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# Enhancement and Scale-Up of β-(1, 3) Glucan Production by *Agrobacterium* sp.

Sri Lakshmi Puliga, Suhas Handa, Sathyanarayana N. Gummadi, and Mukesh Doble

## Abstract

Curdlan is a water insoluble polysaccharide composed exclusively of β-(1, 3) linked glucose residues. *Agrobacterium* sp. is known to produce extracellular curdlan under nitrogen-limited conditions. The purpose of this study was to investigate the effects of pH, amounts of ammonium, sucrose and trace elements, and time the addition of sucrose, ammonium and uracil on the production of curdlan in a shake flask and to further scale-up the process to a 5 L fermentor. A maximum of 48.7 g $L^{-1}$ of curdlan was obtained in a shake flask when 150 and 1.4 g $L^{-1}$ of sucrose and ammonium were used at the initial pH of 6.5. The production was enhanced to 57 g $L^{-1}$ by adding one third of sucrose and 1 g $L^{-1}$ of uracil at the $48^{th}$ h in a fed batch mode. The process was scaled up to a 5 L bioreactor in a batch mode where the oxygen transfer rate was higher (0.192 mg $L^{-1}s^{-1}$) when compared to that in the shake flask (0.096 mg $L^{-1}s^{-1}$). Curdlan production was 58 g $L^{-1}$ in the bioreactor, which was higher than the shake flask under batch conditions (48.7 g $L^{-1}$). The viscosity average molecular weight of the curdlan produced was found to be $1.4 \times 10^5$.

**KEYWORDS:** beta-(1, 3) glucan, curdlan, *Agrobacterium* sp., nitrogen limitation, bioreactor, scale-up

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## Introduction

Curdlan is a water-insoluble polysaccharide composed exclusively of β- (1, 3) linked glucose residues. It is one of the structural macromolecules in the cell wall of higher plants (wound-induced sugar on the plant new cell wall, and callose), yeast (located in the budding scar) and mushrooms (e.g. lentinan). It also plays a role in storing polysaccharide in brown algae (laminarin), euglenoids (paramylon) and fungi (pachyman) [1]. Bacteria belonging to the *Agrobacterium* and *Alcaligenes* species have been reported to produce this extracellular linear β-(1, 3) glucan under nitrogen limiting conditions [2-6]. Harada and coworkers discovered this biopolymer and named it curdlan because it curdled on heating [7]. The production of curdlan has drawn considerable interest because of its unique rheological and thermal gelling properties, which allows its potential use in food products such as jelly, noodles, edible fibers and new calorie reduced products; and as an immobilization support [8, 9]. In 1996, FDA approved this material as a food additive and following which, products based on curdlan were introduced in the US market [10]. Curdlan has been used in drug delivery since it can sustain and control the diffusion of these compounds [11]. Furthermore, researchers developed curdlan sulfate as an agent to inhibit the infection related to human immunodeficiency virus [12]. Takeda Chemical Industires Ltd., Japan, has also used it as an admixture of concrete to enhance its fluidity [13]. Therefore it is important to increase its productivity by manipulating the process conditions and decrease the production cost so that it can compete with other polysaccharides [14]. Lawford *and* coworkers did a major work in designing reactors to enhance its production [5]. With a low- shear system using an axial- flow marine- type propeller, they obtained a yield of 46 g $L^{-1}$ and 64.4 g $L^{-1}$ at an agitation speed of 400 and 600 rpm respectively in a 5.0 L fermentor [21]. Various carbons sources were tested for curdlan production and found that sucrose was best [3]. Curdlan production was also enhanced only under nitrogen limiting conditions [3, 19]. In bioreactor studies, it has been shown that a change in pH from 7.0 to 5.5 under nitrogen limiting conditions is required for maximum production of curdlan [6]. Also, the addition of nucleotide (uracil) after nitrogen limiting conditions found to enhance the curdlan production [23]. Hence, it is important to investigate the effect of sucrose, ammonium, uracil and pH on the curdlan production.

The purpose of this study was to investigate the effect of pH, ammonium, sucrose and trace element amounts and time of addition of sucrose, ammonium and uracil to maximize the curdlan production in shake flask and further scale up the process in a 5 L bioreactor. The rates of cell growth and product formation greatly depended on pH. The exopolysaccharide is produced during the post stationary phase under the nitrogen limited condition, but with the presence of an excess amount of sucrose.

## Materials and methods

### Microorganism and culture conditions

*Agrobacterium sp.* ATCC 31750 (formerly known as *Alcaligenes faecalis subsp. myxogenes*) was used for curdlan production. The organism was maintained in medium contained 20 g $L^{-1}$ sucrose, 5 g $L^{-1}$ yeast extract and 5 g $L^{-1}$ peptone and 20 g $L^{-1}$ of agar at pH 7.0. The seed culture medium contained 20 g $L^{-1}$ sucrose, 5 g $L^{-1}$ yeast extract and 5 g $L^{-1}$ peptone at pH 7.0. The basal fermentation medium contained (per liter): 100 g sucrose, 1 g $(NH_4)_2HPO_4$, 1 g $KH_2PO_4$, 0.5g $MgSO_4.7H_2O$ and 10 mL of trace element solution. The composition of the trace element solution was 5 g $FeSO_4.7H_2O$, 2 g $MnSO_4.H_2O$, 1 g $CoCl_2.6H_2O$ and 1 g $ZnCl_2$ per liter of 0.1 N HCl [19]. All the chemicals unless otherwise mentioned were obtained from HiMedia Laboratories Limited, Mumbai, India and SRL Pvt. Limited, Mumbai, India. Synthesis of curdlan was carried out in a 500 mL conical flask containing 100 mL of this production medium. The seed culture (5%) was grown at 30 $^oC$ and 180 rpm for 17 h in the shake flask and then transferred to the production medium and the batch was run for 120 h and 180 rpm at 30 $^oC$.

