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Exopolysaccharide Yield as a Kinetic Parameter for the Statistical Optimization of EPS Production by *Klebsiella pneumoniae*

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Abstract: *A Klebsiella pneumoniae* strain isolated form a chronically infected patient with urinary tract infection produces an extracellular polysaccharide giving the strain a highly mucoid appearance. Growth characteristics and exopolysaccharide production of this strain was investigated in culture with synthetic medium. For the ultimate aim of optimizing the exopolysaccharide productivity together with the EPS yield ($g/g_{biomass}$) the application of Plackett-Burman statistical experimental design gave conflicting results for ammonium sulphate as the main effect. Moreover, in pH-controlled bioreactor EPS production was not associated with the growth and was maximized at lower growth rate. Furthermore, this study showed that for maximization of EPS production together with EPS yield, ammonium limited fed batch culture in oxygenated bioreactor is the method of choice to be used. Applying this procedure, an increase in the maximum dry weight of cells. EPS yield and EPS concentration (90 fold increase) was obtained compared to a classical batch culture.

Key words: Exopolysaccharide, EPS, klebsiella pneumoniae

INTRODUCTION

Capsules are essential to the virulence of *Klebsiella* (Domenico *et al*., 1994; Williams *et al*., 1983). The capsular material forms thick bundles of fibrillous structures covering the bacterial surface in massive layers (Podschun and Ullmann, 1998). This protects the bacterium from phagocytosis by polymorphonuclear granulocytes, on the one hand (Cortes *et al*., 2002a,b; Lin *et al*., 2003; Campos *et al*., 2004; Lin *et al*., 2006) and prevents killing of the bacteria by bactericidal serum factors, on the other (Sahly *et al*., 2006). Moreover, the Hospital outbreaks of multidrug-resistant *Klebsiella* spp. are often caused by a new type of strain, the extended spectrum beta-lactamases producers (ESBL) (Reish *et al*., 1993; French *et al*., 1996). The incidence of ESBL-producing strains among clinical *Klebsiella* isolates has been steadily increasing over the past several years. Frequencies of up to 40% have been reported in certain regions. Moreover, the available data suggest a further increase in the incidence of ESBL-producing isolates (Burwen *et al*., 1994). As a result, the therapeutic options are becoming limited, so that in the near future there will be an urgent need for hospital infection control measures that counter the spread of ESBL-producing bacteria. Since capsules are produced by almost all clinical *Klebsiella* strains, they have been the obvious vaccine candidates. Moreover, they represent the outermost layer of surface structures in contact with the host milieu and they have been proven to be highly immunogenic and nontoxic (Cryz *et al*., 1984, 1988; Cryz, 1990). Indeed a 24-valent *Klebsiella* CPS vaccine were proven to be the most promising approach for preventing sepsis caused by *Klebsiella* and has already passed phase I human trials. Indeed, CPS was proven to be safe and immunogenic (Cryz, 1990) and EPS-produced by *Klebsiella pneumoniae* was found to possess special pseudoplastic properties and has various potential applications in the medical and chemical fields (Vanhooren and Vandamme, 1999; Kobayashi *et al*., 2000; Ramirez-Castillo and Uribelarrea, 2004).

The kinetics of Exopolysaccharide production by *Klebsiella* were frequently studied by Ohta *et al*. (1981), Mengistu *et al*. (1994), Farres *et al*. (1997) and Garnier *et al*. (2006). Recently, Garnier *et al*. (2006) noticed that *Klebsiella* spp. isolated from activated sludge, produced the EPS material early in the logarithmic phase, after only 4 h of cultivation. This behavior, however, contradicts the data presented by Farres *et al*. (1997) who observed that in a typical batch culture of *Klebsiella* I-714, cell growth is rapidly completed in 6-12 h, while exopolysaccharide production may continue for further 30-48 h. Moreover, in continuous culture studies of *K. pneumoniae*, the rate of polysaccharide synthesis

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was greatest at low dilution rate (Mengistu *et al*., 1994). Indeed and in order to understand the EPS production quantitatively, there is still a need for some kinetics data, especially for those clinical isolates of *Klebsiella*.

