Investigating the mass of Soya Bean Kazein which has the same content of protein with 2,7g Tryptose Phosphate Broth

Extended Essay (Biology)

Supervisor: Ümit Yaşatürk

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Candidate Name: Göksu Cengiz

Candidate number: D1129018

ABSTRACT

Cell cultures are used rather frequently due to its being economic, easy to control and allowing industrial products to yield. The most common area of usage of cell culture is the vaccine production and vaccine of Foot and Mouth Disease is the one of the firsts in this group. The method of vaccine production for the Foot and Mouth Disease is using suspensionof BHK-21(Baby Hamster Kidney) cells with the Glasgow Media. In this media there are two main elements, the cells and the nutrients for the source of energy. As a sorce of nutrient 2,7g Tryptose Phosphate Broth (TPB) is used per liter of a Glasgow media. However TPB is expensive and have some contaminant affects so another vegetal pepton Soya Bean Kazein(TSB) is tried instead of TPB in this experiment. Therefore, this study is searching an answer to the question : “How much Soya bean kazein should be used per liter in order to obtain the same absorbance value on the spectrophotometer with 2.7 g/l tryptose phosphate broth sample which is the standart value used in Glasgow media as an energy source for growth of cells in the vacines of Footh an Mouth Diseases Virus?.”

By using Lowry method and a spectrophotometer the mass of protein in 2,7 g TPB and 0,5-1,0-1,5-2,0-2,5 and 3,0 g TSB was recorded. It was found that 3,0 g TSB can be used in one liter of Glasgow media instead of 2,7 g TPB.

CONTENTS

Introduction

I was first run into the topic of this extended essay when I was helping my mother with her presentation about Foot and Mouth Disease. Viruses of the genus *Aphthovirus* causes the disease, foot and mouth disease, which is a major world problem. Foot and mouth disease often causes extensive epidemics in domestic cattle and swine. Sheep and many species of wildlife are also susceptible. Mortality is low but morbidity is high. In dairy cattles, the febrile disease resulted in the loss of milk production. Today, many countries have either eliminated foot and mouth disease by compulsary slaughter of infected animals or greatly reduced its incidence by extensive vaccination program. Foot and mouth disease virus has seven serotype. (A, O, C, SAT 1, SAT 2, SAT 3, ASIA 1) (Fenner, 408-409) After incubation period of 2-8 days, there is fever, loss of appetite, depression, and a marked drop in milk production. Within 24 hours, drooling of saliva commences, and vesicles develop on the tongue and gums. Vesicles may also be found in the interdigital skin and coronary band of the feet and on the teats. The vesicles soon rupture, producing large denuded ulcerative lesions. In calves up to 6 month of age, foot and mouth disease virus can cause death through myocarditis.(Fenner, 408-409) It was about how to control the disease with vaccination to decrease the fatal effect of the disease on farm animals. (Fenner, 408-409)

I was interested, and searched for detailed information. The influence of the disease was shocking. Due to Foot and Mouth Disease besides the animal deaths each year Turkey loses 89 million dollars due to decrease in efficiency of farm animals.(www.sap.gov.tr)In order to abolish these effects vaccination is absolute solution. For instance, in Argentina Foot and Mouth Disease is controlled and 350 milliondolar damage is blocked by vaccination.(www.wharton,universa,net/index,cfm)

