Int. J. Adv. Pharm. Biotech., 2015; 1(2): 1-8
doi.org/10.38111/ijapb.20150102001

IJAPB

International Journal of Advances in Pharmacy and Biotechnology
Vol.1, Issue-2, 2015, 1-8

Research Article
Open Access

Isolation and Purification of Exopolysaccharides and Exploring its Potential as an Excipient in the Development of Suitable Formulation

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Received: 23 August 2015 Revised: 05 September 2015 Accepted: 29 September 2015

ABSTRACT:
Exopolysaccharides (EPS) are high-molecular-weight polymers, excreted by some microorganisms onto the outside of their cell walls. EPS are primarily composed of carbohydrate and some non-carbohydrate substituents, such as acetate, phosphate, pyruvate and succinate. The study was aimed at isolation of EPS from *pseudomonas* strains and milk samples and also preliminary trials were carried with sea water, sea sediment and soil samples. Based on the trial study *pseudomonas* and milk sample (*lactobacilli)* were selected for EPS production. The *pseudomonas* strains were then subcultured in cetrimide media and lactic acid bacteria strains were subcultured in milk media (50%) supplied with 50% carbohydrate source maintained at 20º C for 72 hours. The EPS obtained from milk sample was highly viscous in nature, good smelling and high yield. The EPS obtained from *pseudomonas* strains didn't show satisfactory yield. The EPS obtained from lactobacilli was characterized and used as gelling agent in formulating a Diclofenac diethylamine gel. The formulated gel was evaluated and compared to marketed formulation for gelling agent, consistency and performance.

Key words: Exopolysaccharides, *Pseudomonas*, *lactobacilli*, Gelling agent.

INTRODUCTION

Bacterial EPSs are biopolymers that are secreted by the cells and form a capsule that remains associated with the cell surface or a slime that is loosely bound to the cell surface. These biopolymers are mainly composed of carbohydrates, with glucose, galactose and mannose being the most common monomers. There has been a marked upsurge of interest and steady increase in the exploitation of microbial exopolysaccharide due to their unique and novel properties. The physicochemical properties of polysaccharide solutions are providing a new insight into the physical structures of these polymers and furnishing the industrialist with clearer indication of their useful properties. The greatest potential of bacterial EPS is related to their use in high value market niches, such as cosmetics, pharmaceuticals and biomedicine, pharmacological, nutraceutical, functional food, herbicides and insecticides in which traditional polymers fail to comply. The increased interest in microbial polysaccharides reflects a growth in the use of water soluble polymers and also an appreciation of the environmental advantages to be gained from use of water soluble rather than solvent based systems[1,2]. The overwhelming diversity of bacterial polysaccharides allows for categorization based on chemical structure, functionality, molecular weight and linkage bonds[3,4]. The diversity of bacterial polysaccharides allows for categorization based on chemical structure, functionality, molecular weight and linkage bonds. Extracellular polysaccharides show considerable diversity in their composition and structure and are synthesized by bacteria of all taxa. Exopolysaccharides occur widely, especially

among prokaryotic species but they are less common among yeasts and fungi[5].

Few microbial genera form components of more complex structures which may be involved in different morphogenetic cycles such as those found in the families *Azotobacteriaceae* and *Myxobacteriaceae*. In each of these bacterial groups, exopolysaccharides are associated with normal vegetative cells and with resting cells in the form of microcysts. The presence of exopolysaccharides associated with microbial cells grown on solid surfaces is frequently recognizable from the mucoid colony morphology. In liquid medium, EPS producing cultures may become very viscous exceptionally, may solidify as a gel. The EPS may form part of a capsulefirmly attached to the bacterial cell surface or it may be observed as loose slime secreted by the microorganisms but not directly attached to the cell. On solid surfaces exposed to aqueous environments, fresh water or the oceans bacterial growth is seen as biofilms in which the microbial cells are associated with large amounts of EPS[1].