Most of studies on *Klebsiella pneumoniae* strains were mainly focused either on their nitrogenase system in nitrogen fixing strains or on the production of 1,3 propanediol, an economically important product that is used in the synthesis of polyurethanes and polyesters (Comaduran *et al*., 1998; Petersen *et al*., 2005; Cheng *et al*., 2004; Lin *et al*., 2005; Wood *et al*., 2005). Both fields of studies were mostly done in anaerobic or microaerobic conditions. Very few studies correlate EPS production and their effect of increasing viscosity as well as the establishment of a microaerobic environment in biofilm and the process of nitrogen fixation. In fact, oxygen interferes with biological nitrogen fixation at three different levels. At the genetic level, oxygen acts by repression of nitrogenase synthesis (Souza *et al*., 1999). Furthermore, oxygen causes irreversible damage to the enzyme. Dinitrogenase reductases (Fe proteins) are more sensitive to inactivation by O_2 than dinitrogenases (Mo-Fe proteins), but the half-lives of bothproteins are a few minutes at the most. Finally, oxygen is responsible for reversible inhibition of nitrogenase activity *in vivo*. Studies on the influence of O_2 on N_2 fixation in *Azotobacter chroococcum* (Drozd and postgate, 1970), Mycobacterium flavum (Biggins *et al*., 1971) and *Azospirillum brasilense* (Zhulin *et al*., 1996; Zhang *et al*., 1997) have shown that the activity can be inhibited to various degrees by excess oxygenation (switch-off). The activity returns rapidly when aeration is lowered (switch-on) without de novo nitrogenase synthesis (Dodsworth and Leigh, 2006). In *Azotobacter vinelandii*, it was shown that compact capsule formation may lower the diffusion of O_2 to the O_2-sensitive nitrogenase and hence lower the stress exerted to cells grown aerobically (Sabra *et al*., 2000, 2001, 2004).

Although statistical experimental design has been widely used in many areas of science and industry and can be adopted on several steps of an optimization strategy, such as for screening experiments or searching for the optimal conditions of a targeted response (Abdel-Fattah *et al*., 2005; Lee and Gilmore, 2005; Wang and Lu, 2005; Lee *et al*., 2006; Zhang *et al*., 2006), it has not yet been adopted for EPS optimization by *Klebsiella*. Considering that this exopolysaccharide can potentially be used commercially, we conducted the optimization procedure for media composition in flask cultures, using factorial design of experiments, for high exopolysaccharide production. Interestingly, the present study shows the failure of factorial experimental design to optimize the EPS production by the clinical isolate of *Klebsiella pneumoniae*. It was also shown that, EPS yield in $g/g_{biomass}$ should be used as a response variable rather than the EPS concentration in the statistical design.

MATERIALS AND METHODS

Microorganism and medium. *Klebsiella* pneumoniae strain K-5, a strain isolated from patient with urinary tract infection was used for all experiments. This strain was stored in nutrient broth containing 25% (vol/vol) glycerol at -20°C. Strain was characterized physiologically and biochemically according to Podschun and Ullmann (1998). A modified EPS medium was used as the basic production medium with the following composition (per liter of deionized water) 7.0 g of K_2HPO_4, 3.0 g of KH_2PO_4, 0.1 g of $MgSO_4$ $7H_20$, 0.1 g of $(NH_4)_2SO_4$, 0.01 g of $CaCl_2$, 0.001 g $FeSO_4$, 0.1 g of NaCl and 10.0 g of glucose (70g L^{-1} glucose was added in experiments done in bioreactor).

Fermentation runs: Fermentations were carried out in a 2 L stirred tank bioreactor (Biostat B, B-Braun Biotechnolgia, Germany) with a working volume of 0.7-1.2 L. The bioreactor was equipped with temperature, pH and agitation speed measure and control unit which was connected to an online writer. Dissolved oxygen tension was measured by an autoclavable pO_2 electrode (ingold Germany). The concentration of carbon dioxide and oxygen in the effluent gas was analysed by a paramagnetic oxygen analyzer (Oxygor, Maihak, Germany) and an infrared carbon dioxide analyser (UNOR, Miahak, Germany).

Off-line analysis: samples were aseptically withdrawn from the bioreactor to determine the optical density (A_{600} nm), cell dry mass,. EPS concentration was determined gravimetrically as well as calorimetrically as described previously (Sabra *et al*., 2000, 2003)

Experimental design and statistical analysis: The Plackett-Burman experimental design was used to evaluate the relative importance of medium components on exopolysaccharide production by *K. pneumoniae* strain (Plackett and Burman, 1946). The Nine independent variables examined in this experiment and their settings are shown in Table 1. The rows in Table 1 represent the 12 different experiments (row No. 13 and 14 represent the trials at which all independent variables are either in excess or in limited amount, respectively) and each column represents a different variable. For each nutrient variable, a high (+) or low (-) concentration was tested.

