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INCREASING OF FOLIC ACID PRODUCTION THROUGH PROTOPLAST FUSION BETWEEN *Lactococcus lactis, Lactobacillus SP* AND *Streptococcus thermophilus*

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ABSTRACT

Two strains were selected i.e *Lactobacillus acidophilus NB1MCC1380, Lactobacillus bulagricus L. B1449* among *Lactococcus lactis NRRL B 23802* and *Streptococcus thermophilus NRC2010* to obtain as a result of protoplasting fusion. the latter strains were known as a good folic acid producer. In this technique polyethylene glycol 1000 and lysozyme enzyme for induction of protoplasting process which was more efficient for time period of 24, 48 hours from protoplasting imitating. All obtained recombinant fusant have been evaluated for folic acid production by using high performance liquid chromatography HPLC. The results showed that the two parameter the retention time of folic acid was 3.7 min and the concentration of folic acid in the fermentation media ranged between (25.7 µg\ L to 42.9µg\L) in the parental strain *Lactococcus lactis* while it was 42.9µg/L. In *Streptococcus thermophilus*, The fusant strains produced the following concentration of folate: Fusant 1 (32.67 µg\L), fusant 2 (25.7 µg\L), fusant 3 (26.04 µg\L), fusant 4 (25.9 µg\ L) respectively.

INTRODUCTION

Folate is an essential compound in the human diet that cannot be synthesized by humans. Human life could not exist without folate that is involved in various essential functions in cell metabolism, such as the synthesis of DNA and RNA. The daily recommended intake (DRI) of folate for an adult is 200/400 µg (EU/USA). For pregnant women 400/600 µg\ l Konings *et al*. (2001) and O'Brien *et al.* (2001). Folate is present in various foods and could be found in fermented dairy products. Still, there are people that suffer from folate deficiency and that may have increased risks for the occurrence of diseases associated with shortage of folate, like neural tube defects, cardiovascular disease, anemia, and some forms of cancer, therfore, it is useful to fortify foods with chemically synthesized folate, or to increase folate levels in fermented foods by using folate synthesizing food-grade using bacteria. The main objective of this research was increase the folate production capacity of lactic acid bacteria by using metabolic engineering pathway modification by genetic engineered protocols and optimization of growth conditions.

The Molecular genetics of dairy lactic acid bacteria, development of techniques of gene transfer, have been rapidly advanced in recent years.

recently Protoplast fusion is one of the most promising methods of gene transfer in both genetically studies and improvement of starter strains for practical use in dairy industries Okamoto *et al.* (1983). The Protoplasts are the cells of which cell walls are removed and cytoplasmic membrane is the outermost layer in such cells. Protoplast could be obtained by specific lytic enzymes to remove cell wall. Protoplast fusion is a physical phenomenon, where two or more protoplasts come in contact and adhere with one another either spontaneously or in presence of fusion inducing agents. By protoplast fusion it is possible to transfer some useful genes. Protoplast fusion an important tools in strain improvement for bringing genetic recombinations. Protoplast fusion has been used to combine genes from different organisms to create strains with desired properties. These are the powerful techniques for engineering of microbial Strains for desirable industrial properties. This technique in the future would be one of the most frequently used research tools for tissue culturists, molecular biologists, biochemical engineers and biotechnological application. Verma *et al*. (2008).

MATERIALS AND METHODS

A- Materials:

1-Strains :

The strains used in this study included the following: *Lactococcus lactis NRRLB 23802*, *Streptococcus thermophilus NRC2010, Lactobacillus bulagricus L.B 1449*, *Lactobacillus acidophilus NBIMCC1380.* this strain was obtained from : Northern Regional Research Laboratory (NRRL), USDA-ARS, Peoria, Illinois, USA, National Research Center, Division Of Microbiology University Of Helsinki, Finland, National Bank For Industrial Microorganisms And Cell Cultures.

2- media :

Lactobacillus strains were growing in MRS media according to De Man *et al* (1960). And GM17 solid selective media for *Lactococcus lactis, Streptococcus thermophilus* according to Atlas (1993). Polyethilin obtained from sigma biochemichal (U. S. A.) Folic acid obtained from sigma biochemichal (U. S. A).