Polysaccharides have a number of applications in the formulation of pharmaceutical products (Table 1). They are incorporated into lotions and gels to impart specific rheological properties to the preparations. The materials used are mainly of plant or algal origin, including alginate but various microbial polysaccharides, including xanthan, may also find such uses. The shear-thinning capabilities of xanthan have caused it to be used in some toothpaste, where it permits the product to be readily squeezed from a tube and regain its viscosity on leaving the container. New gel-forming microbial products such as gellan clearly have potential uses in the pharmaceutical area, as do polymers capable of forming coacervates with gelatin, etc[1].This research interest in bacterial production of exopolysaccharides aims for production of polymers with fine-tuned properties and exploring its potential as an excipient in the development of suitable formulation.

MATERIALS AND METHODS

Materials

King's medium B Base, Cetrimide media, Azotobacter media, MRS media, Glucose, Lactose were procured from Hi Media laboratories, Mumbai. Diclofenac Diethyl amine was obtained from Themis Pharma, Haridwar. Trichloro acetic acid was obtained from Merck specialities Pvt. Ltd., Mumbai; phenol, sulphuric acid, isopropyl alcohol, propyl paraben, methyl paraben, sodium hydroxide and ethanol were of analytical grade.

Isolation and Production of Exopolysaccharides

Strain, media and growth conditions

For the isolation of *Pseudomonas*, various sea sediment samples, marine water samples and soil samples were collected and screened in Cetrimide agar. For the isolation of LAB various marketed milk and curd samples were utilized for the growth of *lactobacilli* on skim milk media (Fig. 1). The pseudomonas strains were then subcultured in cetrimide media and lactic acid bacteria strains were subcultured in milk media (50%) supplied with 50% carbohydrate source (Table2). The bacteria were then screened for their ability to produce exopolysaccharide, based on the colony morphology (Mucous and Ropy).

Isolation of EPS

For the isolation of EPS the media was heated to 90-100ºC to kill the EPS degrading enzymes. The protein and cells were initially precipitated by the addition of 5% (w/v) trichloroacetic acid (TCA) to the culture; the mixture was then stirred for 4 hours. After centrifugation (5000 rpm at 4°C for 20 min), cold ethanol was gradually added to the supernatant and followed by intermediate centrifugation. The exopolysaccharide precipitate was collected after centrifugation at 5000 rpm. EPS production from *pseudomonas* was not enough so as to use it in formulation development; hence EPS obtained from lactic acid bacteria was formulated in gel as a gelling agent.

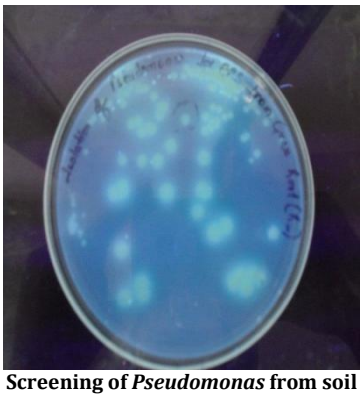
Table 1: Physicochemical and functional properties, main areas of application and market assessment of EPS[2]

EPS	Components	Charge	Molecular weight	Main properties	Main applications	Price (US$)/kg
Xanthan	Glucose Mannose Glucuronic acid Acetate Pyruvate	Anionic	(2.0-50)×10^6	Hydrocolloid High viscosity yield at low shear rates even at low concentrations; Stability over wide temperature, pH.	Foods Petroleum industry Pharmaceutical Cosmetics and personal care products	3 - 5
Gellan	Glucose Rhamnose Glucuronic acid Acetate Glycerate	Anionic	5.0 x 10^5	Hydrocolloid Stability over wide pH range Gelling capacity Thermoreversible gels	Foods Pet food Pharmaceutical gar substitute and gel electrophoresis	55-66
Alginate	Guluronic acid Mannuronic acid Acetate	Anionic	(0.3-1.3) x 10^6	Hydrocolloid Gellling capacity Film-forming	Food hydrocolloid Medicine Surgical dressings Wound management Controlled drug	5-20
Cellulose	Glucose	Neutral	10^6	High crystallinity Insolubility in most solvents High tensile strength Moldability	Foods (indigestible fiber) Wound healing Tissue engineered blood vessels Diaphragms	5.8-12
Dextran	Glucose	Neutral	10^6 - 10^9	Non-ionic Good stability Newtonian fluid behavior	Foods Pharmaceutical industry: Blood volume expander Chromatographic media	N.A.
Curdlan	Glucose	Neutral	5x10^4 - 2x10^6	Gel-forming ability Water insolubility Edible and non-toxic Biological activity	Foods Pharmaceutical industry Heavy metal removal	55
Hyaluronan	Glucuronic acid Acetyl glucosmine	Anionic	2.0x10^6	Biological activity Highly hydrophilic Biocompatible	Medicine Solid culture media	100 000
Succinoglycan	Glucose, Galactose Acetate, Pyruvate 3hydroxybutyrate	Anionic	LMW <5x10^3 HMW >1x10^6	Viscous shear thinning aqueous solutions Acid stability	Food Oil recovery	N.A.
Levan	Fructose	Neutral	3.0x10^6	High water solubility Anti-tumor activity Anti-inflammatory Adhesive strength Film-forming capacity	Food (prebiotic) Feed Medicines Cosmetics Industry	N.A.
GalactoPol	Galactose Mannose Glucose Rhamnose Acetate, Pyruvate Succinate	Anionic	(1.0-5.0) x 10^6	Viscous shear thinning solutions in aqueous media Film-forming Emulsifying capacity Flocculating capacity	Food and feed Cosmetics Pharmaceuticals and medicine Oil recovery	-
FucoPol	Fucose Galactose Glucose Acetate Succinate Pyruvate	Anionic	(2.0-10.0) x10^6	shear thinning Film-forming Emulsifying capacity Flocculating capacity Biological activity	Cosmetics Pharmaceuticals and medicine Oil recovery Source of fucose and fuco oligosaccharides	-