However, as the disease outspread rapidly so large number of vaccine should be produced in short time because of that cell media are used such as Glasgow media with BHK_{21} (Baby Hamster Kidney 21) cells. The media should creat an environment that will lead the cells to survive and grow in variying sizes. In the process of designing a media, the most important factor is that the media should provide the minimum sufficient conditions for the cell and to be avaliable for the cell

type. Chemicals are used in a media as a source of energy for growth, metabolism, moving and protein synthesis. Glucose and glutamin are used as a source of carbon (C), animall cells need ions such as Na^{+}, K^{+},Mg^{+2}, $(SO_4)^{-2}$,$(PO_4)^{-2}$,$(HCO_3)^{-}$,and Cl^{-} for their basic function like osmotic pressure, membrane potential and cofactor for enzymes. Media must have an osmotic pressure between 0,270 and 0,370mOsmol/kg. (Freshney,I. 92) Media must also contain the esential amino acid and vitamin.The percentage of glucose and glutamin in the media should be minimized. Because they cause production of excess amount of ammonia (NH_3) and carestricts the process of cell growth. (Freshney,I. 94) Therefore as a souce of energy, peptons are used. The most abundant pepton is trypthose phosphate broth and lactalbumin hydrolysate. Both of them have disadvantages such as being animal products, hard to be obtained and high costs. Also they can contain enzymatic and bacterial contaminants, TSE (transmissible spongiophorm encephalites) which is the cause of the mad cow. (Yılmaz,Ş.) Although using Glasgow media increases the number of vaccines produced during a constant time period, there are several ways to increase the rate of growth cells and decrease the cost of the vaccine. For example Glasgov media contains adult bovine sera. Sera fills up the absent nutrients in a media. Its ingredients are not well defined yet but it increases the gowth of cell. However, it has several disadvantages such as a high financial cost, can not be obtained in a standart quality also it implies bacterial, viral and fungal contaminants.(Gümüşderelioğlu, M.)

In the media that contain peptons instead of sera the percentage should be %1 to %3 (Key 1975; Kadoi,1984, Saha,1989) In the experiments of Cruz (1998) and Moreira (1995) it was observed that if the percent of sera is dropped below %5 without any addition of other source of energy the growth of cells decrease. Therefore, I decided to serch the affect of pepton percent on the media. While such studies are derived there are less knowledge about how to decrease the cost while increasing the number of obtained cells by changing the composotion of Glasgow media. That is why I decided to chose a study about changing there ‘trypthose phosphate broth’ into ‘soya bean kazein’. Trypthose phosphate broth is a culture containing trypthose, glucuse, NaCl and thiaminium di chloride which is used as protein source. (Freshney,I) Protein sources give energy to the cells for growth. Soya bean kazein is also a source of protein which can correspond the need of cells.

Moreover, soya bean kazein is cheaper and can be obtained faster than trypthose phosphate broth. Thus soya bean kazein may be used in spite of tryphose phosphate broth.

However, in order to claim that the soya bean kazein can be used in spite of trypthose phosphate broth I should know the mass of protein in it relative to trypthose. There are several methods for this but the most common one is the light absorption method. That is why I chose this method. According to the Beer-Lambert Law, absorbance depends on the total quantity of the absorbing compound.(www.mikrobiyoloji.org/tr) So the mass of protein can be measured by the absorbance value by the help of a spectrophotometer. However protein alone in a solution can not give an estimable result so in order to obtain a absorbance value which can be read by a spectrophotometer , there are several methods like Lowry method(Peterson,G,L). In Lowry method there are two major reactions. First one is between Cu^{+2} (found in used solution) and the peptide bonds of the protein which leads a tetradentate Cu^{+2} complex ion. This complex make up the second reaction with one of the reagents in the solution which will give a blue colour. The absorbance value of the blue colour can then be estimated by a spectrophotometer. Then the mass of protein (in milligrams) can be obtained from the standart absorbance-protein chart. (Öztürk, N)

All in all, by comparing the protein value of different masses of the soya bean kazein per liter and the standart mass of tryptose (2.7g/l) I can conclude if soya bean kazein can be used in spite of tryptose as both are the protein structured energy sources in the Glasgow media for the cells to growth.

Consequently, this paper will focus on the research question: **"How much Soya bean kazein should be used per liter in order to obtain the same absorbance value on the spectrophotometer as 2.7 g/l tryptose phosphate broth sample which is the standart value used in Glasgow media as an energy source for growth of cells in the vacines of Footh and Mouth Diseases Virus?."** and will discuss how the experiment done was planned and performed, as well as examing the results obtained by evaluating their accuracy.