B- Methods :

1- Protoplast fusion among Lactic acid Bacteria:-

Bacterial strains were cultured overnight at 30°C in 10 ml of MRS. Cells were harvested by centrifugation, washed in Protoplast buffer(PB), and suspended in 400 ml of protoplast buffer. a portion of the cell suspension was mixed with the same volume of protoplast buffer containing various amounts of lysozyme, and the mixture have incubated at 37°C for 30 min to 4 h with occasional agitation. The appearance of spherical cells, as judged by light microscopy, protoplasts were collected by centrifugation at 2.000 rpm for 20 min and suspended in 2 ml of protoplast buffer. Protoplast suspension (50µl) of each strain was mixed and then 1.5 ml of polyethylene glycol (PEG) solution 50%PEG 6000, 0.6 M sucrose, and 20mM protoplast buffer, 15 mM $MgCl_2$ pH 6.5 was added and mixed immediately. After 2 min at 37°C the

protoplast was recovered by centerfugation and suspended in 2 ml of protoplast buffer, and the suspension was plated with 20 ml in MRS agar. This method was described by Iwata *et al* (1986).

2- determination of Folic acid by HPLC:

Folic acid was determined by HPLC according to the method of Albala- Hurtado *et al.* (1997).

RESULTS AND DISCUSSION

1- Fusant isolats after protoplasting fusion between Lactic acid bacteria strains.

protoplast fusion between *Lactobacillus acidophilus NBIMCC1380*, *Lactobacillus bulagricus L. B1449* with *Lactococcus lactis NRLB23802*, *Streptococcus thermophilus NRC 2010* are shown in Table 1 and figure 1. Of agar plates which showed morphological forms of fusant which were formed by protoblast fusion between different parental strain, the plates showed colony morphology and growth rate on MRS medium, contain a selectable marker and trace of methylene blue for descomestration phosophalation activity to new bacterial strains production of protoplasts of the various strains of Lactic acid bacteria which produce folic acid was investigated by useing various osmatic stabizing solution containing lysozyme and polyethylene glycol PEG 1000, It induced protoplast aggregation followed by fusion of protoplasts of all strains which produced in raffinase (0.3M) succinate (0.5M) molate (0.5M) and lactose all of them were mixed with lysozyme.

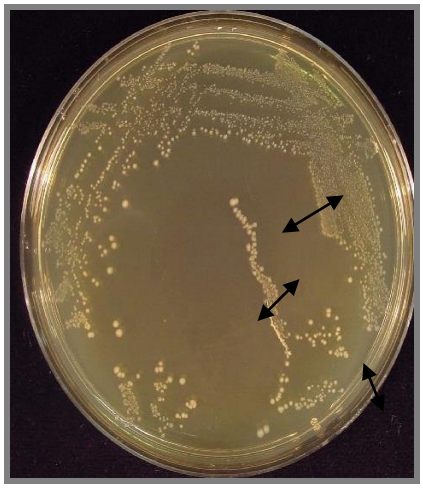

Figure 1:Fusant colony on MRS agar plate

The result showed a decrease in protoplast farming when used lysozyme alone led with MRS media failed to produce protoplast with all the bacterial strains tested. Mixture of lysozyme and mutanolysin which replaced the SDS-10 for the first time to produce protoplast from strains

indicated that. mutanolysin is not sufficient to produce protoplast a Lon with all the Lactic acid bacterial strains. *Streptococcus thermophilusNRC2010* With *L. bulagricus L.B 1449*, *L. acidophilus NBIMCC1380* and *Lactococcus lactis NRRL 23802* with *L. bulagricus L. B 1449,* and *L. acidophilus NBIMCC1380*. grown on a large number of media were evaluated as protoplast regeneration media. GM17 for *Streptococcus thermophilus.* and for *Lactococcus lactis* this media were suitable to regulation of *Lactobacillus sp.* The results obtained from protoplasting protocol has been shown in Table 2 and 3 where the strains were incubated at $37C^0$ for different times 2hour, 6hour, 12 hour , 24hours ,36hours and 48hours

Table 2: Protoplast fusion between *Streptococcus thermophilus NRC2010* With *L. bulagricus L. B 1449*, *L. acidophilus NBIMCC1380* at different times.