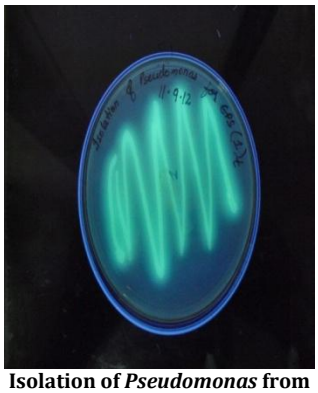
Table 2: Different media and strains used for the growth of EPS

S No.	Media	Strains						
		Pseudomonas fluorescens	*Pseudomonas putida*	*Pseudomonas syringae*	Soil sample	Sea water	Sea sediment	Milk sample
1.	Cetrimide media	+	+	+	-	-	-	NS
2.	King's B medium base	++	+	+	+	+	+	NS
3.	Azotobacter media	-	-	-	+	+	+	NS
4.	Coconut water	-	-	-	-	-	-	+
5.	MRS media	NS	NS	NS	NS	NS	NS	+
6.	Milk media	NS	NS	NS	NS	NS	NS	+++

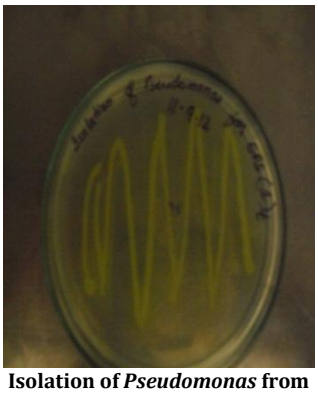
+++ high growth, ++ moderate growth, + less growth, - no growth, NS: Not studied



Screening of *Pseudomonas* from soil (UV 365nm)



Isolation of *Pseudomonas* from soil (UV 365nm)

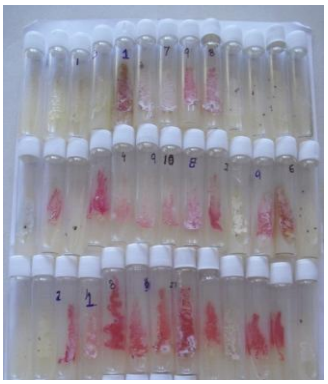


Isolation of *Pseudomonas* from soil

Screening of Pseudomonas from soil

Isolation of peudomonas from coconut water

Isolation of peudomonas from coconut water (UV 365 nm)



Screening of *Azotobacter* from sea sediment

Screening and isolation of LAB from milk samples

Figure 1: Screening & isolation of various Strains for EPS growth

Characterization of exopolysaccharides

The exopolysaccharide obtained were then characterized for Colour, Solubility, pH, Carbohydrates test. Total carbohydrate content was determined by phenol sulphuric acid method, glucose was used as a standard and absorbance determined at 490 nm (Table 3). EPS obtained from milk media (50%) supplied with 50% glucose was showing high production of EPS at temperature 20ºC. The FT-IR spectrum of EPS from *Lactobacillus* was carried out to confirm the identity of exopolysaccharides (Fig.2). FTIR spectra confirmed the presence of functional groups like carbohydrate ring, mannose and D-glucose in pyranose form[6,7].

