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Effects of Some Growth Promoting Substances on the Rate of Growth and Lactic Acid Production by *Bifidobacterium bifidum* in Sterilized Skim Milk

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Abstract

Effect of some growth promoting substances, i.e., yeast extract, fructose, and peptone on the growth rate and acid production by *Bifidobacterium bifidum* in sterilized skim milk at 40°C was studied. Growth promoting substances were added to milk in three different concentrations, i.e., 0.1, 0.3, and 0.5%. The obtained results indicated that the three substances resulted in an increased of growth and lactic acid production by *Bifidobacterium bifidum*. The maximum cell population in the presence of yeast extract reached were $25x10^4$, $12x10^5$ and $21x10^5$ CFU/ml after 12 hours. In the presence of 0.1, 0.3 and 0.5%, respectively. The same effect was found in the presence of fructose and peptone, where the maximum cell population after 12 hours of incubation reached $35x10^4$, $46x10^4$ and $53x10^4$ and $25x10^4$, $51x10^4$ and $40x10^5$ CFU/ml, respectively.

The obtained results showed a direct relationship between the rate of increase in C.F.U and DTA (developed titratable acidity).

Direct relationship could be established between the growth rate of *Bifidobacterium bifidum* in skim milk and the availability of utilizable nitrogenous compound, which might enhance the rate of growth. As it was expected, the highest population would reach in milk supplied with a good source of nitrogenous compounds such as peptone.

***Keywords**: Growth promoting, Bifidobacterium bifidum, Yeast Extract, Peptone, Fructose.*

Introduction

Bifidobacteria are well known as a dominant bacteria found in the intestinal flora of a breastfed infant, and it has been known to provide many physiological properties to the human. Metchnikoff was one of the first to prove that soured milk containing bacteria may have positive effects on intestinal health (Cherie and Glenn, 1998).

Bifidobacterium was first isolated by Tissier (1900). In (2009 and 2012) Turroni *et al*., mentioned that *Bifidobacterium bifidum* is uniquely identified in the human gut and have been shown to represent part of the dominant bacterial members of the gut microbiota of the breastfed infants.

The cultivation of bifidobacteria in a medium like milk is a difficult task compared with that a conventional starter because milk is an artificial medium for growth of Bifidobacterium **(**Hadadji and Bensolture, 2006), and the conditions for cultivations must be strictly anaerobic. Also, they found that if growth promoting substance for bifidobacteria is added to the milk medium, bifidobacteria can be cultivated even in anaerobic conditions.

Yeast extract, peptone, fructose, inulin, fructose oligosaccharides, and galacto-oligosaccharides can be used as growth promoting substances for Bifidobacteria, (Cheric and Glenn, 1998, Hadadji and Bensolture, (2006), Agil *et al*., (2013), Castro *et al*.,(2013a) and Castro *et al*., (2013b).

In 2003, Codex mentioned that a probiotic dairy product should contain at least 6.7 log CFU $g^{-1}$of probiotic bacteria at the time of consumption and in quantity higher than 100 g per day in other words at least 9 log CFU per day.

The present work aimed to compare the effect of some growth promoting substances, i.e., yeast extract, fructose, and peptone on the growth rate and acid production by *Bifidobacterium bifidum* in sterilized skim milk.

Materials and Methods

1- Milk:

Cow's milk used in this study was obtained from the herd of Faculty of Agriculture, Assiut University. As soon as milk arrived at the laboratory it was skimmed by using Alfa-Laval separator operated at 16000 rpm.

Skim milk was divided into 500 ml conical flasks, each flask contained from 150 - 300 ml skim milk, depending on the experiment and the expected time for milk coagulation.

2- Growth promoting substances:

Three growth promoting substances, i.e., yeast extract, fructose, and peptone were added to conical flasks at three different concentrations, i.e., 0.1, 0.3 and 0.5%, before by autoclaving at 121°C/10 minutes.

3- Bifidobacterium bifidum

Bifidobacterium bifidum was obtained from Microbiological Resources Centre (Cairo MIRCEN) Faculty of Agriculture Ain Shams University.