For obtaining and usuing protein assay kits, chemist Nilgün Ödural who is working in ŞAP Enstitute helped me.

Hypothesis:

BHK_{21} (Baby Hamster Kidney) cells are used in the production of vaccine for a fatal animal disease “Foot and Mouthe Dİsease”. These cells should reproduce in Glasgow media in order to reach large numbers. However they need energy source as they can not produce their own energy so 2,7g/l Tryptose Phosphate Broth is used in Glasgow media. However, obtaining Tryptose Phosphate Broth is a long and expensive process. Therefore to avoid these disadvantages using Soya Bean Kazein for energy source is more vital. As both of them are peptons they can be compared by considering their protein content by mass.

It can therefore be hypothesised that **using , 0,5 g, 1,0 g ,1,5 g , 2,0 g, 2,5 g or 3,0 g Soya Bean Kazein in spite of 2,7g Tryptose Phosphate Broth as an energy source, gives the same protein mass which is measured by a spectrophotometer, with a cheaper and shorter process.**

Method Development and Planing

Designing an appropriate method and deciding the suitable variables for supporting or rejecting the hypothesis was not easy. One of them was chosing the protein source instead of Trypthose Phosphate Broth. There were several alternatives for it such as Lactalbumin Hydrolysate. Lactalbumin hydrolysate is a pepton which is produced due to a reaction of lactalbumin and milk proteins under catalisation of pancreatic enzyme as it has high percent of esential amino acites but soya bean kazein was chosen as it is vegetal. (Freshney,I. 95-116)

Secondly, in order to compare these two sources, protein contents are used but obtaining quantitave data was a problem. To solve this Lowry Protein Assay method was used with a spectrophotometer . The procedure involves reaction of protein with cupric sulphate and tartrate in a alkaline solution, resulting in formation of tetradentate cupper- protein complexes. When the folin-ciocalteu reagent is added, it is effectively reduced in proportion to these chrlated cupper complexes producing a water-soluble product whose blue color can be measured at 750nm. (Pierce Biotechnology) Furthermore, 2,7 g/l sample of trypthose phosphate broth was standart. (FMD Protocol) but the gram that should be used for soya bean kazein was not known. It was estimated that the value would be close to the trypthose phosphate broth and it may be less then that, so the values was chosen as 3.0, 2.5, 2.0 , 1.5 , 1.0 and 0.5 g/l. The values were chosen decreasingly so that soya bean kazein would be more preferable as it would be cheaper and matter loss would be minimised .

Before and during the experiment there were several varibles that should be controlled. The features of water and light were the most outcoming ones. The water that was used for preparing the TPB (Trypthose phosphate broth) and soya bean casein (Tryptic soya broth, TSB) solutions were deiyonised in order to prevent any interfering substances coming from water. For every group (2,7 gr TPB; 0,5-1-1,5-2-2,5-3 gr TSB) one liter of deiyonized water was used which is the standart volume for a media. The other characteristics of water that may affect the proteins were pH,conductivity and temprature. (Freshney,I. 41) pH of water was 5,30 which was recorded with a pHmeter and conductivity was 0,40µS/cm(microsimens/centimeter) which was olso recorded with a conductivitymeter. While preparing the media