Formulation of Diclofenac Diethylamine gel

Compatibility studies of Drug and EPS

Prior to the development of the dosage forms the pre-formulation study was carried out. The compatibility studies of drug, polymer and the physical mixture (1:1) of both drug

and polymer were carried out by KBr disc method in the scanning range of 400-4000 cm^{-1} using Fourier Transform Infrared Spectrophotometer (Shimadzu FT-IR 8400-S).

Table 3: Characterization of Exopolysaccharides

Characteristics	EPS from lactic acid bacteria	EPS from pseudomonas
pH	3.6	3.8
Color	Creamy white	yellow
Solubility	Soluble in water, insoluble in organic solvents	Soluble in water, insoluble in organic solvents
Test for Carbohydrates (Molish's test)	Positive	Positive
Total Carbohydrates (Phenol sulphuric acid method)	2 g/L	20 mg/L

Preparation of Diclofenac Diethylamine gel[8]

For the preparation of gel sufficient quantity of water was taken and heated up to 80-90°C, to this methyl paraben and propyl paraben were added. After some time (slightly cool) EPS was added with continuous stirring. Diclofenac diethylamine was weighed and dissolved in isopropyl alcohol until a clear solution was obtained. To the polymeric solution prepared drug solution was added and stirred continuously until the gel was formed. Volume was made up and pH was maintained using 10M NaOH. (Table 4)

Table 4: Composition of topical gel of Diclofenac diethylamine with different concentration of EPS

Ingredients	P1	P2	P3	P4
Diclofenac Diethylamine	1.16	1.16	1.16	1.16
EPS	5	7.5	10	12.5
Isopropyl alcohol	10	10	10	10
Propyl paraben	0.25	0.25	0.25	0.25
Methyl paraben	0.75	0.75	0.75	0.75
Sodium hydroxide	Q.S	Q.S	Q.S	Q.S
Distilled water	Q.S	Q.S	Q.S	Q.S
Total weight	100	100	100	100

Evaluation of Diclofenac Diethylamine gel

Characterization of gel

Gel formulations of exopolysaccharides containing Diclofenac Diethylamine were characterized for appearance, pH, homogeneity, spreadability, Extrudability and viscosity.

Drug content[9,10]

The drug content was determined using Phosphate Buffer (pH 6.8) solution. The absorbance of the solution was measured using UV spectrophotometer. The measurement was done in triplicate and average values were calculated.

In vitro drug release[9,10]

In vitro release studies were performed using dialysis membrane (Hi-media, Mumbai, India) having molecular weight cut off between 12,000–14,000 D. Membrane was activated with 1% of HCl solution for 12 hrs. The dissolution medium used was freshly prepared phosphate buffer pH 6.8. Dialysis membrane, previously soaked overnight, was tied to one end of a specially designed glass cylinder (open at both ends). 5 ml of formulation was accurately placed into this assembly. The cylinder was attached to a stand and suspended in 100 ml of dissolution medium maintained at 37 ± 5ºC so that the membrane just touched the receptor medium surface. The dissolution medium was stirred at low speed using magnetic stirrer. An aliquot of 5 ml of the sample was withdrawn from receiver compartment at predetermined time intervals and replenished with fresh medium. Samples were analyzed by UV-Visible spectrophotometer at a wavelength of 275 nm. Data obtained from *in vitro* release

studies were fitted to various kinetic equations to find out the mechanism of Diclofenac Diethylamine release from gel.

RESULTS AND DISCUSSION

Compatibility studies:

FTIR identification results of Diclofenac Diethylamine indicated the purity the drug also the IR spectra of pure drug, and 1:1 ratio of drug and EPS was identical and did not show any incompatibility, thus it can be suggested that EPS was compatible with the drug (Fig. 3).

Evaluation of Gel

The prepared gel showed creamy white appearance with pH 6.6-6.8. The evaluation parameters for the gel prepared using exopolysaccharide are summarized in Table no. 5

Table5: Evaluation physicochemical parameters of formulation of Diclofenac diethylamine gel

Formulation Codes	Appearance	pH *	Homogeneity*	Spreadability * (gm.cm/sec) Mean±S.D	Extrudability* (gm)	Viscosity * (cps) Mean ± S.D.	Drug Content * Mean ± S.D
P1	Creamy white	6.7±0.2	GOOD	50.12±0.220	16.15±0.27	1200±0.201	87.03±0.121
P2	Creamy white	6.8±0.4	GOOD	40.16±0.112	16.75±0.33	19000±0.020	90.89±0.327
P3	Creamy white	6.7±0.4	GOOD	29.12±0.542	17.10±0.26	35000±0.11	88.831±0.031
P4	Creamy white	6.6±0.3	GOOD	27.11±0.129	17.50±0.39	51032±0.341	90.241±0.0112
Marketed	Creamy white	6.7±00.2	GOOD	30.14±0.112	17.90±0.43	51000±0.118	91.238±0.110

Drug content

The percent drug content of all the topical formulations were found to be in the range 87.03±0.121 to 90.24±0.112 %. This showed that drug was uniformly distributed in the formulated gels.

In vitro drug diffusion studies

Diffusion study of the gel of Diclofenac Diethylamine was performed using dialysis membrane in PBS pH 6.8 as the diffusion medium, the total amount of drug release was observed at different time interval for a period of 120 min. The formulation showed good diffusion (97.34%), given in Fig. 4.

In vitro Drug Release Kinetic

The release data were fitted to various kinetic models in order to calculate the release constant and regression coefficients (R^2) Among the models tested, the drug release profiles for formulations were best fitted with Higuchi Matrix model based on regression coefficients.The linearity of the plot indicated that the release process was diffusion controlled. Thus, the amount of drug released was dependant on the matrix drug load.

CONCLUSION

Exopolysaccharides (EPS) obtained from Pseudomonas and *lactobacilli* were isolated from cetrimide media and different milk samples respectively. The EPS obtained from lactic acid bacteria was highly viscous in nature and further studied for its application in pharmaceutical formulation development. The EPS was used in different concentration and was formulated as gelling agent in Diclofenac Diethylamine gel formulation. The gelling properties of EPS was evaluated and compared with marketed formulation. As the concentration of EPS was increased the viscosity, extrudability, and consistency was found to be increased. All the formulations showed good *in vitro* release profile among which formulation P4 having 12.5% EPS concentration showed maximum release, when compared with marketed formulation it showed equivalent release profile.

Further purification of exopolysaccharides is required which can enhance the properties of EPS. Hence it can be suggested that there is great need to explore the potential of exopolysaccharides as excipient in different formulations such as emulsion, gel, beads, solid dispersions and in cosmetics such as creams and lotions, as it has already been suggested in literature that EPS possess novel and physiochemical properties such as emulsifying, stabilizing, binding, gelling agents, lubricants and thickening agents.