Bifidobacterium was routinely maintained in sterilized skimmed milk fortified with 0.1% yeast extract and stored at 5–7°C.

For the preparation of the inocula, the procedure described by Hassan *et al.* (1989) was adopted. From each stored bacterial culture, 1/10 dilutions were prepared in 250 ml conical flasks, each one contained from 150 ml sterilized skim milk. After overnight of incubation at 40°C, the first non-coagulated flask in which the bacteria were expected to be in the exponential phase of growth was used for inoculating the experimental flasks.

Inoculation was carried out in order to achieve 10^3 to 10^4 CFU/ml at the time of inoculation. Inoculated flasks were incubated anaerobically at 40°C.

4- Sampling:

At the time of inoculation, (zero time) and each two hours intervals up to two hours after milk coagulation in most experiments or up to 48 hours, 15 ml aliquots of each culture was aseptically withdrawn in 25 ml sterilized conical flask.

1 ml of the aliquots was aseptically withdrawn in a test tube containing 9 ml sterilized distilled water to give the first dilution 1/10 for the bacteriological analysis (10^{-1}), mixed gently for 30 seconds, and used for the preparation of the higher dilutions.

5- Bacteriological analysis:

Enumeration of bacteria was carried out on TPY media. Petri dishes were incubated aerobically at 40°C for 5-7 days. Colonies were enumerated visually.

6- Growth media:

- **TPY media**

Selective medium for the isolation and cultivation of *Bifidobacterium* was obtained from (Laboratorios Conda S.A. Madrid Spain).

7- Chemical analysis:

Determination of Titratable Acidity:

Developed titratable acidity was determined according to the method as described in A.O.A.C., (2000) using sodium hydroxide N/9 and phenolphthalein as an indicator. Developed titratable acidity was expressed as the difference between the consumed volumes of NaOH N/9 solution in milliliter and volumes of NaOH N/9 consumed for the same titration of the above milk culture at the beginning of the incubation of cultures. The obtained results were recorded as a percent of lactic acid by weight as lactic acid.

Statistical analysis:

Stander deviation was determined according to Excel (2016).

Results and Discussion

The obtained results for the growth of *Bifidobacterium bifidium* in sterilized skim milk containing 0.1, 0.3, and 0.5% yeast extract at 40°C are presented in Table (1) and Figure (1). The maximum plate counts were $25x10^4$, $12x10^5$ and $21x10^5$ CFU/ml, respectively, after 12 hours of incubation at 40°C.

As it could be seen in Figure 1 that growth according to the rate of increase in colony forming units was steady at exponential rates up to 12 hours of incubation, and it clearly shows the effect of adding yeast extract to medium on the increase with the increase of added yeast extract.

Similar results were also obtained by Heap and Richardson (1985) and Ismail (1990), who detected higher CFU in reconstituted skim milk in the presence of 0.1% yeast extract.

The maximum CFU obtained in the presence of 0.5% yeast extract was $21x10^5$ CFU/ml, which is lower than that obtained by Yerlikay (2014), how detected a maximum CFU of $x10^6 – 10^7$ CFU/ml.

On the other hand, our obtained results are in good agreement with those obtained by Mutai *et al.* (1978), who found that *Bifidobacterium bifidum* grew in milk containing yeast extract reached higher colony counts, compared with milk in the absence of yeast extract, and it was in correspondence with the concentration of added yeast extract.

The obtained results concerning the rate of increase of titratable acidity during the growth of *Bifidobacteria bifidum* in skim milk supplemented with yeast extract are presented in Table 4 and Figure 4. Direct relationship could be established between the growth rate and the corresponding culture acidity. The obtained results are comparable to those obtained by Mutai *et al.* (1978), who found a significant similarity `between the number of viable bacteria and the acidity of the culture during cultivation.