Time\ hour (h)	Total Count of cells cfu/ml	*Streptococcus thermophilus* NRC2010 x *L.bulagricus* L.B 1449(RPFE 202)		*Streptococcus thermophilus* NRC2010 x *L.acidophilus* NBIMCC1380(RPFE 204)	
		Fusant No cfu/ml	Fusant frequency cfu/ml	Fusant No cfu/ml	Fusant frequency cfu/ml
2h	$0.8x10^6$	$8x10^2$	$3.75x10^{-4}$	$2.1x10^2$	$22x10^{-4}$
6h	$2.3x10^6$	$12x10^4$	$5.21x10^{-4}$	$3.5x10^3$	$23.4x10^{-3}$
12h	$3.1x10^6$	$18x10^5$	$5.8x10^{-3}$	$3.4x10^2$	$30.2x10^{-4}$
24h	$3.5x10^6$	$24x10^3$	$6.85x10^{-3}$	$4.5x10^4$	$3.4x10^{-3}$
36h	$4.2x10^6$	$32x10^4$	$7.61x10^{-3}$	$3.2x10^4$	$42x10^{-3}$
48h	$27x10^6$	$36x10^5$	$14x10^{-5}$	$6.3x10^4$	$13x10^{-5}$

Respectively. The result in Table 2 showed an the protoplast fusion between *L. bulagricus* L. B 1449, *L. acidophilus* and *Streptococcus thermophilus* NRC2010 (RPFE 202, RPFE 204) that showed increase the number of fusant by increasing the incubation time between two parental strains. The number of fusant which were formed after 2 hours incubated time were $8x10^2$ C.F.U/ml in the fused (RPFE 202) and the protoplast fusion frequency were $3.75x\ 10^{-4}$ this rate were increased by increasing the incubation time to $36x\ 10^5$ C.F.U/ml at incubation time of 48 hours. The number of protoplasting fusion frequency had been increased by the same rate where it was $14X10^{-5}$ C.F.U / ml, but the number of fusant formed at 2 hours incubation time were $2.1x\ 10^2$ C.F.U/ml in the fused (RPFE 204) and the protoplast fusion frequency were $22x\ 10^{-4}$. This rate was increased by increasing the incubation time to $6.3x\ 10^4$ C.F.U/ml at incubation time at 48 hours where the number of protoplasting fusion frequency had been increased by the same rate were it was $13X10^{-5}$ C.F.U / ml.

On the other hand, the results in Table 3 indicated that fused (RPFE 201), and fused (RPFE 202) were increased as the number of fusant recombinant cell during the incubation time between all parental strains were increased after 2 hours by $25X10^2$ Protoplasting fusion frequency of $10X10^{-3}$ which were obtained from the same results were campered with data after

48 hours number of fusant cell / ml of $19X10^4$ C.F.U / ml. The frequency of protoplasting fusion at same time were $24X10^{-4,}$ but the fused (RPFE 202) showed fusant cell after 2 hours of $42X10^2$ and protoplasting fusion frequency of $22.1X10^{-2}$.

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Table 3: Protoplast fusion between *Lactococcus lactis NRRL 23802* with *L. bulagricus L. B 1449* and *L. acidophilus NBIMCC1380* in different times.

Time\ hour (h)	Total count of cells cfu/ml	*Lactococcus lactis* NRLL 23802 x *L .bulagricus* L.B 1449 (RPFE 201)		*Lactococcus lactis* NRLL 23802 x *L.acidophilus* NBIMCC1380 (RPFE 202)	
		Fusant No cfu/ml	Fusant frequency cfu/ml	Fusant No cfu/ml	Fusant frequency cfu/ml
2h	$2.3x10^5$	$25x10^2$	$10x10^{-3}$	$42x10^2$	$22.1x10^{-2}$
6h	$5.3x10^5$	$18x10^3$	$33x10^{-3}$	$23x10^4$	$51.2x10^{-2}$
12h	$7.1x10^5$	$15x10^3$	$21.1x10^{-4}$	$27x10^4$	$62x10^{-3}$
24h	$8.3x10^6$	$18x10^4$	$2.1x10^{-3}$	$32x10^3$	$14.5x10^{-4}$
36h	$14.2x10^6$	$29x10^4$	$19.2x10^{-4}$	$13x10^2$	$15x10^{-3}$
48h	$6.2x10^5$	$19x10^4$	$24x10^{-4}$	$20x10^3$	$44x10^{-4}$