measuring the conductivity of water is an important step which can not be ommited. Because the ions in the water can produced a compound or give rection with the components of cell media which will change the composition. (Pierce Biotechnology) Temprature was constant all throughthe experiment at 23°C. Because the same rooms is used with airconditioner, so the temperature of water was also 23 °C. Another variable that can affect the protein mass was time. During the pretreatment and Lowry methood instructions were followed which will be explained later. Therefore the time intervals after centrifuge or the time interval inwhich the groups were kept in dark (30 minutes for each) was constant and stated in method. Keeping the groups in dark for 30 minutes was also controlled the light variable because folin reagent corrupts under ligth. (Pierce Biotechnology) Despite the fact that it isn't known much about the affect of the pressure, it was controlled during the experiment as the groups kept in the same place. Moreover all the apparatus, especially the pipettes were same for all groups and trials so that the error coming from the apparatus is stabilized for all groups. Furthermore, following the process resulted badly. Before starting the test tube procedure of Lowry Protein Reagent, kit, which will be explained in appendix-1, the groups were pretreated with Compat-Able Protein Assay Preparation Reagent Set in order to prevent any interfering substance that can change the result. However, this resukted on the contrary and the reagent that was put for preventing interfering, interfere the solution. Although the spectrophotometer can analyse the colour, no colour change can be observed also all of the results were same (see appendix-2) So it become necessary to repeat the experiment. This time pre-treatment was avoided because it was written in the instructions of Compat-Able Protein Assay Preparation Reagent set that since every protein assay method differs with respect to which substances interfere, it may be possible that the usage of pre-treatment may be damaging.

METHOD

MATERİALS

- 8,1 g TPB
- 22,5 g TSB
- Electronic balance (±0,01g)
- 1 L Graduated cylinder (±10 ml)
- Centrifuge(MPW, Medical instruments, High Speed Brushless,350R)
- Spectrophotometer (Bosh and Lomb, Spectronic 2000)
- Vortex mixer (Elektromag, 12V-500mA)
- Disposable tubes and mikrofige tubes
- Scorex authomatic pippettes(2-20µl, 20-200µl, 100-1000µl)
- pHmeter
- Conductivitymeter

2,7gTPB and 0,5-1,0-1,5-2,0-2,5-3,0g Soyabean kazein were measured by electronic scale.All were added 1L deionised water to get a protein solution which is enough for preapering a media.By pHmeter, thermometer and conductivitymeter it was recorded that pH was 5,30,temprature was 23°C and conductivity was 0,40µS/cm for deionised water. For the first experiment groups were pre-treated according to the Compat-Able Protein Assay Preparation method. 100µl of each group were put into a test tube with otomotic pipettes. 500µl Compat-Able Protein Assay Preaperation Reagent 1 was added into each tube and mixed after stand at room temprature for five minutes.Then 500µl reagent 2 was added and mixed after centrifuged at 10000g for ten minutes.After that each tube was inverted and supernatant was removed. Thin layer of protein pellet can be seen on the walls of test tube. In order to dissolve the pellet 100µl of deionised water was added into each tube but all pellet can not be removed so all groups were vortexed than the walls of the test tube were scraped. After that standart procedure of Modified Lowry Protein Assay was used however

ther were no colour change and the results were same for all groups. So the experiment was repeated without pretreatment.