Results presented in Table 2 and Figure 2 show the effect of adding

fructose as growth promoting on the rate of growth of *Bifidobacteria bifidum* in sterilized skim milk at 40°C. It could be seen that the addition of fructose greatly affected the growth rate of *Bifidobacteria bifidum*. The maximum population was $35x10^{4}$, $46x10^{4}$ and $53x10^{4}$ CFU/ml after 12 hours in samples containing 0.1, 0.3 and 0.5% fructose, respectively. The obtained results came in agreement with those obtained by Mitsuka *et al*. (1987) and Hadadji and Bensoltane (2008), who indicated that the fructose resulted in a higher count of *Bifidobacteria bifidum*, which confirm the effect of fructose as a growth promoting substance for these bacteria.

Rate of increase in DTA in cultures was proportional to the percentage of added fructose (Table 5 and Figure 5). The maximum population of *Bifidobacteria bifidum* was $35x10^{4}$, $46x10^{4}$ and $53x10^{4}$ CFU/ml. The corresponding values for DTA were 0.36, 0.38 and 0.39 % in the presence of 0.1, 0.3 and 0.5 % fructose, respectively. These results agreed with the results obtained by Gibson *et al.* (1995), who found that in case of adding fructooligosaccharide to milk, lactic acid production in culture was proportional to the concentrate of the percentage of growth promoting.

Results in Table 3 and Figure 3 show the effect of adding peptone on the growth rate of *Bifidobacteria bifidum* which was greatly affected by concentration of added peptone, and the maximum population reached $25x10^{4}$, $51x10^{4}$ and $40x\ 10^{5}$ CFU/ml after 12 hours of incubation with added 0.1, 0.3 and 0.5 % peptone, respectively .

These results show direct relationship exist between the growth rate of *Bifidobacteria bifidum* in skim milk and the availability of utilizable nitrogenous compound which can enhance the rate of growth, and it was to be expected that it would reach the higher population in milk supplied with a good source of nitrogenous compounds such as peptone. The obtained results are slightly higher than those obtained by Wang and Gibson (1993) and Hadadji and Bensoltane (2006).

Table 6 and Figure 6 show the effect of adding peptone to skim milk on the production rate of lactic acid, which was increased as the percentage of added peptone was increased. In the same time, lactic acid production was proportional to the rate of increase in colony forming units.

After 12 hour of incubation the maximum cell population reached $25x10^{4}$, $51x10^{4}$ and $40x10^{5}$ CFU/ml in samples containing 0.1, 0.3 and 0.5% peptone, the corresponds values for DTA was 0.42, 0.5 and 0.50%, respectively. Which indicate that mil, which was supplemented with 0.5% peptone was markedly superior to milk, contained less percentage of peptone 0.5 substrate for the rate of growth and acid production.

Conclusion

Concerning the results presented in Tables from 1 to 6, at could be observed that yeast extract and peptone had a much higher effect as growth promoting substances for the growth of *Bifidobacterium bifidum* than fructose. Where the maximum CFU after

12 hour of incubation reached $25x10^4$, $12x10^5$ and $21x10^5$ CFU/ml in samples with added 0.1, 0.3 and 0.5% yeast extract, the corresponding values for samples with peptone and fructose were $25x10^4$, $51x10^4$ and $40x10^5$ and $35x10^4$, $46x10^4$ and $53x10^4$ CFU/ml, respectively.

Similar effect was found in the rate of increase in DTA corresponding to the rate of increase in colony forming units, which might prove the existence of direct relation between growth rate and rate of increase in DTA by *Bifidobacterium bifidum.*

These results indicated that a direct relationship might exist between the growth rate of *Bifidobacteria bifidum* in skim milk and the availability of utilizable nitrogenous compound which can enhance the rate of growth, and it was to be expected that it would reach the higher population in milk supplied with a good source of nitrogenous compounds such as peptone.

Table 1. Effect of adding yeast extract on growth rate (Log CFU/ml) of *Bifidobacterium bifidum* cultivated in sterilized skim milk at 40°C.

