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Full Length Research Paper

Genetic mutagenesis through Transposable element 5 (Tn5) to improve beta-D-galactosidase productivity from different bacterial strains

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β-galactosidaseIs an enzyme that catalyzes the hydrolysis of terminal non-reducing β-D-galactose residues in β-D-galactosides, Suicide plasmids considered a good genetic tool for DNA mutagenesis in bacterial origin specially gram negative ones, in this study a group of suicide plasmids carrying transposal element genes Tn5 and Tn7 were used for Beta-D-galactosidase productivity improvement in Escherichia coli strains through mutagenesis stimulation in genomic DNA of recipient cells for plasmid, these plasmids transferred through transconjugation mechanism as both strains related at species level. The results showed that beta-galactosidase productivity was improved in the trans-conjugated isolates which analyzed at DNA level to detect inserted plasmid through beta-galactosidase coding gene carried on it.

Keywords: β-galactosidase, enzyme, catalyzes, hydrolysis, non-reducing β-D-galactose

INTRODUCTION

β-galactosidase Is an enzyme that catalyzes the hydrolysis of terminal non-reducing β-D-galactose residues in β-D-galactosides, conventionally, its main application has been in the hydrolysis of lactose in milk or derived products, particularly cheese whey. More recently, β-galactosidases with transgalactosylation activities (i.e. which can oligomerisegalactosides) have been extensively exploited for the production of functional galactosylated products (**Carla, et al., 2011).**

Many organisms naturally synthesize β-galactosidase, including microorganisms, plant and animal cells **(Husain, 2010).** Traditionally, the β-galactosidases most widely used in industry. The β-galactosidase from Escherichia coli is the most extensively studied but its industrial use is hampered by the fact that it is not considered safe for food applications. Nevertheless, it is commercially available for analytical purposes. Finally, a preparation obtained from Bacillus sp. is also commercialized (**Carla, et al., 2011).**

The lactose hydrolyzing enzyme, β-galactosidase facilitates the reaction between the disaccharide molecules (Lactose) and water, thereby cleaving the oxygen bridge resulting in the production of two simple sugars (Glucose and Galactose). The enzyme has many application in food science including: Low lactose dairy product, Low lactose

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yogurt, Sweetened yogurt, Low lactose concentrate for ice cream, Lactose processing of acid and sweet whey, Food syrups and sweetner manufacture, Lactase treatment during cheese **(Shukla, 1975).** β-galactosidase has been widely used for industrial as well as medical application. In dairy industries, β -galactosidase has been used to prevent crystallization of lactose, to improve sweetness and to increase the solubility of the milk product for lactose-intolerant people and the production of galacto-oligosaccharides for use in probiotic food stuffs **(Gaur *et al.*, 2006; Maksimainen *et al.,* 2011; Guerrero, *et al.,* 2013 and Khedr *et al*., 2013)**.

Recombinant DNA technology can be used to express and optimize the production of interesting β-galactosidases from the most diverse sources in microbial hosts that are recognized for their highly efficient heterologous protein production (**Carla, et al., 2011).**This possibility greatly expands the range of potential applications for β-galactosidases and their economically effective utilization in industrial processes. Modern molecular biology tools combined with bioprocess engineering strategies can be used to optimize protein production, resulting in technically and economically effective enzyme production systems. Besides the wide-ranging properties offered by natural sources, new features – such as reduced product inhibition **(Park and Oh, 2010),** higher product yields **(Gosling et al., 2010)** or secretion signals may be built into specific β-galactosidases using state-of-the-art protein engineering tools.

Bacterial conjugation

Is important not only for bacterial evolution, but also for human health since it represents the most sophisticated form of HGT (horizontal gene transfer) in bacteria and provides, for instance, a platform for the spread and persistence of antibiotic resistance genes **(Norman et al., 2009).** To efficiently counteract the problems associated with antibiotic resistance it is therefore necessary to understand the mobile genetic elements conjugative plasmids (CPs) and integrative conjugative elements (ICEs) that are the vehicles for transfer of antibiotic resistance genes from the large communal gene pool to human pathogenic bacteria (**Günther and Maria 2014).**

