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Optimisation of Exopolysaccharide Production by *Klebseilla pneumoniae* Strain 27F Isolated from Milk Sample

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An exopolysaccharide (EPS) producer was isolated from milk sample by primary screening. It was identified as *Klebseilla pneumoniae* strain 27F on basis of biochemical studies and 16s rDNA gene based analysis. Semi–defined medium composition and other physical parameters viz. pH, temperature and time were varied for high exopolysaccharide production. The influence of variable carbon and nitrogen sources were investigated. *Klebseilla pneumoniae* in semi-defined medium(SDM) at pH 6.5 containing lactose (60g/l), NH_4Cl (17g/l), K_2HPO_4 (2.5g/l), $MnSO_4$ (0.1g/l) and $MgSO_4$ (0.05g/l) under shaker conditions for 6 days at 30^{0}C produced 4.45 g/l of EPS. The other growth parameter like absence of Na-acetate also showed remarkable 2.5 fold increase in EPS yield. HPTLC analysis of EPS from *Klebseilla pneumoniae* strain 27F showed presence of galactose and mannose.

Key words: Exopolysaccharide, *Klebseilla pneumoniae* strain 27F.

Polysaccharides are renewable resources representing an important class of polymeric materials of biotechnological interest, offering a wide variety of potentially useful products to mankind. Exopolysaccharides of microbial origin with a novel functionality, reproducible physico-chemical properties, stable cost and supply, became a better alternative to polysaccharides of algal origin[1]. EPS presents special pseudoplastic properties and has various potential applications in the field of cosmetics and medical science [2-4].

Studies on the effect of growth-limiting substrates on exopolysaccharide synthesis clearly demonstrate that the growth medium composition can dramatically affect the specific rate of polymer synthesis[5]. The amount of carbon substrate converted to polymer by the microbial cells depends on the growth medium composition. Generally, media containing a high C/N ratio are favoured for polysaccharide production. The physical factors of greatest importance are incubation temperature, pH, oxygen tension, agitation speed and incubation time. Chemical factors determining the EPS yield are the C source, N source, C/N ratio, and the presence or absence of other medium components, e.g. salts and vitamins. Nowadays, a lot of effort is put in the optimization of culture conditions to achieve higher yields of these EPS which are already commercially successful[6].

The present work is focussed on the isolation and identification of EPS producer as *Klebseilla pneumoniae* strain 27F from milk sample and study of the effects of different growth parameters (C, N and inorganic salts) and physical factors (temperature, pH and incubation time) on EPS production by the same.

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MATERIALS AND METHODS

Isolation and Identification of EPS producer

Milk sample was screened for EPS producer by observing growth on Luria Bertani agar (LB). The isolate was identified using Bergey's Manual of Determinative Bacteriology, 8th edition [7] and 16s rDNA analysis.

Screening for Growth media

Minimal medium viz. Ashby's lactose broth, M9, SDM[8], YEM medium were screened for maximum growth. Inoculated SDM flasks were kept at static and on shaker at 30^0C for 48h. Growth was determined by checking O.D at 620nm.

Optimisation for EPS production

The optimisation was carried out in SDM medium (Dextrose 20g/l, Tween 80 1ml/l, Ammonium citrate 2 g/l, $MnSO_4$ 0.05g/l, K_2HPO_4 2g/l, $MgSO_4$ 0.1g/l, Na-acetate 5g/l, Yeast N base 5g/l, Bacto–casitone 10g/l pH 6.5 ± 0.2) by studying the effect of media components along with physical parameters. The study was carried out under shaker condition using gyratory shaker at 120rpm (Eltek Company). To study the effect of different C sources, the medium was added with Glucose, Fructose, Xylose, Maltose, Lactose and Galactose (2% each). Incubation period of 2, 4, 6 and 8 days was studied using 2% sugar supporting maximum EPS yield followed by the effect of its variable concentration on EPS production.

The influence of various Nitrogen sources (Ammonium citrate, KNO_3, Bacto–casitone + YNB, $NaNO_3$, NH_4Cl and NH_4NO_3) on EPS yield was also investigated. The SDM medium itself contained both inorganic and organic nitrogen sources e.g. Ammonium citrate and Bacto–casitone +YNB. Inorganic salts were added at a concentration of 0.2%, whereas organic nitrogen sources were added at 1%. The media was formulated in such a way, that the cultures were grown in single nitrogen source either organic or inorganic one at a time, along with the respective control (absence of N source).

The influence of variable concentration of inorganic salts viz. K_2HPO_4, Na–acetate, $MnSO_4$ and $MgSO_4$ were studied along with the control (absence of respective salt).

