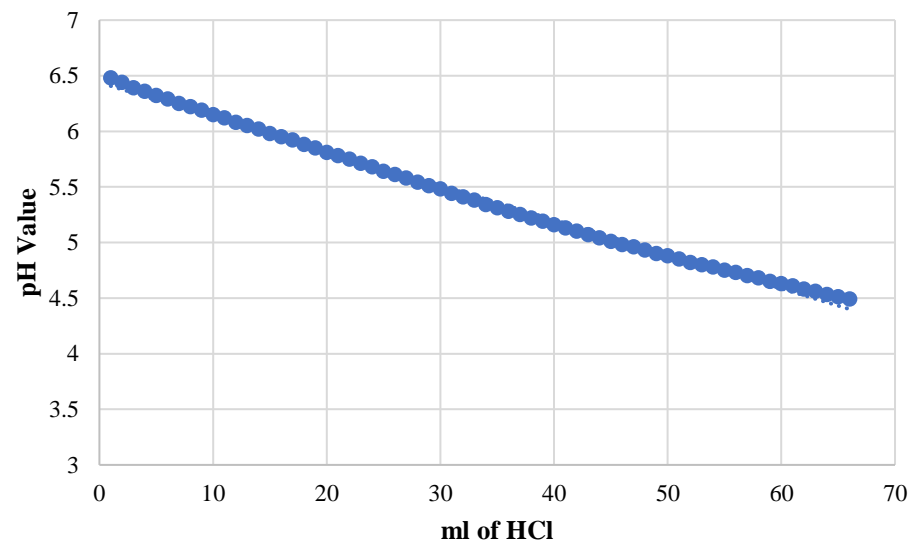


Supplementary Figures S11a, S11b, S11c, and S11d (His: Histidine):

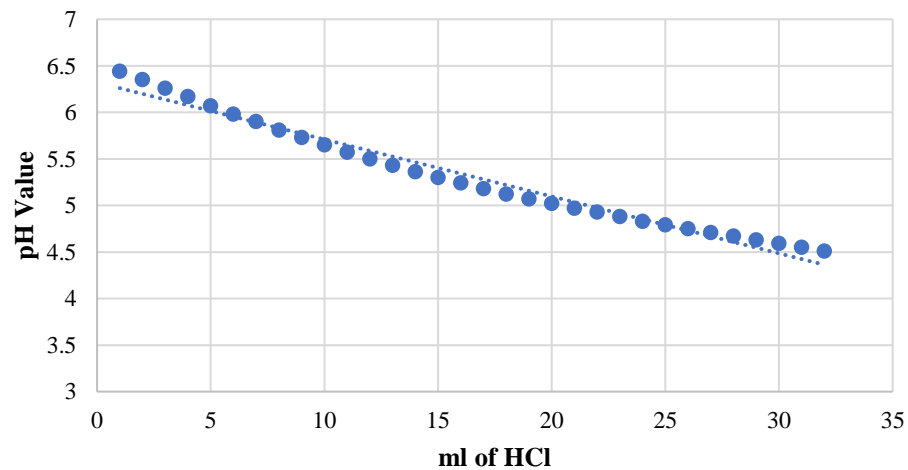
(S11a) Buffer Capacity of KCMaX (5/5)



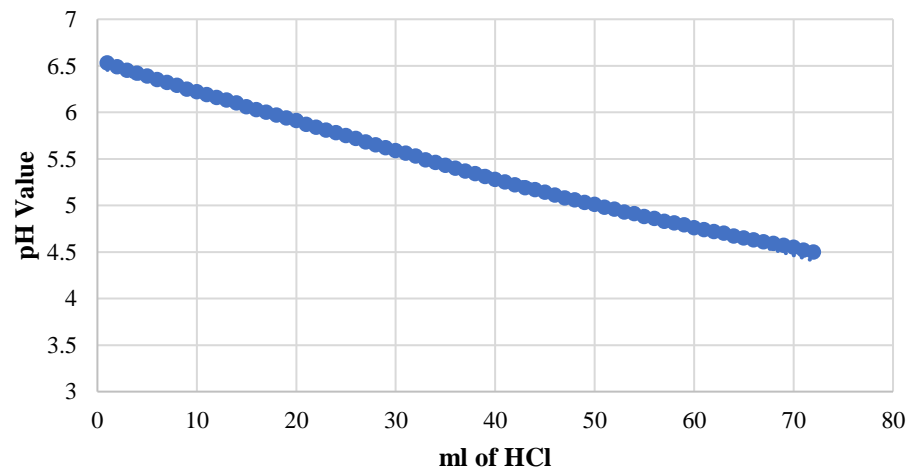
(S11b) Buffer Capacity of KCMaX (5/5) - His