Most plasmid vectors (such as pUC series viz. pUC8, pUC19, p Bluescript, pGEM-T series) carry a short segment of lacZ gene that contains coding information for the first 146 amino acids of β-galactosisdase. The host E. coli strains used are competent cells containing lacZΔM15 deletion mutation. When the plasmid vector is taken up by such cells, due to α-complementation process, a functional β-galatosidase enzyme is produced **(Sambrook *et al*., 1989).**

Suicidal plasmid strains were mobilized to recipient *E. coli* strains by transconjugation to introduce the transposon Tn5. Tn5 as a mutagen which lead to the appearance of different kinds of mutation. Kanamycin-resistant conjugants were selected. Transconjugant colonies were picked and characterized **(Khedr *et al*., 2013).**

Transposons are a powerful tool in molecular biology research and have been widely used to create mutant libraries in a wide range of genera (**Liu et al., 2013)** Such a mutant library is highly valuable as it allows high through-put screening aimed at the identification of genes essential for defined phenotypes (**Ruiz et al., 2013)**. In the present study we describe the implementation of a Tn5-based transposon mutagenesis system in two different bifidobacterial strains, B. breve UCC2003 and B. breve NCFB2258, for which relatively high transformation efficiencies have previously been achieved **(O'Connell et al., 2009** and **Ruiz et al., 2013)**. We also report the creation of a collection of nearly 20,000 transposon insertion mutants in our model strain B. breve UCC2003 which, to our knowledge, represents the first genome-wide random mutagenesis approach for bifidobacteria. Analysis of transposon insertion mutants by Southern hybridization and sequencing of transposon insertion sites confirmed nonbiased transposon insertion events. (**Ruiz et al., 2013)**. Phenotypic screenings for growth deficiencies in certain carbohydrates further allowed the validation of the usefulness of this mutant bank and revealed in most cases a direct and logical correlation between a particular growth-deficient phenotype and the mutation of a specific gene (**Ruiz et al., 2013)**.

MATERIAL AND METHODS

- **Media**

1. L.B. Broth (Laura Bertani Broth) is used for the growth, maintenance and fermentation of Escherichia coli strains used in molecular microbiology procedures.

L.B. Broth, is nutritional rich medium designed by Miller for growth of pure cultures of recombinant strains (Bertani, 1951).

2. Macconkey Agar

medium is a ready medium from (SRL) India-Fulka-biochemika Macconkey agar 1 70143 -500 g. This medium used for differentiate between E. coli strains that lactose-fermenting and others were non-lactose fermenting. This type of media is very efficient in detecting strains with (lac z) and (lac A) which encoding β-galactosidase and lactose permease enzymes, respectively (**Khedr et al., 2013**).

3. M9 minimal medium:

This medium used for detection the strains which can be grown on the minimal medium with lactose as only carbon source and this ability due to its β galactosidase activity

Table (1): Designed primers through Primer3 plus online to amplify Beta-gal gene carried on plasmids

Primers	Sequence
F-primer	3-TTTCCATGTTGCCACTCGCTTT-5
R-primer	3-GATGATGCTCGTGACGGTTAACGC-5

Table (2): PCR reaction mixture for gene amplification

Reagent	**Final concentartion**	**Amount (µl)**
Dream-Taq Buffer (Thermo scientific, formerly Fermentas, Lithuania)	1x	2.5
extracted DNA	40 ng	6
dNTPs	0.2 mM	1
Dream-Taq DNA polymerase (Thermo scientific, formerly Fermentas, Lithuania)	2.5U	2.5
$MgCl_2$	2.5mM	2.5
Primer (F)	20 pmol	1
Primer (R)	20 pmol	1
Deionized distilled H_2O	-	8.5
Total		25

and composed of 12.8 g Na2Hpo4, 3 g KH2po4, 0.5 g Nacl, 10 g NH4cl, 20.0 g Agar 0.49 g Mgso4.7H2o, 0.015 g Cacl2.2H2o, 0.01 g Feso4.7H2o, 0.01 g Thiamine and 2 g Lactose per liter (**Khedr et al., 2013**).