The same results were compared with data showed formally after 48 hours showed that number of fusant were $20x\ 10^3$ C.F.U / ml. and the frequency of protoplasting fusion at same time were $44X10^{-4}$, This results obtained in this investigation agree with the results obtained by Lyang *et al* (1984) who found that high frequency protoplasting and regeneration of protoplasts were in contrast to protoblasts of *Bacillus subtilus*, plating the results indicated that when *Lactobacillus casei* protoplasts on media containing additional SDS and high concentration of gelatin, The autoclaved *L. casei* cell did not increase. On the other hand, the formation and regeneration of protoplast of *L. casei* could be estimated as the suspension could be sampled at various time. diluted in PB or water and plated on MRS medium. Thorne and Barker (1969) Chassy and Giuffrida (1980) with lysozyme and Tomochika *et al* (1980) with mutanolysin have reported that the formation of *L. casei* protoplasts *Streptococcal* protoplasts have been successfully regenerated as reported previously by Gasson (1980) and Kondo *et al.* (1982).

With respect to *L. casei* no reports or methods applicable to the regeneration of protoplasts of *L. casei* were formed. The experiments with various media and conditions that had been described for the regeneration of protoplast of a number of bacterial strains were unsuccessful.

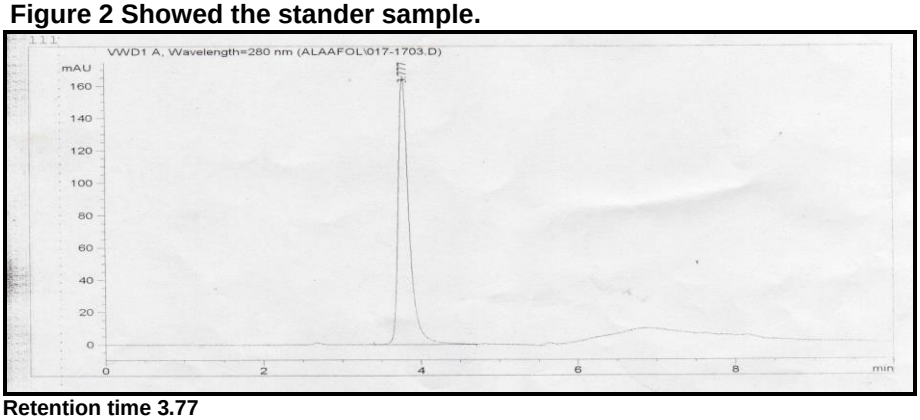
Landman *et al.* (1968), and Miller *et al*. (1967) medicated that regeneration could be achieved by using the minimum times of incubation and the lowest possible concentrations of enzymes. The produced protoplasts could be regenerated with more than 10% frequency in a complex medium containing 0.3 raffinose. It showed be emphasized that for any given strain, higher frequencies might be obtained by carefully optimizing which were experimental parameters. It was clear that even though the

methods described were applicable to *Lactobacillus* strains, there exist optimal conditions of protoplast preparation and probably optimal media for regeneration of each individual strains, It was noticed that *L. casei* protoplasts behave quit differently than some other bacterial protoplasts In the respect, Akamastu and Sekiguchi (1981) found that plasma expanders such as dextran facilitated the regeneration of *B. subtilis* protoplasts, Also Allcok *et al* (1982) found that $MgCl_2$ and $CaCl_2$ increased the stability of *C. acetobutylicum* protoplasts but decreased the regeneration frequency, In this study, divalent metal salts were very stimulatory to regeneration where regulation may depend on a series of factors such as the presence of residual primer the preservation of enzymatic activity of the wall biosynthetic system and the ability of the strain to develop in a rich hypertonic medium.