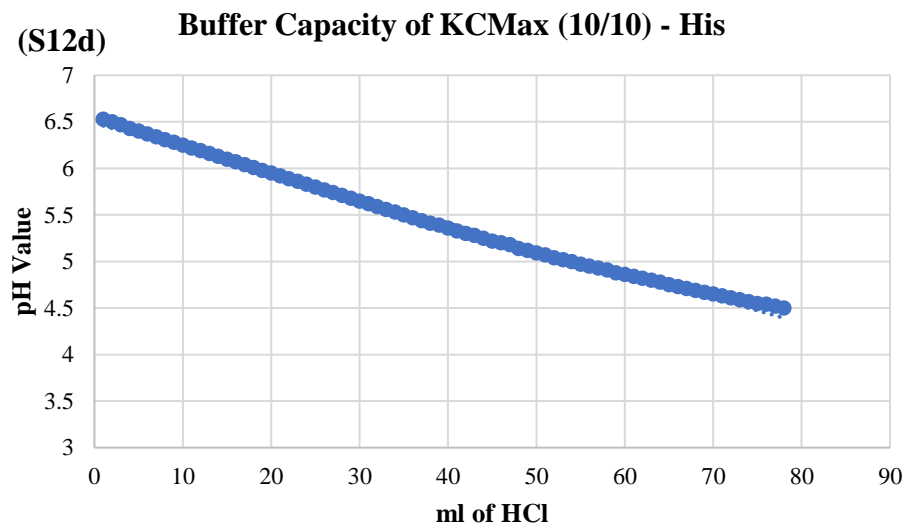
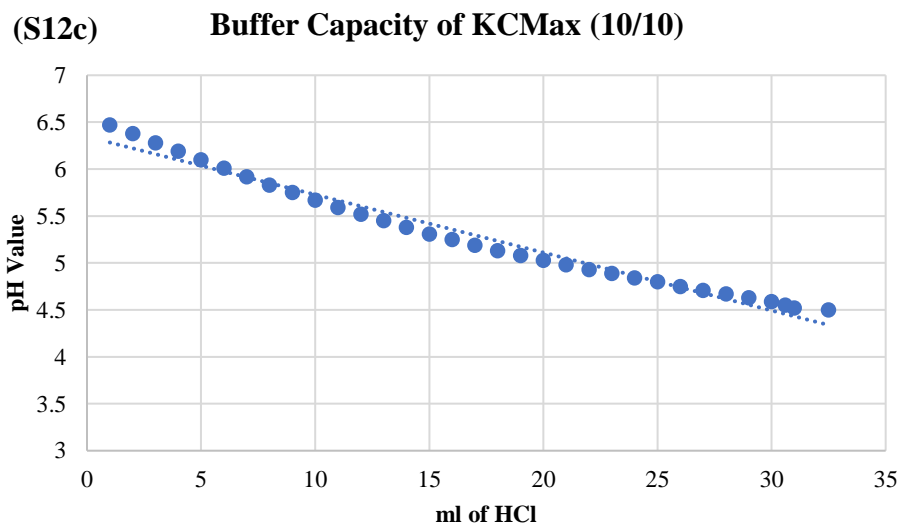
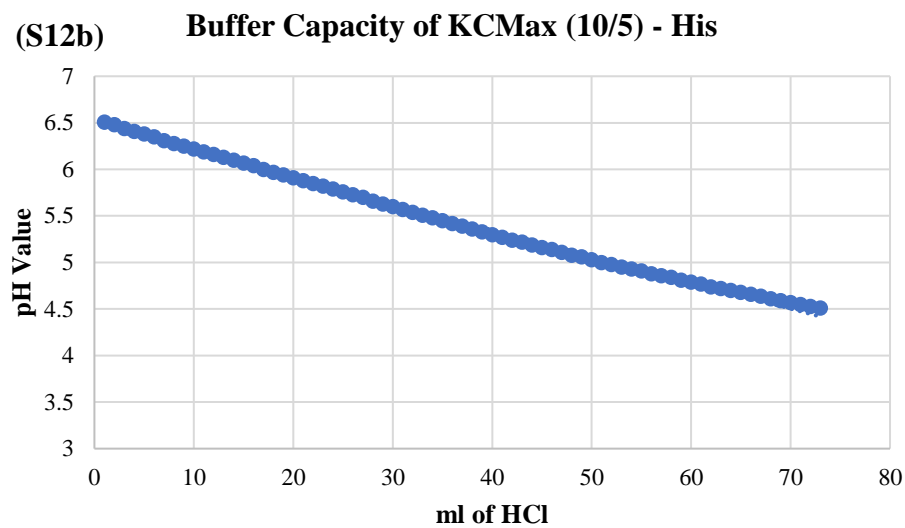
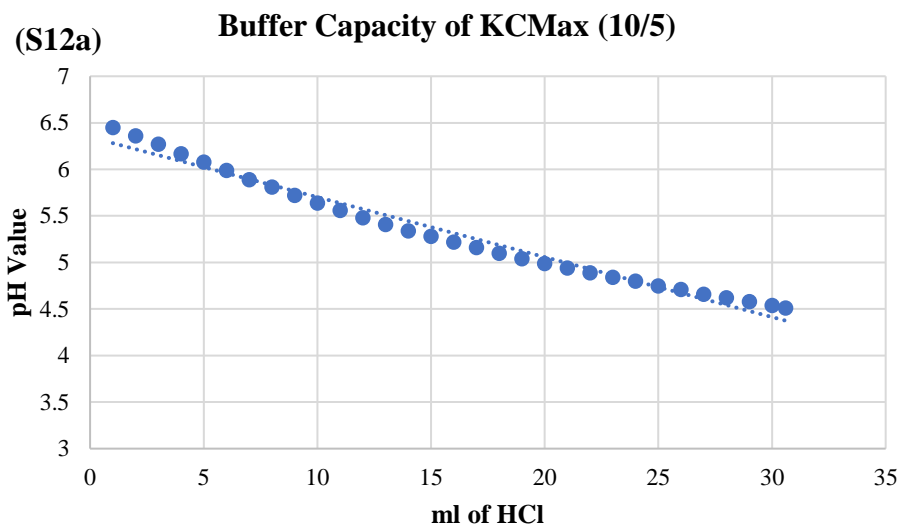
(S11c) Buffer Capacity of KCMaX (5/10)