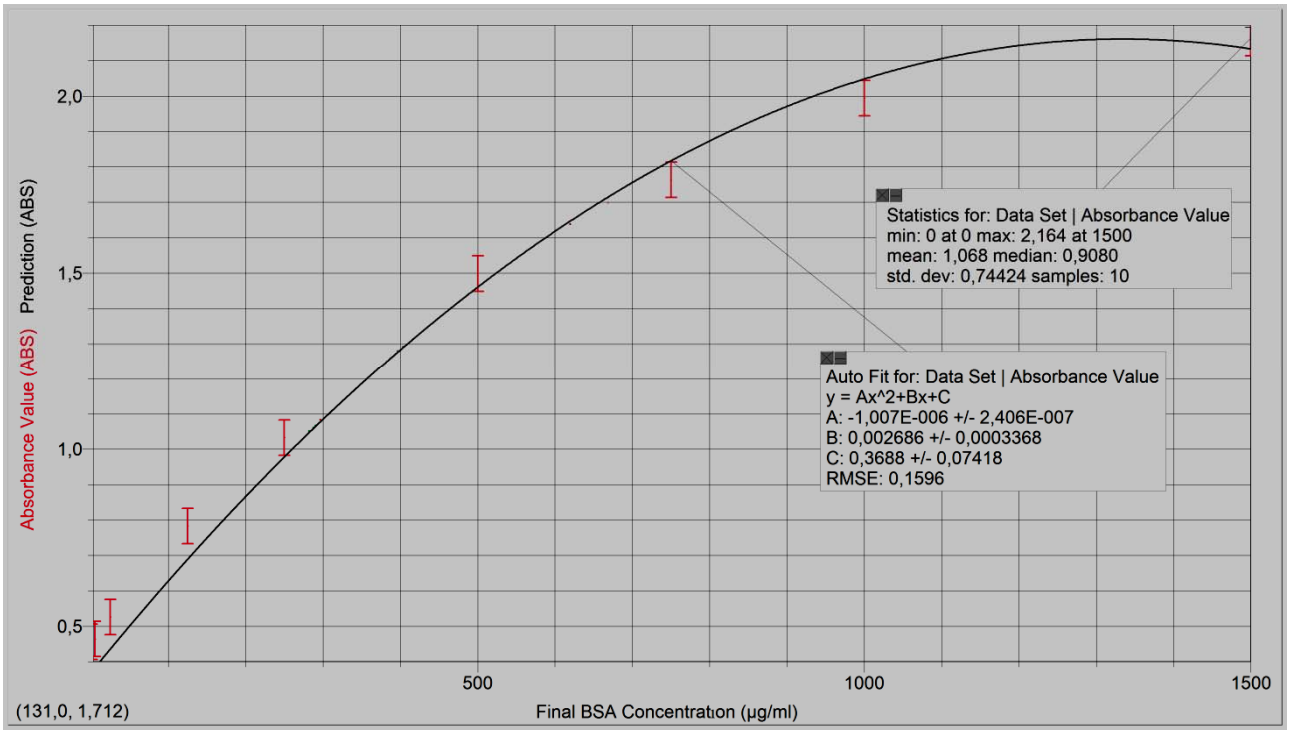
In the second experiment the groups were directly treated with Modified Lowry Protein Assay Reagent Kit method. 200µl of each group is replicated into test tube with a pipette (all were labelled before). Then 1ml Modified Lowry reagent, which is alcaline-copper reagent, was added and mixed and groups were incubated exactly 10 minutes in a cupboard in which no light can pass at room temprature (23°C). Then 100µl 1X Folin –Ciocolteu reagent is added and the groups were vortexed in order to mix the contents. After that groups were incubated for 30 minutes under same condition. Then the absorbance of blue colours of all the samples caused by the Cu^{+2} ion which is coming from given reagents, were measured with spectrophotometer set to 750nm(nanometer) and zeroed with a cuvette filled only with deionised water. The whole procedure was reapeated third times in order to minimise any error made. After that in order to get standart protein absorbance graph for experiment, Modified Lowry Protein Assay Method is repeated for the groups shown in table 1 and graph - 1 is obtained in logger-Pro. By using this graph the protein mass in the 2,7gTPB and 0,5-1,0-1,5-2,0-2,5-3,0g soya bean kazein experimental groups, is recorded. Then these data is analysed first by using Anova test in Excell and then as the protein masses of 2,7g TPB and 3,0 g soya bean kazein is so close to eachother t-test is used for analysing them. All the results of these statistical analysis are qualified in conclusion.

Vial	Volume of Diluent (µl)	Volume and source of BSA (µl)	Final BSA Concentration (µg/ml)
A	250	750 of stock	1500
B	625	625 of stock	1000
C	310	310 of vial A dilution	750
D	625	625 of vial B dilution	500
E	625	625 of vial D dilution	250
F	625	625 of vial E dilution	125
G	800	200 of vial F dilution	25
H	800	200 of vial G dilution	5
I	800	200 of vial H dilution	1
J	1000	0	0

TABLE 1:Table for the necessary data for the preparation of Diluted Albumin(BSA) standards for the Graph-1. Dilution sheme.