To evaluate the effect of pH and temperature, the culture was subjected to range of pH 4.5 - 7.5 and temperature 30^0C, 37°C, and 55°C respectively.

Quantification and analysis of EPS

Extraction of EPS was carried out using solvent precipitation method[9]. Extracted EPS was purified by dialysis using dialysis bag of 2kDa, and quantified (Dried EPS g/l = Weight of empty tube with dried EPS powder – Initial weight of empty tube). The polysaccharide nature of extracted EPS was determined by Molisch test. Carbohydrate from extracted EPS was estimated by phenol-sulphuric method[10].

EPS was precipitated using 100ml of medium, dried, weighed and dissolved in 30 ml D/W (pH – 7.0). The solution was dialyzed for 72h against D/W (pH – 7.0). After dialysis EPS was precipitated from the dialyzed sample and the resultant precipitate was weighed. Equal volume of 1N HCl is added to the EPS solution, vortexed, and autoclaved at 10psi/30minutes. The resultant solution was diluted 1:2 and then used as unknown for sugar analysis.10µl of the samples and 5µl of the standard sugars (1 mg/ml) were applied on silica gel TLC plates (Merck, Darmstadt, Germany). Sample application was done by CAMAG Linomat 5 auto sampler at a speed of 15 µl/sec. Solvent system was n-Butanol : acetic acid : D/W (2 : 1 : 1). Spots were revealed by diphenylamine-aniline-phosphoric acid (DPA) reagent followed by drying the plate and heating at 110° C for 15 minutes, till colour develops. Scanning was done by CAMAG TLC Scanner 3 at a speed of 100 mm/s and data resolution of 100 µm/ step. Wavelength used was 580 nm. Various components of EPS are identified by comparing with the R_f values of std sugars. Standards used were (1mg/ml) each of Galactose, Xylose, Rhamnose and Mannose.

Adhesive property

EPS obtained from *Klebsiella pneumoniae* strain 27F showed sticky property, thus, was checked for adhesive nature. To determine the adhesive property, extracted EPS was applied on small pieces of papers and dried.

RESULTS AND DISCUSSION

Isolation and identification

Milk sample was screened for glistening colonies on Luria Bertani media followed by capsule staining. The isolate was identified using

morphological, cultural and biochemical characteristics according to Bergey's Manual of Determinative Bacteriology, 8th edition as *Klebsiella pnuemoniae* and strain 27F from 16s rDNA analysis.

Selection of production media for EPS production

No growth was observed in Ashby's lactose broth and M9 minimal medium suggesting that *Klebsiella pnuemoniae* strain 27F required additional nutritional supplements. SDM supported high growth yield (0.31 $O.D_{620}$) comparatively less on YEM medium (0.2 $O.D_{620}$). Substances such as amino acids and vitamins present in the organic nitrogen sources stimulate the synthesis of biopolymers. SDM consists of YNB which is a source of vitamins and minerals while Bacto-Casitone as a source of amino acids[8, 11]. The yeast extract in YEM interferes with analysis of EPS due to the presence of mannan. Thus, SDM was selected for further optimisation studies.

Optimisation for EPS production

Shaker condition were found to be effective for maximum growth (0.65 $O.D_{620}$) as compare to static (0.09 $O.D_{620}$) in 2 days. Increase in broth viscosity from fast-growing micro-organisms, high biomass and biopolymers lead to oxygen limitation and broth heterogeneity act as diffusion barrier[12]. Thus, further analysis was carried out under shaker condition.

Exopolysaccharide are produced during high C/ N limiting ratio[6], so the media was optimised in such a way that the growing cultures were subjected to high C sources under limited N conditions. The *Klebsiella* genus is able to increase the production of exopolysaccharides under nitrogen limitation and excess of carbon source conditions[13-14]. The growing cultures were subjected to different monosaccharide (Glucose, Fructose and Xylose) and disaccharide (Maltose, Galactose and Lactose) to investigate the sugar supporting maximum EPS yield. The type of carbon source has a huge influence on EPS productivity and may also affect the composition of EPS[15]. Levan is an exopolysaccharide containing a $(2 \rightarrow 6)$ linked backbone with a single β-D-fructose at the C-1 position every seven residue, in case of *Bacillus licheniformis*[16]. The study on *Sporobolomyces salmonicolor* strain AL_1 and AL_{36} revealed that considerable amounts of polysaccharide was synthesised on fructose containing medium than glucose containing medium[5]. In *K. oxytoca*, lactose was the preferred source for maximum biopolymer production since it was isolated from milk where lactose is the main carbon source and the organism is best adapted for its utilization[11]. In current study also, lactose supported the maximum EPS yield for *K. pneumoniae* strain 27F (Table 1) since it was isolated from milk sample.