2- HPLC Folic acid analysis

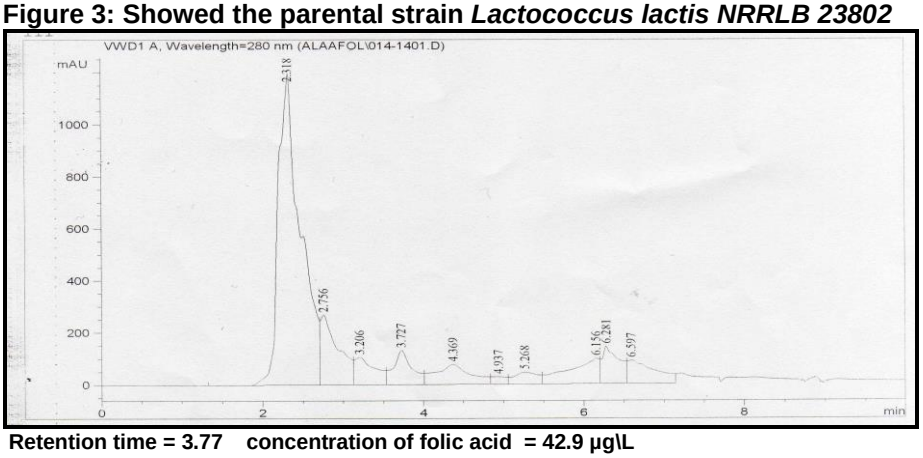
folic acid vitamin or folate was obtained using technique of Albala – Hurtado *et al* (1997) HPLC technique has the ability to determine the small amount of folic acid in growth media in which the Lactobacillus strains are grown. And we It was found in the following Table 4, 5 and Figures 2, 3, 4, the production concentration of folic acid by parental strain and fusant that determinate by HPLC as the followed.

Figure 2 Showed the stander sample.



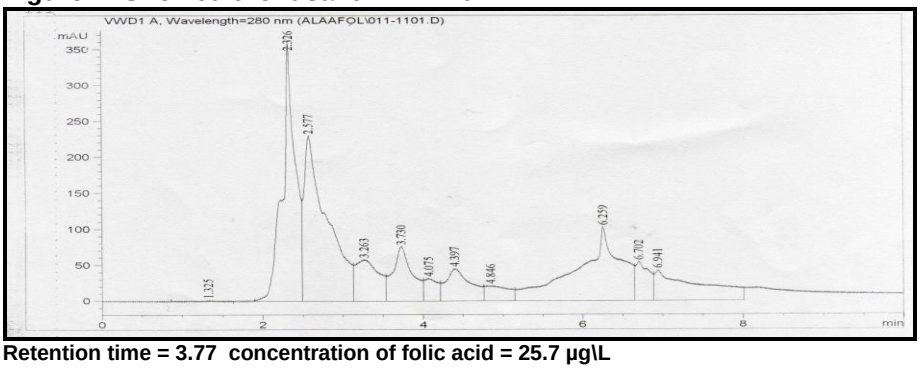
Retention time 3.77

Figure 3: Showed the parental strain *Lactococcus lactis NRRLB 23802*



Retention time = 3.77 concentration of folic acid = 42.9 µg\L

Figure 4: Showed the fusant RPFE 201



Retention time = 3.77 concentration of folic acid = 25.7 µg\L

Folate in an essential component in the human diet and it's involved a cofactors. in many metabolic reaction and folates produced by many species of bacteria included lactic acid bacteria..as seen in Table 4, 5 Table Shows that the parental strain *Streptococcus NRC 2010* was produce about 38.9 µg\ L, the another parental strain *Lactococcuss lactis NRRLB* 23802 produces 42.9 µg\ L, and the the fusant RPFE 203 was 25.9 µg\ L, but the fusant RPFE 204 produces 26.04 µg\ L, and fusant RPFE 201gives 25.7 µg\ L, and the fusant RPFE 202 gives 32.67 µg\ L, The two strains of *Lactobacillus bulagricus*, *Lactobacillus bulagricus* don't have the ability to produce folic acid, This result was in agreement with results obtained by sybesma *et al* (2003).