The graph obtained from the solutions in this table is shown below.

Graph 1 : Graph of standart Absorbance value vs Final BSA (Diluted Albumin). Drawn by using Logger-Pro.



DATA SET	
BSA (µg/ml)	**Absorbance value (ABS)**
1500	2,164
1000	1,994
750	1,764
500	1,499
250	1,033
125	0,783
25	0,526
5	0,464
1	0,456

0	0

Table2: Data set for the graph which contains the absorbance values obtained from the groups shown in table-1 in order to obtain a standart protein-absorbance chart for the rest of the experiment.

RESULTS

TPB and TSB	TRIALS	Absorbance Value (ƛ-750,0nm) (ABS)	Protein amount (BSA) (µg/ml)
2,7 g TPB	1	1,64	620
	2	1,64	620
	3	1,70	668
0,5 g TSB	1	0,83	185
	2	0,84	186
	3	0,82	179
1,0 g TSB	1	1,09	302
	2	1,07	290
	3	1,08	296
1,5 g TSB	1	1,28	396
	2	1,29	404
	3	1,26	387
2,0 g TSB	1	1,47	505
	2	1,47	505
	3	1,50	524
2,5 g TSB	1	1,59	579
	2	1,59	579
	3	1,55	552
3,0 g TSB	1	1,76	699
	2	1,79	726
	3	1,73	673

TABLE 3: The results of the experiment read from the graph above. TSB=tryptose soya broth, TPB= Trypthose phosphate broth

Statistical Analysis-1

Groups	Standart Deviation	Minimum	Maximum	Mean of protein amount (BSA) (µg/ml)
2,7 g TPB	27,7	620	668	636
0,5 g TSB	3,7	179	186	183
1,0 g TSB	6,0	290	302	296
1,5 g TSB	8,5	387	404	395
2,0 g TSB	10,9	505	524	511
2,5 g TSB	15,5	552	579	570
3,0 g TSB	26,5	673	726	699

Table 4: Table for the “Standart Deviation”, “Minimum” and “Maximum” values and the “Mean” of protein amounts of 2,7gTPB and 0,5-1,0-1,5-2,0-2,5-3,0g soya bean kazein. Values are calculated between trials.

Examples of calculations:(shown for group 1-(2,7 g TPB))

Mean= $(x_1+x_2+x_3+\ldots+ x_n) / n$

$= (620+620+668) / 3 = 636$

Standart Deviation= $\sqrt{[\sum x^2 – (\sum x)^2/n)] / n}$

$= \sqrt{[(620^2 + 620^2 + 668^2) – (620+620+668) / 3] / 3}$

$= 27,7$

($\sum x^2$ means sum of value x^2 , $\sum x$ means sum of value x, n means sample size)

Statistical Analysis-2

H_0: There is no statistically significant difference between the mean of the protein mass ((BSA) (µg/ml)) of 2,7gTPB and 0,5-1,0-1,5-2,0-2,5-3,0g soya bean kazein

H_1: There is statistically significant difference between the mean of the protein mass ((BSA) (µg/ml)) of 2,7gTPB and 0,5-1,0-1,5-2,0-2,5-3,0g Soyabean kazein

ANOVA

Sourcof variance	*SS*	*df*	*MS*	*F*	*P-value*	*F-crit*
Between groups	629511,1429	6	104918,5238	375,4113137	1,22193E-14	2,847725996
In the groups	3912,666667	14	279,4761905			
sum	633423,8095	20				

Table 5: Results of Anova Test(single group) for the groups of experiment ((2,7 gr TPB; 0,5-1-1,5-2-2,5-3 gr TSB)

Result of Anova: As the P-value which is 1,222 x 10^{-14} is smaller than alpha value (0,05) it can be said that there is a statistically significant difference between groups.