Sampling time (hour)	Percentage of added yeast extract					
	0.1		0.3		0.5	
	CFU/ml	Log CFU/ml	CFU/ml	Log CFU/ml	CFU/ml	Log CFU/ml
0	10 x 10^4	5.00	15 x 10^4	5.17	30 x 10^4	5.47
2	13 x 10^4	5.11	32 x 10^4	5.50	64 x 10^4	5.80
4	16 x 10^4	5.20	55 x 10^4	5.74	11 x 10^5	6.04
6	20 x 10^4	5.30	80 x 10^4	5.90	16 x 10^5	6.20
8	23 x 10^4	5.36	99 x 10^4	5.99	19 x 10^5	6.27
10	24 x 10^4	5.38	11x 10^5	6.0٤	20 x 10^5	6.30
12	25 x 10^4	5.40	12 x 10^5	6.08	21 x 10^5	6.31*
24	83 x 10^4	5.91	13 x 10^5	6.11*	17 x 10^5	6.23
26	70 x 10^4	5.84*	10 x 10^5	6.00	14 x 10^5	6.15
SD		0.30		0.31	0.28	

*Coagulated sample.

Table 2. Influence of adding fructose on the growth rate (Log CFU/ml) of *Bifidobacterium bifidum* cultivated in sterilized skim milk at 40°C.

Sampling time (hour)	Percentage of added fructose					
	0.1		0.3		0.5	
	CFU/ml	Log CFU/ml	CFU/ml	Log CFU/ml	CFU/ml	Log CFU/ml
0	5 x 10^4	4.69	8 x 10^4	4.90	10 x 10^4	5.00
2	8 x 10^4	4.90	13 x 10^4	5.10	16 x 10^4	5.20
4	12 x 10^4	5.08	19 x 10^4	5.27	24 x 10^4	5.38
6	17 x 10^4	5.23	27 x 10^4	5.43	33 x 10^4	5.51
8	22 x 10^4	5.34	34 x 10^4	5.53	40 x 10^4	5.60
10	28 x 10^4	5.45	41 x 10^4	5.61	47 x 10^4	5.67
12	35 x 10^4	5.54	46 x 10^4	5.66	53 x 10^4	5.72
24	40 x 10^4	5.60	51 x 10^4	5.70	58 x 10^4	5.76
26	45 x 10^4	5.65	55 x 10^4	5.74	61 x 10^4	5.78
SD		0.33		0.29		0.27

Table 3. Effect of adding peptone on the growth rate (Log CFU/ml) of *Bifidobacterium bifidum* cultivated in sterilized skim milk at 40°C

Sampling time (hour)	Percentage of added peptone					
	0.1		0.3		0.5	
	CFU/ml	Log CFU/ml	CFU/ml	Log CFU/ml	CFU/ml	Log CFU/ml
0	70×10^3	4.84	72×10^3	4.85	25×10^4	5.40
2	82×10^3	4.91	10×10^4	5.00	30×10^4	5.48
4	10×10^4	5.00	16×10^4	5.20	40×10^4	5.60
6	13×10^4	5.11	25×10^4	5.39	50×10^4	5.69
8	16×10^4	5.20	35×10^4	5.54	64×10^4	5.80
10	20×10^4	5.30	44×10^4	5.64	80×10^4	5.90
12	25×10^4	5.40	51×10^4	5.70	40×10^5	6.60
24	10×10^5	6.00*	13×10^5	6.11	16×10^6	7.20
SD		0.37		0.40		0.62

*coagulated sample

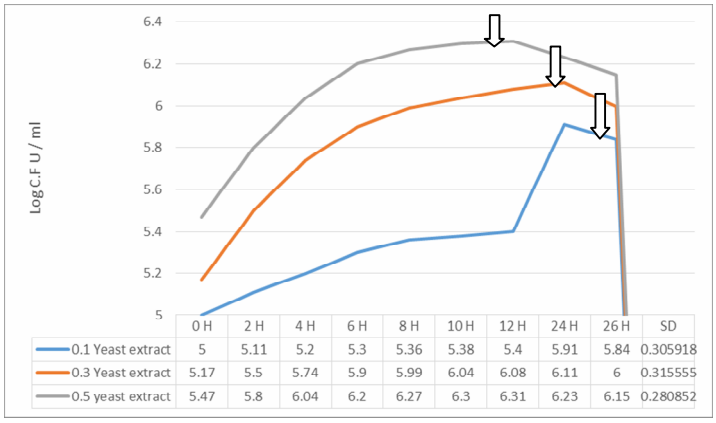


Figure 1: Effect of adding yeast extract on growth rate (log CFU/ml) of *Bifidobacterium bifidum* cultivated in sterilized skim milk at 40°C.