- **Enzyme assay:**

β-galactosidase activity was determined using o-nitrophenyl β-D-galactopyranoside (ONPG) as a substrate. Unless otherwise specified, β-galactosidase activity was assayed at 40°C by incubating 20 µL of suitably diluted enzyme with 480 µL of 22 mM o-nitrophenyl β-D-galactopyranoside (oNPG) in 50 mM phosphate buffer pH 6.5 as the substrate for 15 **(Volkin and Klibanov 1989).**The reaction was stopped by adding 750 µL of 0.4 M Na2CO3 and the o-nitrophenyl (ONP) released was determined by reading the increase in absorbance at 420 nm. One unit of β-galactosidase activity (U) was defined as the amount of enzyme releasing 1µmol of ONP from ONPG per minute under the given conditions (**Khedr et al., 2013** and **Princely, et al., 2013).**

- **Bacterial Tran conjugation:**

Overnight cultures of donor and recipient strains were diluted 50-fold in LB liquid medium. Both strains were incubated at 37 ºC with shaking to O.D. 0.40-0.60 at 600 nm. Donor and recipient cultures were combined in a ratio of 1:10 (v/v). Transconjugants were selected on medium supplemented with Kanamycin (Kmr) and Gentamycin (Gm) **(Khedr et al., 2013).**

- **Genomic DNA extraction**

Genomic DNA and plasmid isolated by using Alkaline Method Kit **(Khalil, 2011).** In an eppendorf, 1.5 ml from overnight culture were taken, centrifuged at 8,000 x g for 1 min, pellet was kept and 250 µl of solution A was added, mixed by pipetting. Then 250 µl of solution B was added and mixed by moving up and down three times. Then 250 µl of solution C was added and centrifuged at 13,000 x g for 5 min. Finally, the upper phase was removed into new eppendorf. After extraction of the DNA samples, an appropriate amount was transferred (about 25 µl) of each sample to a fresh eppendorf and 5µl of loading buffer was added (**Khedr et al., 2017).**

- **Beta-gal PCR detection and amplification**

Beta-gal gene coding functional Beta-D-galactosidase enzyme was detected and amplified by two specific primers as in **Table (1).**

Table (3): Bacterial strains used in this study

	code	genotype	Reference
1	109	*Escherichia coli* JM109	Khedr et al., 2013
2	7	*Escherichia coli* DH5α-7-lacZΔM15	Khedr et al., 2013
3	1	*Escherichia coli* k-12-1	Khedr et al., 2013
4	555	*Bacillus thuringiensis* 5	Khedr et al., 2013
5	888	*Bacillus thuringiensis* 8	Khedr et al., 2013
6	999	*Bacillus subtilis* strain M	Khedr et al., 2013
7	2021	*Escherichia coli* with Psu2021plasmid	Khedr et al., 2013
8	3411	*Escherichia coli* k-12-lacZΔM15	Khedr et al., 2013
9	111	*Bacillus subtilis* strain 111	Khedr et al., 2013
10	4	*Escherichia coli* DH5α-4 harboring pYV02 with Tn5 and GenR	Khedr et al., 2013
11	2	*Escherichia coli* DH5α-5-lacZΔM15	Khedr et al., 2013
12	6	*Escherichia coli* DH5α-6 harboring PUC18-LacZ ΔM15	Khedr et al., 2013
13	8	*Escherichia coli* DH5α-8harboring PUC18-LacZ ΔM15	Khedr et al., 2013
14	10	*Escherichia coli* DH5α- harboring pGDT4 with Gent^{R+}	Khedr et al., 2013
15	5011	*Bacillus stearothermophilus 5011*	Khedr et al., 2013
16	12	*Escherichia coli* k- harboring pGDT4 carrying Tn5 - and Kanamycin^{R+}	Khedr et al., 2013
17	13	*Escherichia coli* k- harboring pGDT4 carrying Tn5-Kanamycin^{R+}	Khedr et al., 2013

Table (4): Plasmids used in this study

Plasmid	Selectable marker
PUC18	Ampicillin complete beta-gal gene
pGDT4	Kanamycin OR Gentamycin partial beta-gal gene
Psup202	Neomycin partial beta-gal gene

RESULTS

1. Screening for Beta-galactosidase producing bacteria

Different seventeen bacterial strains **(Table 3)** were obtained from (**Khedr, et. al., 2013)** who tested for their enzyme productivity both qualitatively and quantitively on whey agar medium and LB flasks respectively. Strains 10, 12, 4 and 13 were harboring suicide plasmids with transposons Tn5 and Tn7, while strain 2021 harboring psup 202 plasmid with complete b-galactosidase gene. (Khedr, et. al., 2013) modified all these plasmids **(Table: 4)**