Statistical Analysis-3:

H_0: There is no statistically significant difference between the protein mass ((BSA) (µg/ml)) of 2,7gTPB and 3,0g Soyabean kazein

H_1: There is statistically significant difference between the protein mass ((BSA) (µg/ml)) of 2,7gTPB and 3,0g Soyabean kazein

t-Test: Two-Smaples Assuming Unequal Variances

	2,7 g TPB	*3,0 g TSB*
mean	636	699,3333333
Variance	768	702,3333333
observations	3	3
hypothesized Mean difference	0	
df	4	
t Stat	-2,860784026	
P(T<=t) one-tail	0,022946578	
t critical one-tail	2,131846782	
P(T<=t) two-tail	0,045893155	
t critical two-tail	2,776445105	

Table 6: Results of t-Test for the groups of experiment ((2,7 gr TPB; 0,5-1-1,5-2-2,5-3 gr TSB)

Result of t-test: As the P-value is equal to the alpha value (0,05) it can be said that there is no statistically significant difference between two groups. In this result “P(T<=t) two-tail” is used and “0,045893155” taken in 3 significant figures which leads to 0,05.

Conclusion Evaluation

Aim of this experiment is to investigate the the mass of vegetal protein, soya bean kazein containing same mass of protein with the energy source of Glasgow media, Tryptose Phosphate Broth. In order to find out the protein percent, Lowry Method is used but before starting the protein analysis the solutions containing 2,7g tryptose phosphate broth, 0,5 g, 1,0 g, 1,5 g, 2,0 g, 2,5 g and 3,0 g soya bean kazein is pretreated with Pierce-pre-Treatment Reagent Set because it is thought that the solutions can contain interfering substances that may give reaction with the protein analyse kit and that will change the results. However, when protein analysis did to these groups no colour change can be observed. In spite of this, absorbance values of the groups are detected under 750nm wavelength in the spectrophotometer, but almost all the values are same. Therefore it is concluded that there is no interfering substance in the solutions and the protein analysis repeated without pre-treatment. This time the results are acceptable as there is a significant colour change. These absorbance values then turned into the mass of protein in µg/ml by using graph 1(Absorbance value vs Final BSA (Diluted Albumin)). For 2,7 g TPB, mean protein mass is 636, for 0,5 g TSB 183, for 1,0 g TSB 296, for 1,5 g TSB 395, for 2,0 g TSB 511, for 2,5 g TSB 570 and for 3,0g TSB 699 µg/ml.

The hypothesis of the experiment which indicates that one of the groups will contain the same mass of protein with the 2,7g of TPB is proved by using different statistical analysis. First of all the values of standart deviation is so small compared to the protein masses, which shows that all the trials are close to the mean value. Secondly, Anova test is done between all groups by using the trials.

H_0:There is no statistically significant difference between the protein mass((BSA) (µg/ml)) of 2,7gTPB and 0,5-1,0-1,5-2,0-2,5-3,0g soya bean kazein

H_1: There is statistically significant difference between the protein mass ((BSA) (µg/ml)) of 2,7gTPB and 0,5-1,0-1,5-2,0-2,5-3,0g soya bean kazein

In the result of the Anova test it is seen that there is a statistically significant difference between the groups as the P- value which is$1,222x10^{-14}$ is below the Alpha value (0,05). So H_0 is rejected and H_1 is accepted. However this is not enough for proving the hypothesis. Therefore, another statistical analysis is done, the t-test.

T-test is done between 3,0g TSB and 2,7 g TPB groups because from the table4 it is seen that the mean values of these two groups are close to each other. T-test is done between the trials.

H_0: There is no statistically significant difference between the protein mass((BSA) (µg/ml)) of 2,7gTPB and 3,0g soya bean kazein.