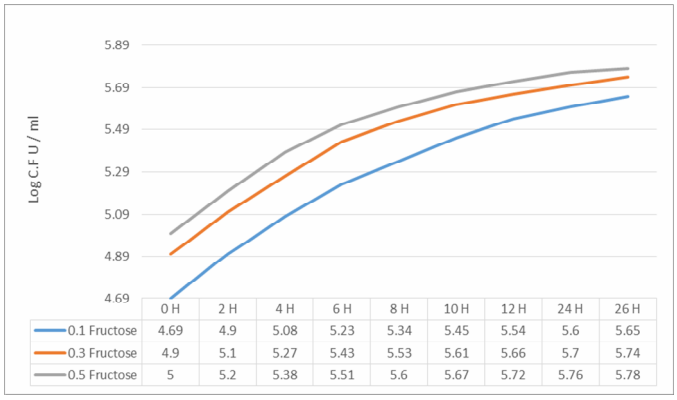
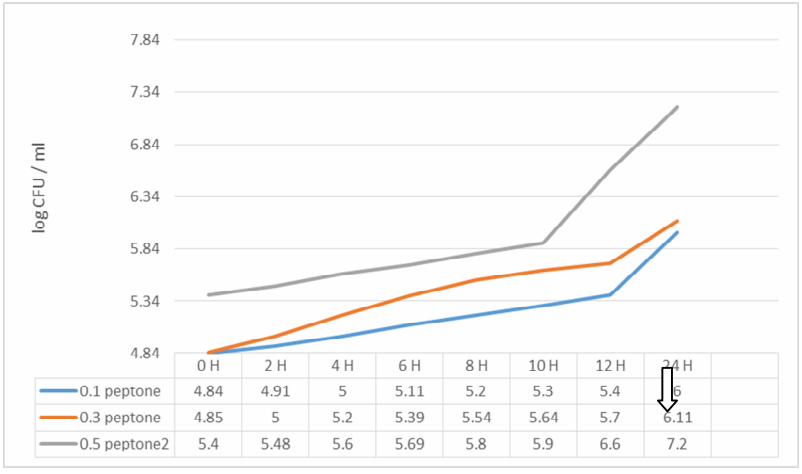


Figure 2: Effect of adding fructose on the growth rate (log CFU/ml) of *Bifidobacterium bifidum* cultivated in sterilized skim milk at 40°C.



Coagulated sample

Figure 3: Effect of adding peptone on the growth rate (log CFU / ml) of *Bifidobacterium bifidum* cultivated in sterilized skim milk at 40°C.

Table 4. Effect of adding yeast extract on the rate of increase in D.T.A (%) during the growth of *Bifidobacterium bifidum* cultivated in sterilized skim milk at 40°C.

Sampling time (hour)	Percentage of added yeast extract					
	0.1		0.3		0.5	
	%DTA	Difference in DTA	%DTA	Difference in DTA	%DTA	Difference in DTA
0	0.25	0.00	0.26	0.00	0.27	0.00
2	0.27	0.02	0.28	0.02	0.29	0.02
4	0.28	0.03	0.29	0.03	0.30	0.03
6	0.29	0.04	0.30	0.04	0.31	0.04
8	0.35	0.10	0.37	0.11	0.40	0.13
10	0.40	0.15	0.43	0.17	0.44	0.17
12	0.44	0.19	0.45	0.19	0.49*	0.22
24	0.70*	0.45	0.72*	0.46	0.73*	0.46
26	0.71	0.46	0.73	0.47	0.74	0.47

*Coagulated sample.

Table 5. Effect of adding fructose on increasing rate in D.T.A (%) during the growth of *Bidobacterium bifidum* cultivated in sterilized skim milk at 40°C.