Table 1: Plakett-Burmann experimental design matrix for the evaluation of the relative importance of selected nutrients and their tested levels for EPS production by *Klebsiella pneumoniae*

	Independent variables								
Variables level tested									
-1	0.1	0.02	0.02	0.0	0.00	0.000	10	75	0
0	10.0	0.10	0.10	0.1	0.01	0.001	10	50	0
+1	10.0	0.40	0.40	1.0	0.10	0.010	30	25	1
Plakett-Burmann experimental design matrix									
Trials	Phosphate ($g\ L^{-1}$)	$MgSO_4$ ($g\ L^{-1}$)	NaCl ($g\ L^{-1}$)	Ammonium ($g\ L^{-1}$)	$CaCl_2$ ($g\ L^{-1}$)	$FeSO_4$ ($g\ L^{-1}$)	Glucose ($g\ L^{-1}$)	Aeration (mL)	Trace element (mL)
1	1	-1	1	-1	-1	-1	1	1	1
2	1	1	-1	1	-1	-1	-1	1	1
3	-1	1	1	-1	1	-1	-1	-1	1
4	1	-1	1	1	-1	1	-1	-1	-1
5	1	1	-1	1	1	-1	1	-1	-1
6	1	1	1	-1	1	1	-1	1	-1
7	-1	1	1	1	-1	1	1	-1	1
8	-1	-1	1	1	1	-1	1	1	-1
9	-1	-1	-1	1	1	1	-1	1	1
10	1	-1	-1	-1	1	1	1	-1	1
11	-1	1	-1	-1	-1	1	1	1	-1
12	-1	-1	-1	-1	-1	-1	-1	-1	-1
13	1	1	1	1	1	1	1	1	1
14	-1	-1	-1	-1	-1	-1	-1	-1	-1

The main effect of each variable was determined with the following Equation:

$$\text{Variable main effect} = (M_{i+}-M_{i-})/N$$

Where M_{i+} and M_{i-} are the EPS ($g\ L^{-1}$) or EPS ($g\ g^{-1}$) in trials. The independent variable (i) was present in the high and low concentrations, respectively and N is the number of trials divided by 2. A main effect figure with a positive sign indicates that the high concentration of this variable is near to optimum and a negative sign indicates that the low concentration of this variable is nearer to optimum.

RESULTS AND DISCUSSION

Diazotrophic growth enhances exopolysaccharide yield ($g/g_{Biomass}$) but not the exopolysaccharide concentration ($g\ L^{-1}$) by *K. pneumoniae* strain in flask cultures: Preliminary results showed that, the presence of initial supply of nitrogen substrate in the EPS medium gave an increased biomass concentrations and growth rates with an accompanying increase in gum production rates (data not shown). However, final gum concentrations are not necessarily increased (0.5, 0.55 and 0.6 $g\ L^{-1}$ at ammonium sulphate concentration of zero, 0.1 g and 0.5 $g\ L^{-1}$ ammonium sulphate, respectively). Instead the exopolysaccharide yield ($g_{polysaccharide}/g_{biomass}$) increased drastically under condition of nitrogen fixation. Indeed, under conditions permitting diazotrophic growth, extracellular polysaccharide material may play an important role in mass transfer limitation of the toxic level of high-oxygen concentration toward the O_2-sensitive nitrogenase (Sabra *et al*., 2000, 2001). This oxidative stress exerted under diazotrophic conditions may also explain the poor growth and lower specific growth rate obtained under this condition. Therefore, for further medium optimization a 0.1 g ammonium sulphate was used to be the basal concentration in the statistical optimization experiment.

In order to screen the most important environmental factors responsible for the best polymer production, a fractional two factorial Plackett-Burman design was used (Plackett and Burman, 1946; Lee *et al*., 2006). This method was used as one of the most popular applied statistical designs to bioprocessing (Liu *et al*., 2003; Wang and Lu, 2005). All trials were performed in triplicates and the averages of EPS and biomass concentration observation results were treated as the responses. Factors chosen for the statistical study was the nutritional components of the EPS medium and the addition of trace elements together with the effect of aeration of the culture in twelve combinations organized according to the Plackett-Burman design matrix described in Table 1.