Among seventeen strains, four were the best producer (1, 4, 6 and 10) and strain no. 6 is the best producer with 30 IU/ml after 24h of incubation. Two strains 5011 and 999 showed neither enzyme activity on LB nor growth on whey agar medium **(Table 5)**

2. Bacterial transconjugation

Three strains (10, 12 and 13), carrying suicidal plasmid used as donors. Suicidal plasmids were mobilized to *Escherichia coli* strains 6 and 8 as recipient strains by transconjugation to introduce the transposons Tn5 and

Table (5): Beta-galactosidase activity of the tested strains and qualitative screening on whey agar plates

Strain	whey plates	enzyme activity IU/ml
1	WG	7.93
2	G	10
4	WG	7.5
6	WG	12
8	WG	4.98
10	WG	12.78
109	G	0.77
7	G	0.6
12	G	10.4
13	WG	11.81
5011	NG	0
999	NG	0
111	G	0.66
3411	G	0.89
2021	G	1.17
555	G	7.16
888	G	7.20
*WG: well grown 5-10mm, G: poor grown1-5mm, NG: not grown		

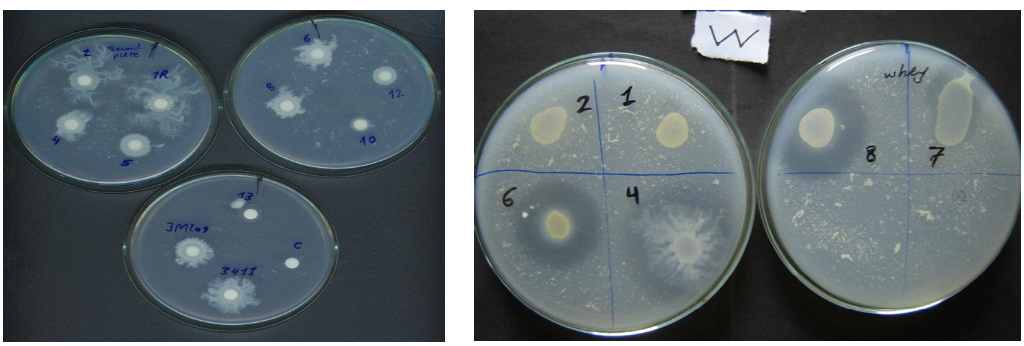


Figure (1): screening of bacterial strains on whey agar plates as a qualitative method for enzyme production.

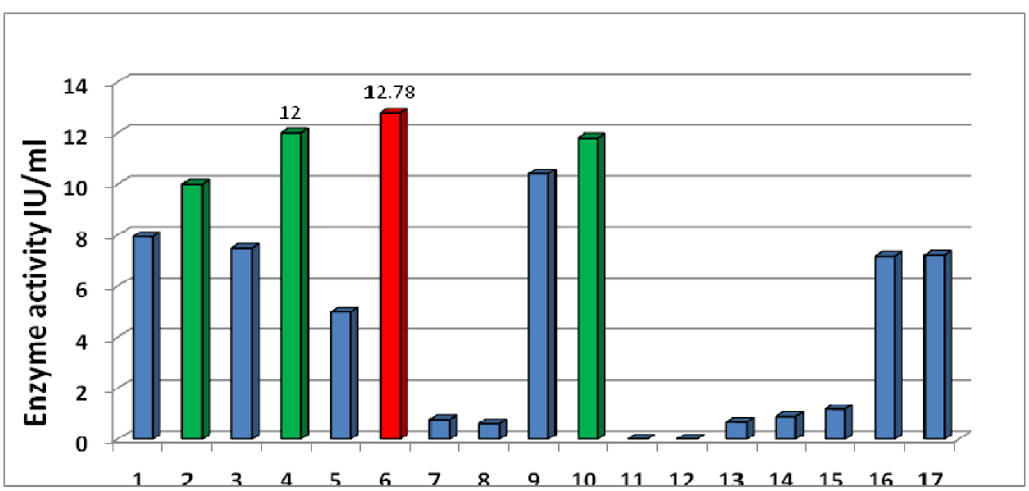


Figure (2): Enzyme productivity (IU/ml) of seventeen bacterial strains on LB after 24h of incubation.

Table (6): Enzyme productivity from transconjugated isolates and their parents after 24 h of incubation in LB at pH7 and 37^{o}C

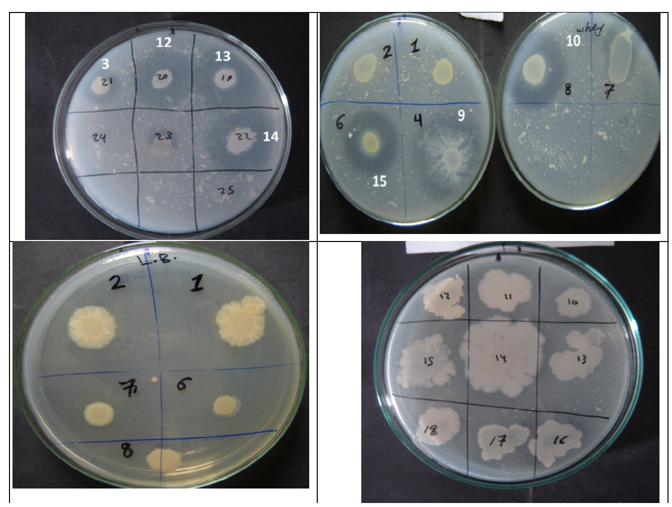
Isolated trans conjugant	Tn5 donor	Tn5 recepient	Enzyme activity U/ml
1	10	6	35
2	10	6	44
3	10	6	37
4	10	6	40
5	10	6	34
6	10	6	45
7	10	8	40
8	12	8	45
9	12	8	46
10	12	6	39
11	12	6	36
12	12	6	47
13	13	6	49
14	13	6	45
15	13	8	34
Donor	10		11
	12		9
	13		23
Recipient		6	33
		8	30

Tn7. Transposon as a mutagen can lead to the appearance of different kinds of mutation.

Transconjugants were carried out on medium supplemented with Kanamycin (Km), Gentamycin (Gm) and Neomycin (Nm). Negative controls were prepared by plating donor and recipient strains separately on selective medium. Kanamycin-ampicillin resistant transconjugants colonies were picked and characterized.

Selected transconjugants were tested for their ability to grow in the present of lactose as a sole carbon source to prove its β-galactosidase production. Also transconjugants were grown in fermentation liquid medium. ONPG were used to assay β-galactosidase production.

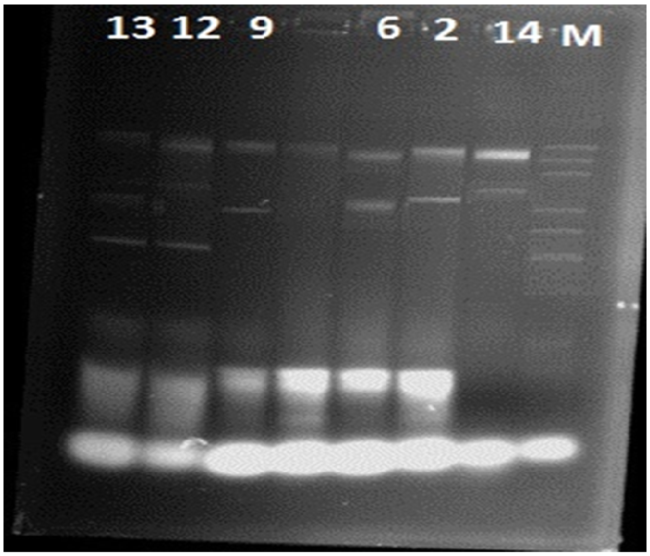
Six transconjugants (2, 6, 9, 12, 13, and 14) out of fifteen showed their maximum Beta-galactosidase productivity after 24 hours which reflect the same behavior of their parental strains (Recipient and donors). While nine transconjugants in addition to their parental strains (Recipients) prove their maximum β-galactosidase productivity after 48 hours **Table (6).**



3. Molecular isolation and amplification Beta-gal gene

The best producer trans-conjugated isolates were tested for detection of both two harboring plasmids (one from donor and another from recipient), Beta-gal gene was amplified through thermo cycler PCR and designed two primers **(Table 1)** and the expected PCR product size 450-500 bps.

Six trans conjugates 2, 6, 9, 12, 13, and 14 were used as a template for isolation and amplification of Beta-gal gene



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