Incubation time was also studied to know

Table 1. Effect of carbon and incubation time on EPS yield by *Klebseilla pneumoniae* strain 27F

1a		1b		1c	
2% sugars	EPS (g/l)	Days	EPS (g/l)	Lactose (%)	EPS (g/l)
Glucose	0.34	2	1.8	2	1.8
Fructose	1.12	4	2	4	2.4
Xylose	1.48	6	2.3	6	3
Maltose	1.48	8	1.6	8	0.21
Galactose	0.36				
Lactose	1.8				

1.a - SDM medium with different sugars, 1.b - SDM medium with 2% Lactose,
1.c - SDM medium incubated for 6 days

Table 2. Effect of nitrogen source on EPS yield by *Klebseilla pneumoniae* strain 27F

N sources	EPS (g/l)
Control	No growth
Ammonium citrate	1.3
Casitone + Yeast N base	3
KNO_3	1
$NaNO_3$	0.4
NH_4Cl	3.3
NH_4NO_3	2.3

the time under which maximum EPS can be extracted from growth medium. Carbon sources in low concentration were not suitable for exopolysaccharide synthesis because the carbon was almost all used for biomass production, seen in case of *Sporobolomyces salmonicolor*[5]. The growing culture was then subjected to increasing concentration of the respective sugar and 6% lactose gave maximum EPS yield. The lactose 6 % (w/v) concentration at incubation period of 6 days was found to support highest EPS production (3g/l) for *K. pneumoniae* strain 27F (Table 1). The EPS production in a 5% sucrose containing medium *Sporobolomyces salmonicolor* AL_1 was found to be maximum than 3% for 7days[5]. For the production of polysaccharides, instead of conventional batch fermentation, two-step processes have been proposed to promote bacterial growth in the first step and polysaccharide production in the second. To achieve the objective of both stages, several approaches of operational and nutritional conditions have been studied[17-18].

For some EPS-producing bacteria, such as *Xanthomonas, Pseudomonas* and *Rhizobium* species nitrogen limiting conditions result in increased EPS production[19]. The high EPS yield was supported by inorganic source for *K. pneumoniae* strain 27F i.e. NH_4Cl (Table 2). The NH_4^+ plays a role in nitrogen metabolism in a form which is easily incorporated into organic cell components i.e biomass. The highest exopolysaccharide yield and viscosity of the culture broth were obtained with a 0.25% concentration of ammonium salts by *Sporobolomyces salmonicolor*[5]. The *Klebsiella* genus is able to increase the production of exopolysaccharides under nitrogen limitation and excess of carbon source conditions[20]. In this study EPS was produced during N limiting condition.

Other media components mainly K_2HPO_4, $MnSO_4$, $MgSO_4$ and Na-acetate were studied by varying their concentration. EPS production under absence of respective component was also checked (control). Absence of K_2HPO_4 showed no growth seems to be essential for either growth and/or EPS production but its presence also did not show much effect on EPS yield (Table 3). In *Klebsiella aerogenes*, exopolysaccharide is produced from glucose under nitrogen, sulfate, and phosphate limitation but not under carbon limitation[21]. Na-acetate, $MgSO_4$ and $MnCl_2$ appeared to be essential for growth[22]. Increase in EPS yield was observed in absence of Na-acetate (Table 3). Absence of $MnSO_4$ has affected growth, and hence EPS production which showed $MnSO_4$ is essential either for growth and/or EPS production. $MnSO_4$ of 0.01% (w/v) has found to be effective (Table 3c). High demands for Mn^{2+} have been observed for Pediococci and this phenomenon has been studied in great details in *Lactobacillus plantarum*.