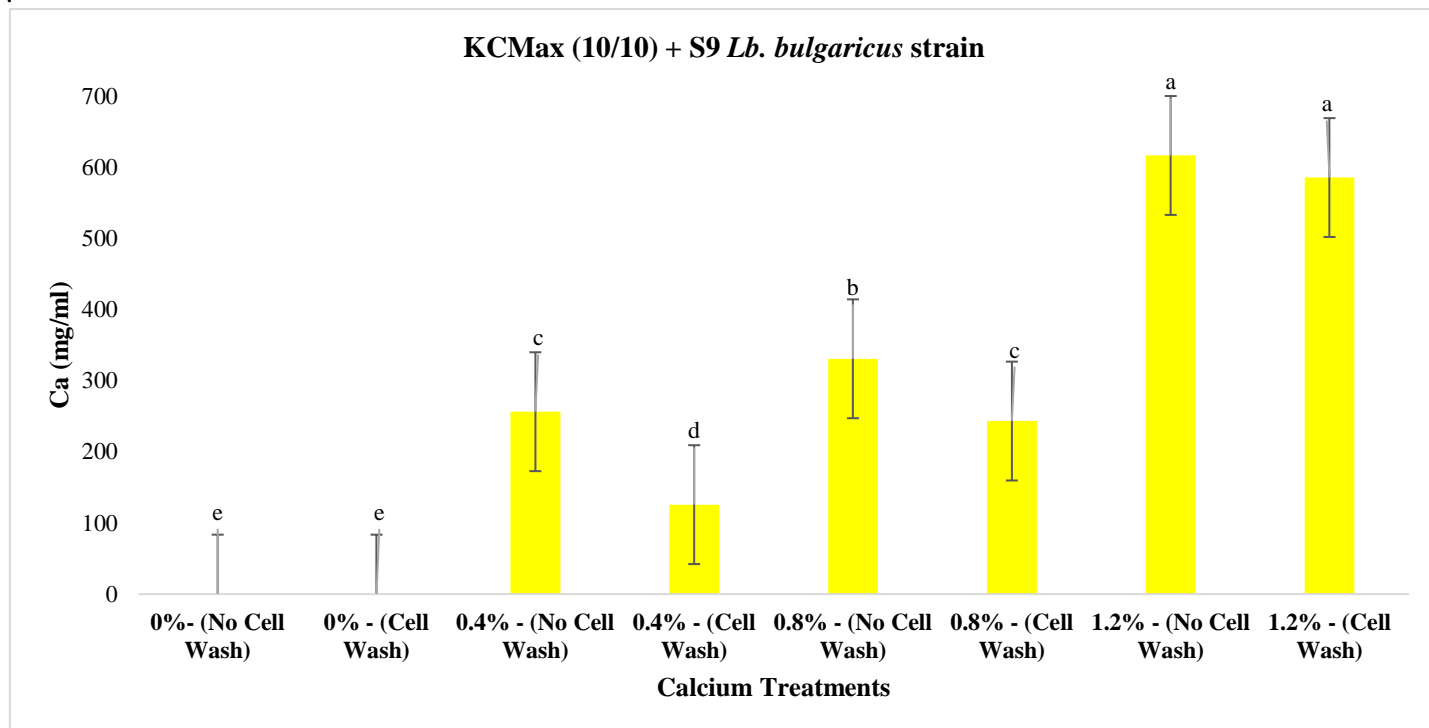
(S11d) Buffer Capacity of KCMaX (5/10) - His



Supplementary Figures S12a, S12b, S12c, and S12d (His: Histidine):



Supplementary Figure S13:



Supplementary Figure S14:

