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Development and Characterization of Engineered Commensal Bacteria for the Delivery of Enzymes

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The present study was aimed at development and characterization of engineered commensal bacteria for site-specific targeting of enzymes. Separation of plasmid (pJR_2 and GB_4) and transformation in *Lactobacillus acidophilus* was carried out using a reported method. Azo polymers were synthesized using water-soluble and water- insoluble monomers belonging to acrylate series. The synthesized polymers were characterized for physical appearance, solubility and film forming properties. Effect of microbial flora of colon on polymers was seen by incubating the glass cover slips coated with azo polymers in sterilized nutrient broth containing freshly voided human fecal culture suspension. Colon-specific drug delivery system was developed by mixing the bacterial pellet with skimmed milk and then coating these granules with azo- and pH-sensitive polymer. Lowering of galactose concentration and α-oxoglutaric acid concentration were monitored to *in vitro* characterize *E. coli* (56) and *E. coli* (797), respectively. *In vivo* screening of developed oral, colon-specific formulation, azo polymer coated granules of engineered *E. coli* (56) producing galactokinase (GK) and engineered *E. coli* (797) producing glutamate dehydrogenase (GDH) was performed on rabbits. D-galactose, which is the substrate for enzyme galactokinase and ammonium chloride, was administered intraperitoneally to all rabbits. The lowering of blood galactose level and urea level was observed with respect to variable time interval.

Although oral route is the most attractive, acceptable, natural and convenient for administration of drugs yet oral delivery of peptides and proteinous drugs is limited due to barriers such as enzymatic degradation in the gastrointestinal tract, low epithelial permeability and instability under formulation conditions[1]. The colon has recently received great attention as a potential site for the delivery of pharmaceutical entities[2-4]. Colon targeting may be achieved in several ways such as the use of prodrugs[5,6], pH dependent coatings, microbially degradable polymers[7] and azo polymers[8,9]. Since it is known that azo functions can be reduced in the colon[10] a lot of novel polymers containing azo groups either in the polymeric backbone[11] or in the crosslinks[12,13] have been synthesized. The site-specific delivery to the colon would be beneficial for the delivery of proteins, which are degraded by the digestive enzymes in the stomach and small intestine.

Cellular systems like commensal bacteria may offer the way of protein delivery through gastrointestinal tract mucosa via site-specific drug delivery system. In order to provide site specific targeting of protein release, a new live vector system, namely, recombinant commensal bacteria have been developed[14]. They have been evolved to colonise a specific niche such as the oral cavity, the gut, the urogenital tract or the rectum. *E. coli* is the normal resident of large intestine. In the present study genetically engineered live vector system, recombinant commensal bacteria, *E. coli/ Lactobacillus acidophilus* producing/releasing, an enzyme

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(GK/GDH) for site specific targeting of an enzyme, packed as granules and coated with polymers which are helpful in lowering blood substrate level, has been investigated. After disintegration of granules the recombinant strain will express heterologous proteins on their surface or secret into the colon. These engineered bacteria will use conserved pathway to express and anchor their surface protein to colon and provide prolonged delivery of an enzyme[14].

This approach can be used to park a recombinant commensal at a specific location where it can colonise, grow and subsequently secrete a biological active protein (e.g. a hormone or an enzyme) to produce a desired pharmacological effect at the correct local physiological site.

MATERIALS AND METHODS

Ampicillin was generously supplied by M/s Esteem Pharmaceuticals, Sagar. Ethidium bromide, L. B. broth, peptone, agar, agarose were procured from HIMEDIA, New Delhi. ATP, lysozyme, NADH, D-galactose and galactose oxidase were procured from Sigma, St. Louis, USA. All other reagents were of analytical grade and water was double distilled.

Selection of bacterial strain and growth media:

Lactobacillus acidophilus (447), *E. coli* (56) and *E. coli* (797) were the bacterial strains selected for present study[15] (Table 1). Special features of bacterial strains are presented in Table 2. Various growth media containing different quantities of various nutrients were tried for growing bacterial strains and two growth media were selected. A medium (growth medium A) that has been found suitable for *Lactobacillus acidophilus* (447) consisted of skimmed milk (100 g), tomato juice (100 ml), yeast extract (5 g) and distilled water (1 l). Tomatoes were filtered and left over night at 10°. The pH was adjusted to 7.0. Optimum growth medium for *E. coli* (56) and *E. coli* (797) (growth medium B) consisted of beef extract (1 g), yeast extract (2 g), peptone (5 g), sodium chloride (5 g), agar (15 g), ampicillin (25 mg) and distilled water (1 l).

Separation of plasmid and transformation:

The method reported by Chakrabarti and Ghose[15] was used for separation of plasmid and transformation. It consisted of following steps: *E. coli* carrying pJR_2 (MTCC 797) in Luria Bertani (LB) medium was grown overnight at 37°. *E. coli* culture (1.5 ml) was transferred to an Eppendorf tube and spun for 3 min at 10 K. The supernatant was discarded and the pellet was resuspended in 300 µl of Trans Element Solution (TES) and transferred to a 1.5 ml Eppendorf tube. Lysozyme solution (100 µl) was added and incubated at 37° for 30 min with intermittent shaking. 200 µl of 0.3M alkaline sodium decodyl solution was added, vortexed well, incubated at 55° for 30 min and cooled in a water bath to room temperature. Acid phenol: chloroform (600 µl) was added, vortexed well and spun at 10 K for 10 min. The upper aqueous phase (700 µl) was transferred into a new Eppendorf tube and 70 µl of 3 M sodium acetate (unbuffered) and 700 µl of isopropanol were added. It was mixed well and kept at

TABLE 1: BACTERIAL STRAINS SELECTED FOR PRESENT STUDY.

MTCC NO.	BACTERIA	INCUBATION TIME (H)	INCUBATION TEMPERATURE (0^0)	GROWTH MEDIUM	SUB-CULTURING (D)
447	*Lactobacillus acidophilus*	24	37	A	30
797	PjR_2 (in *Escherichia coli*)	24	37	B	30
56	GB_4 (in *Escherichia coli* SF_6)	48	37	B	30

TABLE 2: SPECIAL FEATURES OF SELECTED BACTERIAL STRAIN.

MTCC No.	Name of bacteria	Special features
447	*Lactobacillus acidophilus*	Good strain for transformation
797	PjR_2 (in *E.coli*)	PJR_2 (cloned glutamate Dehydrogenase genes into PVC_8 from *Neurospora*), Amp (R)
56	GB_4 (in *E.coli* SF_6)	Plasmid GB_4, Amp (R), Plasmid contains *S. cerevisiae* galactokinase gene

room temperature for 5 min and spun at 10 K for 10 min. The supernatant was discarded and the pellet was dissolved in 270 µl of TES. 30 µl of 3M-sodium acetate (pH 5.8), 3 µl of 1M magnesium chloride and 700 µl of absolute ethanol were added, mixed well and kept at -20° for 2 h and spun at 10 K for 10 min. The supernatant was discarded and the pellet was dissolved in 500 µl of TES and 10 µl was analyzed on 1% agarose gel.

Transformation of pJR_2 in *Lactobacillus acidophilus*:

LB medium (20 ml) was inoculated in a 100 ml flask with 200 µl of overnight culture of *Lactobacillus acidophilus* (1:100) and grown at 37° with shaking till the value of O.D590 was reached at 0.2. The resultant culture (1.5) ml was removed into a tube pre-chilled to 0° in an ice water bath and centrifuged at 4000 rpm for 5 min at 4° and drained out completely. The pellet was resuspended in 0.5 ml ice-cold, 0.5 M $CaCl_2$, incubated at 0° for 35 min followed by centrifugation at 3000 rpm for 5 min at 4°. The pellet was resuspended in 150 µl of ice-cold 0.5 M $CaCl_2$ and incubated at 0° for 1 h. The suspension was divided into three tubes, 5 µl and 1 µl DNA were added to the first and second tube, respectively, while third tube served as control. The samples were incubated at 0° for 1 h heat shocked at 42° for 2 min and immediately removed to ice-water (0°). LB medium (500 µl) was added to each tube and incubated at 37° under stationary conditions (to allow phenotypic expression of the drug resistance genes) in growth media containing ampicillin, for 1 h. It was centrifuged at 1000 rpm for 5 min. Most of the liquid was drained off leaving behind approximate 100 µl. The pellet was resuspended on selective plates. Similar methods were used for the separation and transformation of plasmid GB_4

Synthesis of polymers and characterization:

In the present study the azo polymers were synthesized and azo polymers and Eudragit polymers were used to coat the dosage forms (granules) for site specific delivery of commensal bacteria to colon.

Selection of monomer:

The water-soluble and water-insoluble monomers belonging to acrylate series were selected for the present study. Water-soluble monomer, acrylic acid and water-insoluble monomer methyl methacrylate in different ratios (1:3, 1:1 and 3:1) were used for synthesis of azopolymer.

Synthesis of azopolymers:

The azopolymers were synthesized by bulk polymerisation technique. The monomers were taken in a specific ratio in a flask and azobisisobutyronitrile (2 ml) was used as the initiator and methyl orange (0.1 ml of 2%, in ethyl alcohol) was used as the cross-linker. The flask was attached with a water condenser and a stirrer. The temperature was maintained at 70±2° using constant temperature bath. The contents were stirred until high viscosity was attained. The polymerization was stopped by the addition of hydroquinone solution (10 ml of 0.02%w/v solution in ethanol) as the polymerization inhibitor. The polymers were purified by dissolving in respective solvent and crystallized by evaporation of solvent. Finally, the polymers were dried in a vacuum oven at 60°.

Characterization of polymers:

The synthesized copolymers and Eudragit-S 100 polymer were characterized for physical appearance, solubility, film forming properties and effect of microbial flora on colon. The synthesized co-polymer and Eudragit-S 100 copolymer were observed for physical appearance such as hardness and colour. A weighed quantity (2 g) of the copolymer was mixed with 100 ml of solvent for determination of solubility. The mixture was kept for a period of 24 h with intermittent shaking at room temperature and the extent to which the co-polymer dissolved in a particular solvent was determined.

For determination of film forming properties, 2% solution of co-polymers in organic solvents containing 2% and 5% plasticizers (dibutylphthalate) were transferred in a petridish containing mercury as substrate, the area of the film cast being controlled by a glass ring. Each petridish was covered by an inverted glass funnel of suitable diameter to protect the film from atmospheric moisture and to control the rate of solvent evaporation, which could affect the appearance of film. These films were visually observed for their appearance.

Effect of microbial flora of colon on polymers was seen by using the glass cover slips coated with the prepared azo polymers by dipping in the solution of polymers and evaporating the solvent. These cover slips were dipped in sterilized nutrient broth containing freshly voided human faecal culture suspension and incubated at 37±1° for 2-8 d. After incubation, the cover slips were washed and observed under optical microscope.

Development of colon-specific drug delivery system:

In the present work, bacterial pellets were prepared and coated with the azo and pH sensitive polymers to release

bacteria selectively in the colonic environment. An overnight 5 ml bacterial culture was considered to be optimum for delivering enzymes in to the colon. Accurately measured bacterial culture (5 ml) was centrifuged at 600-800 rpm for 15 min. Supernatant was discarded and the pellet was mixed with skimmed milk. These granules containing bacteria were coated with the polymers.

Coating of granules:

A 2.0% w/v solution of azo and Eudragit-S 100 copolymers was prepared in respective organic solvents. Granules were coated aseptically six times by dipping into the solution of the copolymers to ensure the formation of a uniform and thin coating over the granules. The film was allowed to dry in the air and stored in well-closed containers for further studies.

***In vitro* characterization:**

In vitro release studies may provide valuable information of the product behaviour *in vivo*. *In vitro* release of enzymes was carried out in an overnight bacterial culture of *E. Coli* (56) and *E. Coli* (797) producing GK and GDH, respectively. Further, the studies were extended to *Lactobacillus acidophilus* containing the plasmid for the expression/production of GK and GDH.

***In vitro* release of GK:**

An overnight culture (5 ml) of *E. Coli* (56) was taken to study the release of GK. Galactose (100 mg), the substrate for enzyme (galactokinase) was dissolved in distilled water. ATP (0.1 mmol) and $MgSO_4$ (0.1 mmol) were dissolved in the above solution. An overnight culture of *E. Coli* (56) was transferred into it and the pH of test solution was immediately adjusted to 7.0. The solution was kept in a metabolic shaker at 25° for 1 min. After this, the test solution was dipped in a water bath at 70° for 5 min. The test solution was centrifuged (5°) at 3000 rpm for 10 min. Supernatant (0.5 ml) was taken out and diluted to 100 ml and was analysed spectrophotometrically for the lowering of galactose content. The similar treatment was applied with an overnight culture of *Lactobacillus acidophilus* containing plasmid for the expression of galactokinase. The galactose concentration was measured spectrophotometrically at 515 nm.

***In vitro* release of GDH:**

An overnight culture (5 ml) of *E. Coli* (797) was taken for studying the *in vitro* release of GDH. One hundred milligrams of α-oxoglutaric acid (α-ketoglutaric acid), the substrate for the enzyme GDH was dissolved in distilled water. NADPH (0.1 mmol) and ammonium chloride (0.1 mmol) were dissolved in the solution containing α-oxoglutarate. An overnight culture of *E. Coli* (797) was transferred into the above solution and the pH of test solution was immediately adjusted to 8.3. The test solution was kept in a metabolic shaker at 30° for one min and then dipped in a water bath at 70° for 5 min. The solution was centrifuged (5°) at 3000 rpm for 10 min. After centrifugation, 0.5 ml of supernatant solution was diluted to 10 ml and analyzed spectrophotometrically for the lowering of substrate (α-oxogultarate) concentration. The above treatment was also applied to an overnight culture of engineered *Lactobacillus acidophilus* containing plasmid for the expression of GDH. The lowering of substrate (α-oxoglutarate) concentration was measured spectrophotometrically at 525 nm.

***In vivo* evaluation:**

The *in vivo* studies of a designed formulation are very important criteria in successful development of a drug carrier system. In the system designed for targeted delivery of enzyme, the blood levels of substrate for that enzyme were measured. The *in vivo* evaluation of formulations was conducted on rabbits.

***In vivo* evaluation of formulations containing GK:**

Rabbits of either sex weighing 2.5±0.25 kg were divided into four groups, the first group serving as control. The second group was administered with ampicillin (Amp), (100 mg), while the third and fourth groups were taken as non-ampicillin (Namp1 and Namp2) treated animals. One millilitre of 10 mg/ml D-galactose, which is substrate for enzyme galactokinase, was injected intraperitoneally to all rabbits. The formulation azopolymer coated granules of *E. coli* (56) (AEGK) producing GK, was administered orally to groups second and third. The fourth group was administered with formulation, Eudragit-S 100 coated granules of *E. coli* (56) (EEGK) producing GK. The lowering of blood galactose level in rabbits was determined with respect to variable time interval. The above said treatment was repeated for formulations; ALGK and ELGK containing engineered *Lactobacillus acidophilus* having the plasmid for the expression/production of GK.

***In vivo* evaluation of formulations containing GDH:**

Rabbits were divided into four groups as above. One millilitre of 10 mg/ml ammonium chloride injection was given intraperitoneally to all rabbits. The formulation azopolymer coated granules of *E. coli* (797) (AEGDH) producing GDH was administered orally to second and third groups respec-

tively while the fourth group was administered with formulation, Eudragit-S 100 coated granules of *E. coli* (797) (EEGDH) producing GDH. The lowering of blood urea level in rabbits was monitored with respect to variable time interval by using Technicon Autoanlyser (RA 50, USA). The above treatment was iterated for formulations ALGDH and ELGDH containing engineered *Lactobacillus acidophilus* bearing plasmid for the expression/production of GDH.

RESULTS AND DISCUSSION

The site-specific delivery of drug may be achieved by coating the drug with polymers, which can release the drug exclusively in the colon. Therefore azopolymers were synthesized for the coating of dosage form to release drug at the colon. These polymers were synthesized by bulk polymerization technique using various ratios of water-soluble and water-insoluble monomers. Azobisisobutyronitrile was used as an initiator and methyl orange was used as a cross-linker because the azo bonds (cross-links) are cleaved by azoreductase enzyme of microflora (azobacters) present in the colon.

The bulk polymerization technique had the advantage that the product was obtained in a high state of purity. The synthesized copolymers were characterized for physical appearance (Table 3) and solubility studies (Table 4). The physical characteristics indicated that the higher molecular weight copolymers had hard and tough nature while the lower molecular weight copolymers were soft and tacky. The synthesized polymers showed good film forming properties in the presence of plasticizer, dibutylphthalate.

The solubility studies revealed that copolymers of methylmethacrylate and acrylic acid were best soluble in toluene: methanol mixture (80:20) where as Eudragit-S 100 was best soluble in acetone. Solubility studies indicate that Eudragit-S 100 is insoluble in acidic medium but soluble in the buffer of pH 7.0 (Table 4). The microbiological studies showed that the human faecal culture containing azobacters were capable of and responsible for breaking the polymeric azo bonds. Human faeces was used as a source of microorganisms to minimize the contact of the microflora with oxygen and had the advantage of being suspended quickly in the medium under anaerobic conditions. The long incu-

TABLE 3: PHYSICAL APPEARANCE OF CO-POLYMERS.

Co-polymer	Physical appearance
Poly (AA-Co-MMA) 1:3	Hard, crystalline transparent solid
Poly (AA-Co-MMA) 1:1	Crystalline, tough solid, orange coloured
Poly (AA-Co-MMA) 3:1	Reddish-orange sticky mass
Eudragit- S 100	Crystalline white coloured powder

TABLE 4: SOLUBILITY OF CO-POLYMERS.

Co-polymer	Ether	EtOH	Benzene	Acetone	Toluene	Toluene: Methanol (80:20)	Buffer pH 6.0	Buffer pH 7.4
Poly (AA-Co-MMA) 1:3	-	+	-	+	-	+++	-	-
Poly (AA-Co-MMA) 1:1	-	+	-	+	-	+++	-	-
Poly (AA-Co-MMA) 3:1	-	+	-	+	-	+++	+	-
Eudragit-S 100	+	+	+	+++	+	+	+	+++

+++ indicates highly soluble (1 to 10 parts of solvent) while + indicates partially soluble (30 to 100 parts of solvent) and - indicates insoluble (more than 1000 parts of solvent).

bation time of 2-8 d was required due to the fact that the *in vitro* tests on polymer films were carried out with a relatively dilute suspension of microflora, in contrast to the very concentrated population in the colon, where reduction of the azo bonds could occur within minutes.

The synthesized azo polymer and Eudragit-S 100 were used to coat the granules prepared by bacteria and skimmed milk. Granules were dipped in copolymer solutions six times to ensure the uniform coating of copolymer.

In vitro studies were performed on the culture media as azopolymers and Eudragit-S 100 coated granules, which released bacteria at specific environmental and pH conditions. ATP and NADP were mingled with the solution as coenzyme. ATP acts as energy currency for exchange of energy between exergonic and endergonic reactions. NADP removes electrons and hydrogen ions from reduced substrate. Mg^{++} acts as cofactor. It is assumed that these metal ions function in combination with enzyme, and hence regarded as inorganic coenzyme. Sometimes both, a cofactor and a coenzyme (organic) are required before an enzyme becomes active[16].

The test solution was adjusted to different pH (7.0 and 8.3) and temperature (25° and 30°) for determining the amount of enzyme produced. Therefore enzymatic reaction was allowed for only one min. After one min the test solutions were kept in a water bath at 70° for denaturing the enzyme, which terminated the enzymatic reactions. Enzymes being proteins, therefore exposure towards high temperature denatures them by destroying their tertiary and quaternary configuration. Test solution was centrifuged in a refrigerated centrifuge to avoid the effect of heat generated during centrifugation and for suspending the bacteria and other particulate material.

In case of *E. coli* (56), 14.9±1.48 % lowering of galactose concentration was observed, while it was 12.4±1.49 % in case of engineered *Lactobacillus acidophilus* producing GK (Table 6). In case of *E. coli* (797), 14.4±1.13 % lowering of α-oxoglutarate concentration was observed, while it was 9.8±1.40 % in case of engineered *Lactobacillus acidophilus* producing GDH (Table 7). The engineered *Lactobacillus acidophilus strain* showed low production of GK and GDH (low *in vitro* activity) as compared to engineered *E. coli* strains indicating lack in expression/production of enzyme.

For *in vivo* studies the formulation containing viable engineered *E. coli* and *Lactobacillus acidophilus* were used.

TABLE 5: EFFECT OF PLASTICIZERS ON FILM FORMING PROPERTIES OF POLYMER.

Co-polymer	2% DBT	5% DBT	2 %TGT	5 % TGT
Poly (AA-Co-MMA) 1:3	+	++	+	+
Poly (AA-Co-MMA) 1:1	++	++	+	++
Poly (AA-Co-MMA) 3:1	++	+++	++	++
Eudragit- S 100	+	++	+++	++

+++ indicates best film forming property, ++ indicates good film forming property and + represents poor film forming property.

TABLE 6: *IN VITRO* RELEASE STUDIES FOR GK.

Bacterial strain	Amount of substrate (mg/ 100 ml)	Amount of substrate remaining after 1 min (mg)	Amount of substrate consumed (mg)	Percent lowering in substrate concentration	I.U.* produced in 5ml overnight culture
E.coli(56)	100	85.1±1.55	14.9±1.48	14.9±1.48	82.7±1.87
Engineered *Lactobacillus acidophilus*	100	87.6±1.46	12.4±1.49	12.4±1.49	68.8±1.41

*GK: 1 unit will convert 1.0 μM of D-galactose to galactose 1- phosphate per min. at pH 7.0 at 25°. Each value represents mean±SD (n= 3).

TABLE 7: *IN VITRO* RELEASE STUDIES FOR GDH.

Bacterial strain	Amount of substrate (mg/ 100 ml)	Amount of substrate remaining after 1 min (mg)	Amount of substrate consumed (mg)	Percent lowering in substrate concentration	I.U.* produced in 5ml overnight culture
E.coli (797)	100	85.6±1.24	14.4±1.13	14.4±1.13	98.6±0.68
Engineered *L. acidophilus*	100	91.2±1.45	9.8±1.40	9.8±1.40	69.0±1.12

*GDH: 1 unit will reduce 1.0 μM of α-oxoglutarate to 1-glutamate per min at pH 8.3 at 30° in the presence of ammonium ions and NADPH. Each value represents mean±SD (n= 3).

These formulations were given orally to experimental animals (rabbits). Coating of azopolymers and Eudragit-S 100 made the formulation specific for colon delivery.

Among the four groups of experimental animals, one group was treated with ampicillin. The dose of ampicillin (100 mg) partially destroyed the normal flora present in the colon and could facilitate the establishment of engineered commensal bacteria at the desired site. Subsequently a dose of enzyme substrate (galactose and ammonium chloride) was given to all animal groups. Enzyme concentration does not affect the reaction rate while in equilibrium state[7]. Therefore the equilibrium was disturbed by intraperitoneal dose of substrate. The blood substrate level was monitored at time intervals of 6, 12 and 18 h. It takes 1-2 h for material to pass through stomach[9].

The close observation and interpretation of these data reveal that azopolymer coated formulation showed good results in Amp rabbit in comparison to azopolymer and Eudragit-S 100 coated formulations in Namp1 and Namp2 animals, respectively. After 18 h 46.6±1.84 % lowering of blood galactose level was observed in Amp animals (AEGK), while it was 35.1±1.32 % and 19.8±0.95 % in Namp1 (AEGK) and Namp2 (EEGK) animals, respectively (Tables 8 and 9). Similarly after 18 h, 38.1±1.56 % lowering of blood urea level was observed in Amp (AEGDH) animals, while it was 29.6±1.24 % and 27.0±1.48 % in Namp1 (AEGDH) and

TABLE 8: PERCENTAGE LOWERING OF GALACTOSE IN COMPARISON TO CONTROL ANIMAL AFTER ADMINISTRATION OF AZOPOLYMER COATED FORMULATION.

Blood galactose level in control animal (mg/dl) after			Formulation	Blood galactose level in Amp animal (mg/dl) after			Blood galactose level in Namp1 animal (mg/dl) after		
6 h	12 h	18 h		6 h	12 h	18 h	6 h	12 h	18 h
0	11.34±0.87	17.0±0.72	AEGK	1.52±0.32	35.27±1.77	46.58±1.84	1.53±0.28	16.94±1.15	35.14±1.32
0	11.34±0.87	17.0±0.72	ALGK	0.0	14.13±1.08	31.07±1.64	0.0	12.15±0.98	27.06±1.32

Each value represents mean±SD (n= 3).

TABLE 9: PERCENTAGE LOWERING OF GALACTOSE IN COMPARISON TO CONTROL ANIMAL AFTER ADMINISTRATION OF EUDRAGIT-S 100 COATED FORMULATION.

Blood galactose level in control animal (mg/dl) after			Formulation	Blood galactose level in Namp2 animal (mg/dl) after		
6 h	12 h	18 h		6 h	12 h	18 h
0	11.34±0.87	17.0±0.72	EEGK	2.93±0.19	12.97±0.56	19.81±0.95
0	11.34±0.87	17.0±0.72	ELGK	1.55±0.38	10.88±0.57	17.79±0.69

Each value represents mean±SD (n= 3).

TABLE 10: PERCENTAGE LOWERING OF UREA IN COMPARISON TO CONTROL ANIMAL AFTER ADMINISTRATION OF AZOPOLYMER COATED FORMULATION.

Blood urea level in control animal (mg/dl)			Formulation	Blood urea level in Amp animal (mg/dl)			Blood urea level in Namp1 animal (mg/dl)		
6 h	12 h	18 h		6 h	12 h	18 h	6 h	12 h	18 h
0	11.34±0.87	17.0±0.72	AEGDH	0.0	30.51±0.98	38.14±1.56	0.0	18.97±0.57	29.55±1.24
0	11.34±0.87	17.0±0.72	ALGDH	0.0	26.54±1.32	29.59±0.88	0.0	15.26±1.11	27.87±1.96

Each value represents mean±SD (n= 3).

TABLE 11: PERCENTAGE LOWERING OF UREA IN COMPARISON TO CONTROL ANIMAL AFTER ADMINISTRATION OF EUDRAGIT-S 100 COATED FORMULATION.

Blood urea level in control animal (mg/dl) after			Formulation	Blood urea level in Namp2 animal (mg/dl) after		
6 h	12 h	18 h		6 h	12 h	18 h
0	11.34±0.87	17.0±0.72	EEGDH	3.45±0.25	17.56±0.84	26.98±1.48
0	11.34±0.87	17.0±0.72	ELGDH	2.43±0.28	14.36±1.04	23.25±0.89

Each value represents mean±SD (n= 3).

Namp2 (EEGDH) animals respectively (Tables 10 and 11). This could be due to the possibility that ampicillin might have partially destroyed the bacteria present in the colon. This might facilitate the establishment of commensal bacteria at the desired site e. g. colon. In Eudragit-S 100 coated granules, most of the granules might have released the commensal bacteria in terminal ileum and proximal part of large intestine due to degradation of formulation at neutral pH of ileum, which are not desired sites for establishment of commensal bacteria. No activity was seen in first 6 h that may be due to the long gastric retention (transition) time. In first 6 hr slight lowering of blood substrate level was observed in Namp2 animals indicating partial dissolution of Eudragit-S 100 coated granules in the small intestine.

Engineered *E. coli* containing formulations (AEGK, AEGDH, EEGK and EEGDH) showed better results in comparison to engineered *Lactobacillus acidophilus* containing formulations (ALGK, ALGDH, ELGK, ELGDH,). After 18 h 46.6±1.84 % lowering of blood galactose level was observed in Amp animals (AEGK) while it was 31.1±1.64 % in Amp animals administered with formulation ALGK (Table 8). Similarly 38.1±1.56 % lowering of blood urea level was observed (AEGDH) while it was found to be 29.6±0.88 % in another animal (ALGDH) that could be due to lesser expression/production of enzyme on the surface of engineered *Lactobacillus acidophilus* (Table 10). It could also be possibly due to the poor adaptation of *Lactobacillus acidophillus* in colon based on the observation that *E. coli* count is more than that of *Lactobacillus acidophillus* in colon.

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