Table 3. Effect of inorganic salts on EPS yield by *Klebseilla pneumoniae* strain 27F

3a		3b		3c		3d	
K_2HPO_4 (%)	EPS (g/l)	Na-acetate (%)	EPS (g/l)	$MnSO_4$ (%)	EPS (g/l)	$MgSO_4$ (%)	EPS (g/l)
Control	0.0	Control	6.4	Control	-	Control	1.4
0.15	1.83	0.25	4.2	0.0025	1.75	0.005	1.85
0.2	1.85	0.5	4.35	0.005	1.95	0.01	0.55
0.25	1.88	1	4.4	0.01	2.15	0.015	1.2

Table 4. Effect of pH and temperature on EPS yield by *Klebseilla pneumoniae* strain 27F

pH	EPS (g/l)	Temperature °C	EPS (g/l)
4.5	No growth	30	4.45
5.5	No growth	37	3.55
6.5	4.45	55	No growth
7.5	2.9		

EPS concentration was raised more than 30- fold by combining best condition with higher concentration of Mn^{2+} (5mg/l) in the respective formulated medium[23]. Alginate biosynthesis in *Pseudomonas aeruginosa* is stimulated in the presence of Ca^{2+} and Mn^{2+} ions due to enhancement of growth and subsequent EPS synthesis. Mg^{2+} ions are required for activating

certain enzymes leading to EPS synthesis[24]. Presence and absence of $MgSO_4$ did not affect EPS production (Table 3).

Both pH and temperature play a crucial role in EPS synthesis. The growth medium composition can also indirectly affect the polymer yield, for example, by governing the pH change which can occur during fermentation without pH control[5]. The extreme pH profiles of the medium (pH 2.0-3.0 or pH $\geq$10) inhibited not only the process of microbial growth but also the biosynthesis of extracellular polymers. It was particularly observed in *Aureobasidium pullulans* where the optimal pH profile of the medium for the EPS production oscillated between pH 5.5 and pH 6.5[25]. In this study, acidic pH has found to inhibit the growth of the cultures while neutral pH has not only supported growth but also increased the EPS yield. The actual medium pH 6.5 has proved to be efficient for EPS production (Table 4).

It was observed that 30°C supports maximum yield for *K. pneumoniae* strain 27F (Table 4). The optimal cultivation temperature for the production of most EPS molecules was estimated between 26°C and 31°C. This dependence also was confirmed by the results concerning the optimization of EPS compound production by *Streptococcus salivarius* cells[25]. In case of *K. pneumoniae* strain 27F, one unusual thing was observed that the colour of EPS changed to brown at 37°C whereas it remained colourless at 30°C. Thus, the temperature has some chromogenic effect on EPS produced which can be one of the parameter yet to be studied. Since this property and/or parameter is not been studied until now.

Quantification and analysis of EPS

The greatest loss during recovery of crude EPS is probably due to the method of harvesting the EPS, in which only the bottom layer of precipitated material was centrifuged following ethanol precipitation[19]. Thus, efficient separation of soluble EPS was done by solvent-precipitation method using single volume of iso-propanol. Propanol found to be the most effective precipitating agent, being followed by isopropanol[9]. The Molisch test was carried out on dried EPS, the general test which confirms the presence of carbohydrate[26].

Dialysis was employed for purification of EPS and characterisation was done to check the presence of sugars and also chemical analysis was carried out. Dialysis can be used to remove a low molecular weight sugars (glucose and lactose) prior to quantification of EPS to avoid interference from other medium components including beef extract, peptones and yeast extract[19].

Quantification of carbohydrate content from obtained EPS was done by phenol-sulphuric method. The carbohydrate content was found to be 152µg/ml for EPS from *K. pneumoniae* strain 27F. HPTLC analysis performed showed that *K. pneumoniae* strain 27F synthesized EPS made up of mannose and galactose units. *Klebsiella pneumoniae* K63 produces exopolysaccharide (EPS) which is composed of D-galactose, L-fucose and D-galacturonic acid, with some of the hydroxyl groups being acetylated[4].

Adhesive property

EPS from *K. pneumonia strain* 27F was found to possess sticky nature, thus was checked for adhesive properties. Polysaccharides, such as xanthan gum, are produced by culturing microorganisms, e.g. of the *Xanthomonas* genus[27]. *Klebsiella* possess ability for biofilm formation[28]. Thus, the sticky nature might be useful for the organism for adherence. But at the same time, this nature of the EPS can be explored for commercial application as bio-adhesives. The EPS from *Klebsiella pnuemoniae* has various potential applications in the field of cosmetics[20]. By effective characterisation and study of EPS, the polymer can be modified to bioadhesive of better quality.

CONCLUSION

Thus, *Klebseilla pneumoniae* strain 27F was optimised for production of high EPS yield (4.45g/l) in SDM medium which has an industrial application as bioadhesives. The optimised medium for high EPS yield consists of Lactose (60g/l), Tween 80 (1ml/l), NH_4Cl (17 g/l), $MnSO_4$ (0.1g/l), K_2HPO_4 (2.5g/l), $MgSO_4$ (0.05g/l) pH 6.5 at 30^{0}C for 6 days at 120 rpm.

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