Sampling time (hour)	Percentage of added fructose					
	0.1		0.3		0.5	
	%DTA	Difference in DTA	%DTA	Difference in DTA	%DTA	Difference in DTA
0	0.26	0.00	0.27	0.00	0.28	0.00
2	0.27	0.01	0.29	0.02	0.30	0.02
4	0.28	0.02	0.31	0.04	0.32	0.04
6	0.30	0.04	0.32	0.05	0.33	0.05
8	0.32	0.06	0.33	0.06	0.34	0.06
10	0.34	0.08	0.35	0.08	0.36	0.08
12	0.36	0.10	0.38	0.11	0.39	0.11
24	0.42	0.16	0.44	0.17	0.46*	0.18
26	0.43	0.17	0.45*	0.18	0.46	0.18

Table 6. Effect of adding peptone on the increasing rate in D.T.A (%) during the growth of *Bidobacterium bifidum* cultivated in sterilized skim milk at 40° C.

Percentage of added peptone						
0.1			0.3		0.5	
Sampling time (hour)	%DTA	Difference in DTA	%DTA	Difference in DTA	%DTA	Difference in DTA
0	0.27	0.00	0.28	0.00	0.29	0.00
2	0.29	0.02	0.30	0.02	0.31	0.02
4	0.30	0.03	0.32	0.04	0.33	0.04
6	0.31	0.04	0.34	0.06	0.35	0.06
8	0.34	0.07	0.36	0.08	0.37	0.08
10	0.37	0.10	0.43	0.15	0.44	0.15
12	0.42	0.15	0.50*	0.22	0.50*	0.21
24	0.72*	0.45	0.77*	0.49	0.79*	0.50
26	0.72	0.45	0.77	0.49	0.79	0.50

*coagulated sample

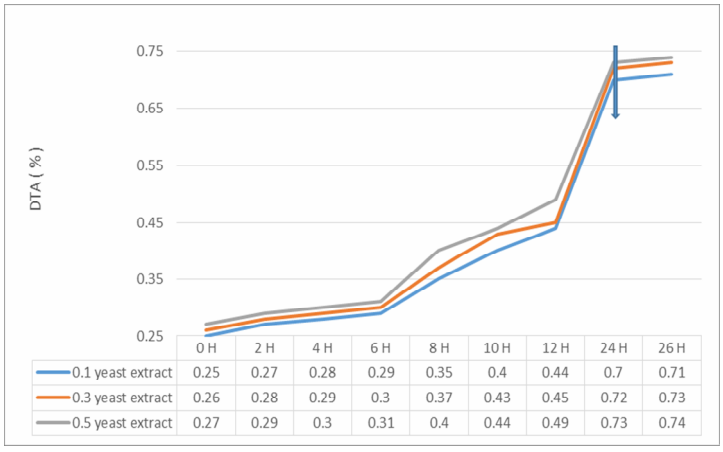


Figure 4: Effect of adding yeast extract on increasing rate in D.T.A (%) during growth of *Bidobacterium bifidum* cultivated in sterilized skim milk at 40°C

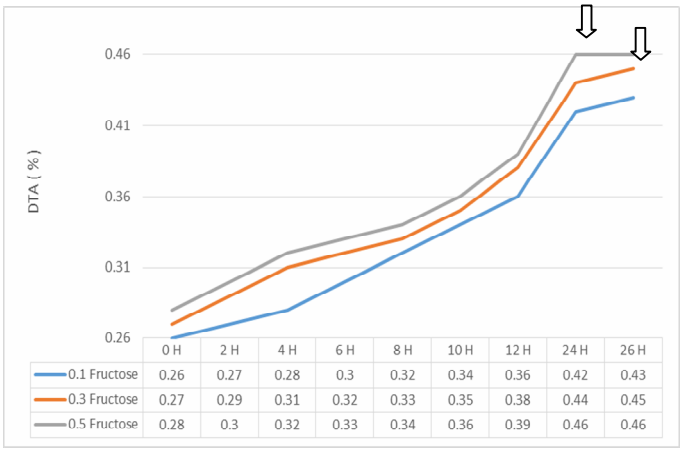


Figure 5: Effect of adding fructose on increasing rate in D.T.A (%) during growth of *Bidobacterium bifidum* cultivated in sterilized skim milk at 40°C