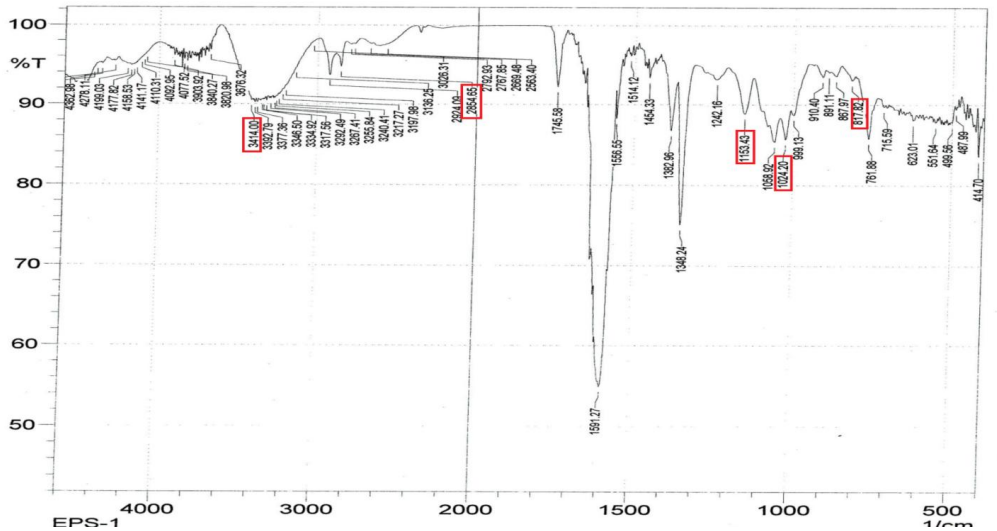


Figure 2: FTIR spectra of EPS from LAB

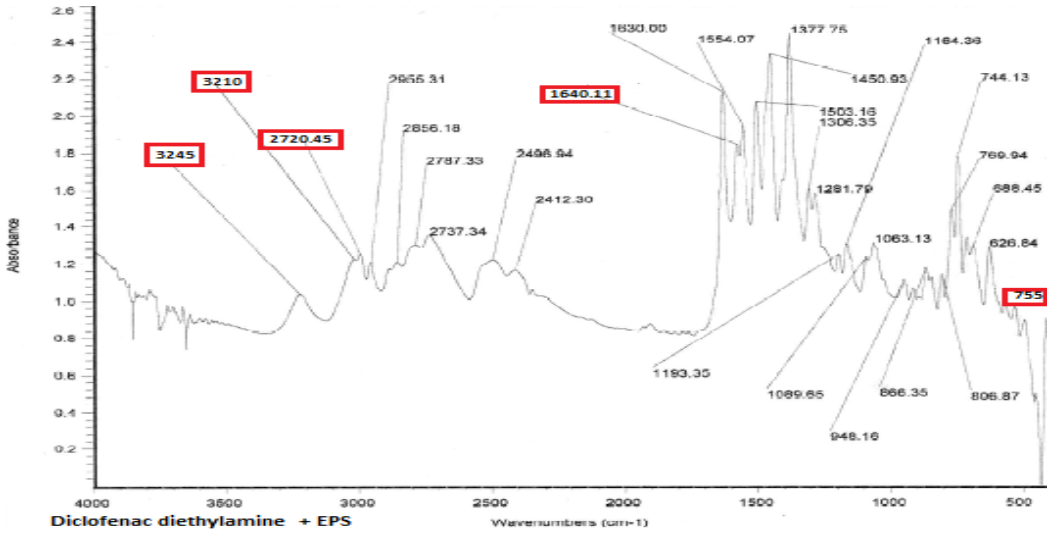


Figure 3: FTIR spectra of Diclofenac Diethylamine + EPS

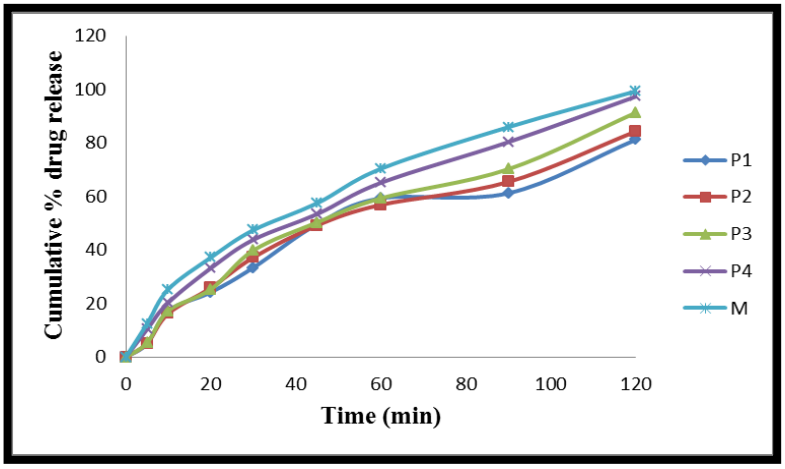


Figure 4: Cumulative drug release profile of all the formulations

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How to cite this article:

Gadad A.P., Sharma Vani, Darbha N.S., Dandagi P.M. and Singh Renu. Isolation and Purification of Exopolysaccharides and Exploring its Potential as an Excipient in the Development of Suitable Formulation. *Int. J. Adv. Pharm. Biotech.*, 2015; 1(2): 1-8.