Specific growth rate for *K. pneumoniae* was calculated based on the initial rate data form different environmental condition in flasks (after 6 h cultivation). This represents conditions of negligible gum concentration and unaltered environmental conditions suitable to get the most accurate growth rate response toward the different conditions applied in flasks. However, EPS concentration and consequently the EPS yield were estimated after 48 h.

Interestingly and regarding the effect of ammonium sulphate and phosphate levels, the EPS yield as main effect data contradict that of EPS concentration as the main effect (Fig. 1). Since the objective of this screening factorial experiment was to get a general picture of how the response variable (EPS concentration) is affected by changes in the different factors or to find the over-all combination of factor levels which gives a maximum value of the response variable. In this case and for the further optimization of exopolysaccharide production by *Klebsiella pneumoniae*, one of two different approaches had to be chosen, the first one was to optimize the overall exopolysaccharide production in term of g L^{-1}. The second approach was for the optimization of the polysaccharide yield in term of g/g_{bimass}. The mutual optimization of both of them seems to be invalid.

The same trend was also noticed in xanthan production by *Xanthomonas compestris* under different levels of nitrogen substrate (Letisse *et al*., 2003). Bulk broth viscosity effects or slime layer accumulation around cells inhibiting diffusion of substrates were reported to explain such results in xanthan production. However in cultivation with *K. pneumoniae*, the viscosity of the batch culture medium didn't increase over 30 mPas. Yet, capsule formation is common in *K. pneumoniae*, especially when grown under nitrogen limitation (Podschun and Ullmann, 1998). Undoubtedly, the nature of product whether growth associated product formation or growth non associated product formation plays here a crucial role. It is obvious that there must be a relationship between the amount of cells that is available and the amount of exopolysaccharide that will accumulate in a particular environmental condition. From Fig. 1, it was obvious that for best EPS yield, a limitation in both phosphate and ammonium sulphate is advantageous. Definitely, nutrient limitations have an adverse effect on the bacterial growth rate. Therefore getting a relation between the growth rate and the exopolysaccharide yield of *Klebsiella pneumoniae* might be interesting to further classify the EPS production kinetics.

With the aim of getting a relationship between the specific growth rates and the EPS yield, the main effect of the most significant variables for EPS yield, namely ammonium sulphate, glucose, phosphate, trace element and magnesium sulphate concentration was plotted against the specific growth rate at the same conditions. As can be depicted form Fig. 2, the EPS overproduction is associated with periods of nonbalanced growth which is physiologically characteristics of secondary metabolism. Nevertheless, more detailed and controlled studies in bioreactor should be performed to ascertain such assumption.

Production of exopolysaccharide in pH-controlled bioreactor: Although *Klebsiella* are known to be extremely sensitive to oxygen when grown diazotrophically, very few studies dealt with EPS production under this condition. Therefore, with the final aim of increasing the overall concentration as well as the EPS yield, a detailed kinetic study was performed in controlled bioreactor. Three different fermentations were performed using EPS medium with ammonium sulphate concentrations of 0, 0.1 and 1 g L^{-1}. Samples were taken regularly from bioreactor and analysed for dry weight and EPS production.

Interestingly, cultures with higher concentrations of ammonium sulphate recorded the fastest onset of the stationary phase due to oxygen limitation (5 h compared to 7 h and 12 h at 1, 0.1 and 0 ammonium sulphate concentration, respectively) This was also observed from the CO_2 production rate which peaked shortly before the onset of the stationary phase (data not shown). Higher biomass level indeed has higher oxygen consumption demand, especially when the cell is not further oxygen sensitive. At low and intermediate ammonium sulphate concentrations, the growth limitation was obviously for nitrogen requirement causing lower biomass concentration under such conditions (Fig. 3). For those cultures, oxidative stress of a functional nitrogenase system may cause the limitation of growth and consequently the onset of stationary phase. Moreover, in agreement with flask cultures, higher EPS yield was obtained under condition supporting unbalanced growth with cells growing diazotrophically. Under such conditions cells are under stress of nitrogenase inactivation by oxygen, which explains the lower biomass yield. Certainly, the formation of capsule around the cell may help the nitrogenase protection as described in *Azotobacter vinelandii* growing under nitrogen fixing conditions (Sabra *et al*., 2000, 2001). Unfortunately, this high EPS yield was not accompanied with a high biomass yield and therefore, the overall EPS concentration (0.54 g L^{-1}) was not satisfactory. Ideally, ammonium limitation together with oxidative stress and higher biomass yield will result in higher EPS concentration and EPS yield.