Coagulated sample

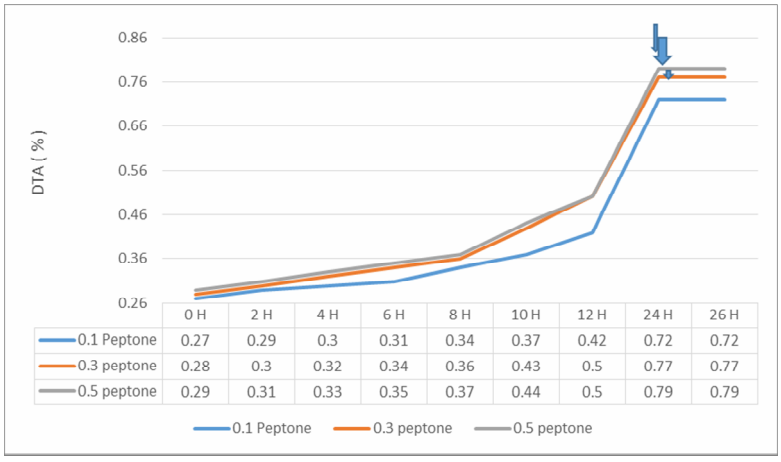


Figure 6: Effect of adding peptone on increasing rate in D.T.A (%) during growth of *Bidobacterium bifidum* cultivated in sterilized skim milk at 40°C.

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تاثير بعض العوامل المحفزة للنمو علي معدل نمو وانتاج الحموضة لبكتريا *Bifidobacterium bifidum* في اللبن الفرز المعقم

ايمان عبد الحميد ، داليا جمال كامل ، ياسر عبد العزيز وعلى اسماعيل حسن

قسم الألبان – كلية الزراعة – جامعة أسيوط

الملخص

لقد تمت دراسة بعض العوامل المحفزة للنمو مثل مستخلص الخميرة والفركتوز والببتون علي معدل نمو بكتريا *Bifidobacterium bifidum* وانتاجها للحموضة في اللبن الفرز المعقم.

تم اضافة المواد المحفزة للنمو للبن بثلاث تركيزات مختلفة (٠،١% و ٠،٣% و ٠،٥%).

وقد وجد ان هذه المواد أدت الي زيادة معدل النمو لبكتريا *Bifidobacterium bifidum* وزيادة في انتاجها للحموضة.

وتم الحصول علي النتائج التالية:

في العينات التي تحتوي علي مستخلص الخميرة كانت القيمة القصوي التي تم الحصول عليها لعدد الخلايا بعد ١٢ ساعة من التحضين $25x10^4$ ، $12x10^5$ ، $21x10^5$ مل/خلية في العينات التي تحتوي علي ٠،١ % و ٠،٣% و ٠،٣ % علي التوالي.

وقد وجد تاثير مشابه في العينات المضاف اليها الفركتوز والببتون حيث كانت القيمة القصوي لعدد الخلايا بعد ١٢ ساعة من التحضين كما يلي:

$35x10^4$ ، $46x10^4$ ، $53x10^4$ في حالة الفركتوز. اما في حالة الببتون كانت: $25x10^4$ ، $51x10^4$ ، $40x10^5$ مل/خلية علي التوالي.

النتائج السابقة أثبتت وجود علاقة مباشرة بين معدل نمو الخلايا ومعدل تطور الحموضة.

بمقارنة النتائج التي تم الحصول عليها وجد انه تحتمل وجود علاقة مباشرة بين معدل نمو بكتريا *Bifidobacterium bifidum* في اللبن الفرز والمركبات النيتروجينة المتاحة التي تم اضافتها ويمكن التوقع انها تسببت في ارتفاع تعداد الخلايا في اللبن المدعم بمصدر جيد من المركبات النيتروجينية مثل الببتون.