H_1: There is statistically significant difference between the protein mass ((BSA) (µg/ml)) of 2,7gTPB and 3,0g soya bean kazein.

In the result of t-test it is seen that there is no statistically significant difference between the protein content of these two groups as the “two tail P-value” is below Alpha value(0,05).So H_0 is accepted and H_1 is rejected. Therefore, it can be concluded that 3,0g TSB can be used instead of 2,7g TPB which proves the hypothesis and it can be said that the experiment is successful as the results reached the aim.

Although the experiment is successful and the hypothesis is proved by the statistical analysis some improvements can be done. For instance, from the table4 it can be seen that the protein content of 2,7 g tryptose phosphate broth is between the protein content of 2,5g and 3,0 g soya bean kazein. In order to find the exact value that can be used in a Glasgow media, experiment can be repeated for the groups between these two values, such as 2,5g -2,6g -2,7g -2,8g -2,9 and 3,0 g of TSB. Moreover, the groups are compared due to their protein content but the solutions are prepared with only distilled water. The experiment can be repeated with the same groups by using the Glasgow Media ingredients because TSB may give reaction with one of those ingredients which may change the protein value. Also, those media can be used in a culture of cells and observed under electromicroscobe for controlling the cell reproduction rate by counting the number of cells. (Project of Şükran Yılmaz)

If these improvements are done the results can be more accurate.

Appendix-1

Test Tube Procedure for Sample Pierce- Pre-Treatment

1. Dispense 100 µl of each sample or diluted protein standart to be treated into a test tube.

2. Add 500µl of Compat-Able™ Protein Assay Preparation Reagent 1 to each tube.Mix each tube and allow the tubes to stand at room temperature for at least five minutes.

3. Add 500µl Compa-Able™ Protein Assay Preparation Reagent 2 to each tube. Mix each tube and centrifuge at 10000 g for at least 5 minutes.

4. Invert each tube and discard the supernatant. Blot the open end of the inverted tube on clean paper towelling to completely remove supernatant. If needed, a pipette can be used to carefully remove excess liquid.

5. Dissolve protein pellet in original sample volume (100µl) of ultrapure water.

6. Protein analyzes begins.

(Modified Lowry Protein Assay Kit Instructions)

Appendix-2

	TRIALS	Absorbance Value (λ-750,0nm) (ABS)	Protein amount (BSA) (µg/ml)
2,7 g TPB	1	0,504	-
	2	0,524	-
	3	0,490	-
0,5 g TSB	1	0,519	-
	2	0,515	-
	3	0,491	-
1,0 g TSB	1	0,506	-
	2	0,510	-
	3	0,502	-
1,5 g TSB	1	0,510	-
	2	0,508	-
	3	0,477	-
2,0 g TSB	1	0,512	-
	2	0,526	-
	3	0,515	-
2,5 g TSB	1	0,518	-
	2	0,499	-
	3	0,521	-
3,0 g TSB	1	0,512	-
	2	0,521	-
	3	0,516	-

Table 7: Results after pre-treatment done

Appendix-3

Inorganic Compounts	**Amino Acide**	**Vitamin**
$CaCl_2$	L-Cystine	Folic acide
NaCl	L-Tyrosine	Choline chloride
KCl	L-Arginine	Nicotineamide
$MgSO_4$	L-Histidine	Ca-Pantotenate
NaH_2PO_4	L-Isoleucine	Pyridoxal
Glucose	L-Leucine	Riboflavine
Ferro Nitrat	L-Lysine	
Phenol Red	L-Methionine	**Pepton**
$NaHCO_3$	L-Threonine	Tryptose Phosphate Broth
	L-Phenylalanine	
	L-Tryptophane	
	L-Valine	
	Inositol	
	L-Glutamine	

Table 8:Ingradients of Glasgow Media

Appendix 4

Groups of the experiment before protein analysis

Blue colour of groups after protein assay method is done

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