Optimized nitrogen-limited fed batch culture for EPS production by *Klebsiella pneumoniae*: From the previous results; it seems that maximizing EPS concentration by *Klebsiella pneumoniae* can not be realized in ordinary batch cultures. That is in order to get high biomass concentration; growth at excess amount of ammonium sulphate should be achieved. This has a negative effect on the EPS yields and consequently on EPS

concentration. This actually represents a major drawback in the statistical optimization approach of medium components for EPS production by *Klebsiella*. On the other hand, this is one case in which fermentation technology has an advantage. Growth at nutrient limitation by different microorganisms has long been used in fed batch culture without the loss of cell viability. Moreover, substrate limitation was the technical method used to avoid oxygen limitation in high cell density cultures (Lukondeh *et al*., 2005). Feeding rate of 0.014 g L^{-1}*h was chosen which is comparable to the ammonium sulphate uptake rate in batch culture (Fig. 4) with 0.1 ammonium sulphate depicted from data in Fig. 3. Parallel to the first CO_2 production peak (after 6 h), the bacterial growth rate gave a maximum of 0.4 h^{-1}. This actually the case in which the addition rate for ammonium sulphate exceeds the N-demand by the organism and consequently, the concentration in the bioreactor will increase and consequently the specific growth rate (0-6 h). Thereafter, due to the increased biomass concentration, the addition rate is ultimately less than the biomass demand and thus the ammonium sulphate concentration will decrease. This in turn will cause the decrease in the specific growth rate and finally nitrogen limited fed batch culture will be obtained. Under such phase of N-limitation the EPS yield is maximized despite the prevalence of high biomass concentration which explains the higher EPS concentration obtained (Fig. 4). The dissolved oxygen tension, on the other hand, remained unlimited and hence a constant oxidative stress was exerted on *Klebsiella* cells. Moreover, Fig. 5 clearly shows that the production kinetics of EPS material is maximized at lower growth rate. This again ascertains the nature of the secondary metabolites production pattern of this biopolymer and solve the deviation in some author opinions about the nature of EPS production kinetics (Farres *et al*.,1997; Garnier *et al*., 2006).

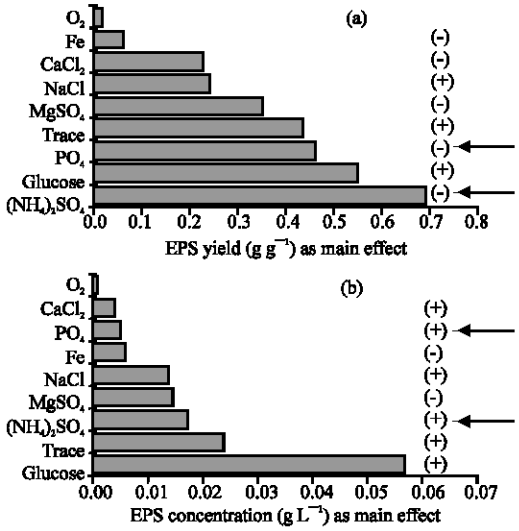

Fig. 1: Exopolysaccharide yield ($g/g_{biomass}$) (a) and concentrations (g L^{-1}) (b) as the main effect in flask cultures as influenced by different components levels of EPS medium

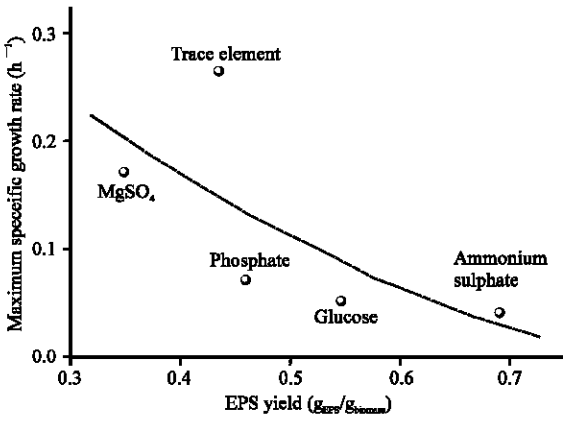

Fig. 2: Maximum specific growth rate as a function of exopolysaccharide yield by *Klebsiella pneumoniae* grown in flask cultures