Table 4: Production of folic acid in fermented media by recombinant in protoplast fusion bacterial strains between *Streptococcus thermophilus NRC2010* With *L. bulagricus L. B 1449*, *L. acidophilus NBIMCC1380*

Strains	Folic acid µg/L
Parental strain *Streptococcus thermophilusNRC2010*	38.9
Parental strain *L. bulagricus L.B 1449*	0.0
Parental strain *L. acidophilus NBIMCC1380*	0.0
Fusant RPFE 203	25.9
Fusant RPFE 204	26.04

Table 5: Production of folic acid in fermented media by recombinant in protoplast fusion bacterial strains *Lactococcus lactis NRLLB 23802* with *L. bulagricus L. B 1449*, and *L. acidophilus NBIMCC1380*

StraiEns	Folic acid µg/L
Parental strain *Lactococcus lactis NRLLB 23802*	42.9
Parental strain *L. bulagricus L.B 1449*	0.0
Parental strain *L. acidophilus NBIMCC1380*	0.0
fusant RPFE 201	25.7
Fusant RPFE 202	32.67

He studied the production of folic acid by several species and strains from lactic acid bacteria such as. *Lactococcus, Lactabacilli, Streptococcus, Leuconostoc*, These bacteria were screened for interacellular and extracellular folate production where the highest folate level was detected in an aerobically grown lactic acid bacteria *L. lactis* 291µg\l and *Streptococcus thermophilus* B119 were (214 µg\l). In general, *Lactobacillus* strains didn't produced folate. Rao *et al* (1984) said that some fermented milk products especially yoghurt contains even large amounts of folate up to 110 µg\l as found in yoghurt. This high level is a direct result of the production of additional folate by lactic acid bacteria in the yoghurt. Of the two lactic acid bacterial species in yoghurt, *Lactobacillus bulagricus* and *Streptococcus thermophilius*, only the latter is reported to produce folate by large amount and the *Lactobacillus bulagricus* did not produc folate, It could be indicated that protoplast fusion technique would improve the production of folic acid by lactic acid bacteria. Thus, protoplast fusion is a good technique when compared with genetic protocols which were used in modification bacterial genome such as matting and by addition to some moleculer genetic tools uses in genetic recombination of bacteria. Moleculer cloning using expression vector and many other of genetic tools, as good as protoplast fusion technique which have characterized by a highly stability under different growth condition for long time.

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زيادة انتاج حمض الفوليك عن طريق دمج البروتوبلاستات بين سلالات*Lactococcus lactis*, *Lactobacillus sp* and *Streptococcus thermophilus* .

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تم اختيار سلالتين من بكتريا حمض اللاكتيك *Lactobacillus bulagricus,* *Lactobacillus acidophilus* مع كلا من السلالتين *Lactococcus lactis* , *Streptococcus thermophilus* لإجراء دمج بروتوبلاستى لتحسين إنتاجية حمض الفوليك حيث من المتعارف علية أن السلالتين الأخيرتين لهما القدرة على إنتاج حمض الفوليك وتم استخدام هذا التكنيك باستخدام كل من البولى ايثلين جليكول وانزيم الليزوزيم على فترات 24و 48 ساعة حيث تكون أعلى فترةمن الافضل للحصول على البروتوبلاستات وقد تم اختبار كل الاتحادات الجديدة والآباء لقدرتهم على إنتاج حمض الفوليك بواسطة استخدام جهاز التحليل الكمى الكروماتوجرافى السائل HPLC وكانت النتائج المتحصل عليها عند زمن 3.7 دقيقة حيث كان تركيز حمض الفوليك فى بيئة النمو ما بين 25.7 ميكروجرام / لتر و 42.9 ميكروجرام / لتر حيث يكون تركيز الحمض فى سلالة الاب *Lactococcus lactis 42.9* ميكروجرام / لتر بينما فى سلالة الأب الأخرى*Streptococcus thermophilus* كانت 38.9 ميكروجرام / لتر وكانت نسبة التركيزفى الاتحادات الجديدة كالاتى الاتحاد الأول 32.67 ميكروجرام / لتر و الاتحاد الثانى 25.7 ميكروجرام / لتر و الاتحاد الثالث 26.04 ميكروجرام / لتر و الاتحاد الرابع 25.9 ميكروجرام / لتر على التوالى .

قام بتحكيم البحث

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