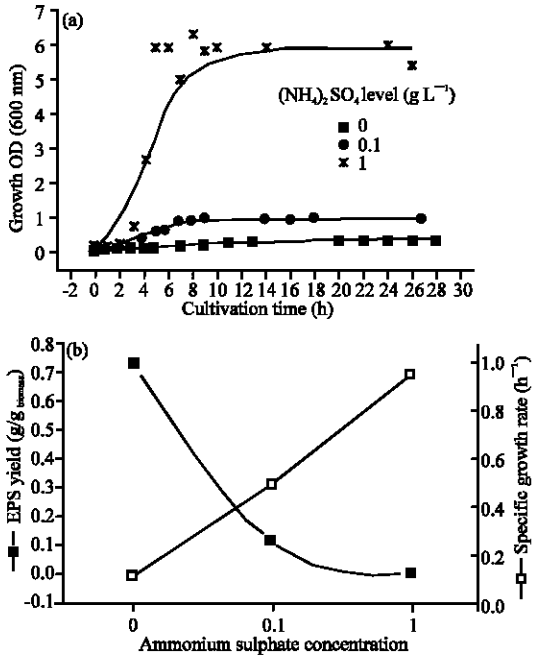

Fig. 3: Growth behavior of *K. pneumoniae* at different ammonium sulphate concentration in pH controlled batch cultures (a) and the dependency of EPS yield and growth rate on these level (b)

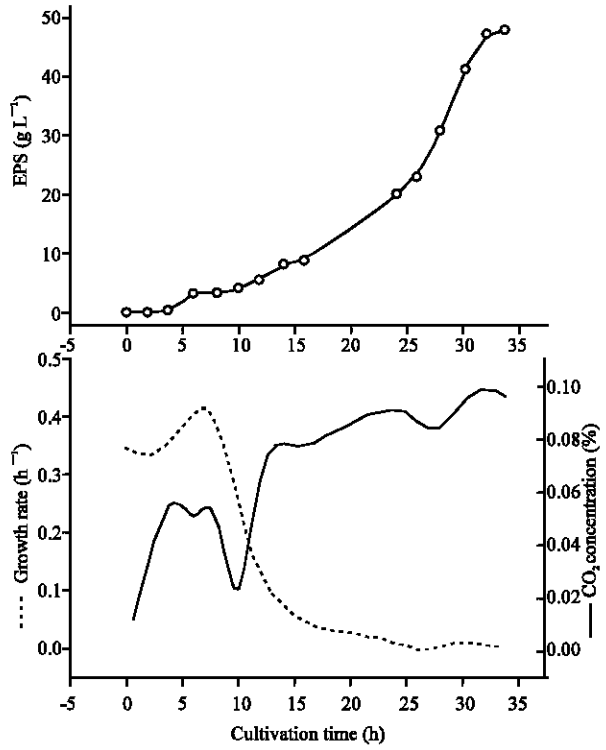

Fig. 4: EPS concentration (a), CO_2 production rate and the bacterial growth rate (b) as a function of time in a fed batch culture with constant feeding rate of 0.04 g L*h ammonium sulphate

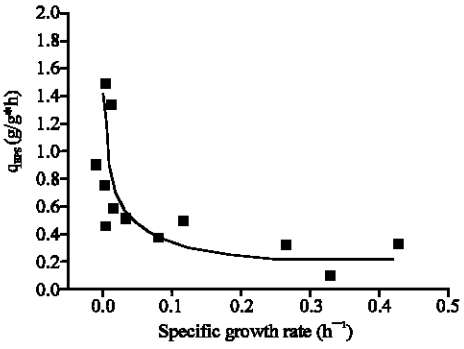

Fig. 5: The relation between specific EPS production rate in g/g*h and the specific growth rate in a fed batch culture with constant feeding rate of ammonium sulphate

In summary, the nitrogen limited fed batch culture did give the maximized overall EPS concentration (48 g L^{-1}) representing more than 90 fold increase over the conventional batch culture. This increase in EPS concentration was also evident when the bacterial cultures were subjected to low-speed centrifugation (14000 rpm) as described by Lai *et al.* (2003). Batch culture precipitated much faster than that of the fed batch culture. In fact samples from fed batch culture failed to precipitate even for longer period (45 min) at the same speed. Higher EPS concentration was accompanied with an increased biomass concentration that reached 10 fold increase. Finally, the exopolysaccharide yield was also relatively high which reached a value of 15 g g^{-1}. Indeed more studies are still needed for the further optimization of the feeding rate and the type of feeding strategy for the maximization of EPS by the clinical isolates of *Klebsiella pneumoniae.*

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