### Shake flask experiments

The effect of various initial concentrations of sucrose (100, 150 and 180 $gL^{-1}$), ammonium (0.50, 1.0, 1.4 and 1.8 g $L^{-1}$), uracil (0.5, 1.0 and 1.5 g $L^{-1}$) and trace element solution (10, 13 and 15 mL/L) and pH (from 5.5 to 7.5) on cell growth and curdlan production were tested in the shake flask. Uracil was added at the $48^{th}$ h. In addition, one experiment was carried out by adding uracil (1.0 $gL^{-1}$) and sucrose (50 g $L^{-1}$) at the $48^{th}$ h when the ammonium in the production medium was depleted (the initial sucrose in this experiment was 150 g $L^{-1}$). The effect of addition of various concentrations of (2, 5, 8 and 20 mM) Reactive Oxygen Species (ROS) inducing agent namely, hydrogen peroxide ($H_2O_2$) at the $36^{th}$ h on cell growth and production was also studied. The volumetric mass transfer coefficient ($K_La$) in the fermentor was measured by static gassing out method.

### Bioreactor studies

Production of curdlan was also carried out in a 5 L fermentor of 24.13 cm diameter (BIOFLO 110 Modular Benchtop Fermentor/ Bioreactor, New Brunswick Scientific Co., INC., Edison, U.S.A) equipped with a 6 bladed 9.25 cm diameter turbine agitator, a dissolved oxygen analyzer and a pH controller. The seed culture (240 mL) which was grown at 30 $^oC$ for 17h in the shake flask was transferred to the fermentor containing 2.4 L of the fermentation medium. Culture

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temperature was controlled at 30 $^{o}$C, while the agitation speed and aeration rate were maintained at 600 rpm and 0.5 vvm respectively. The initial pH of the medium was adjusted to 6.5 and maintained at that value by continuously dosing alkali (4 N NaOH). When nitrogen was depleted in the medium, the pH was manually adjusted to 5.5 using acid (3 N HCl) and the batch was run for 120 h.

**Estimation of curdlan and cell dry weight**

The concentration of cells and curdlan were determined by measuring their dry weight. 10 mL of the sample was centrifuged at 10,000 rpm for 15 min at 4 $^{o}$C. Pellet consisting of cells and curdlan was washed twice with 0.01 N HCl and harvested by centrifugation. The curdlan was solubilized by adding 15 mL of 2 N NaOH at 30 $^{o}$C for 3 h. The cells were separated by centrifugation at 10,000 rpm for 15min at 4 $^{o}$C. The curdlan present in the supernatant was precipitated by the addition of an appropriate amount of 2.0 N HCl. Both cells and curdlan were washed thrice with water and dried to a constant weight in a hot air oven (Gambaks Instruments Co., Chennai) at 50 $^{o}$C for 24 h and then lyophilized (Virtis Benchtop 4K Freeze Dryer, SP Industries, New York) [19].

**Analytical methods**

Sucrose concentration was determined using DNS method [15]. Ammonium ($NH_4^+$) concentration was determined by the Indophenol method [16]. Curdlan was quantified by the fluorescence dye binding micro assay using Aniline Blue dye [1].

**Characaterization of Curdlan**

FTIR (Fourier transform Infra Red) spectroscopic analysis of curdlan was performed by the KBr disc method [20]. Approximately 300mg of dry KBr and 5 mg curdlan was mixed and ground to a fine powder. Some of the ground mixture was transferred into a die which was placed on the anvil below the plunger of the pellet making machine and 10-16 kpsi pressure was applied on the plunger for 1-2 min. The pellet was pushed out and placed in the FTIR spectrophotometer holder (Spectrum one: Perkin Elmer, USA) and the transmittance was measured from 450 to 4000 $cm^{-1}$ at a resolution of 4 $cm^{-1}$.

Molecular weight of curdlan was determined by dilute solution viscometry. Curdlan samples were weighed and then dissolved in 0.3 N NaOH to get various concentrations of 0.25, 0.5, 1, and 2% (w/v). The sample solution was then poured into the viscometer (Ostwald Viscometer, Barnstead Int'l/ ERTCO, Ottawa) and the time taken for the liquid level to drop from point A to B (marking

in the viscometer) was noted with the help of a stopwatch (efflux time) at 25 $^{o}$C and 0.3 M NaOH served as blank. The efflux times were noted for each concentration and the experiments were repeated thrice. A graph was prepared between the reduced viscosity ($\eta_r$) (on the Y axis) and the concentration of the solution (on the X axis), where the reduced viscosity is given by the following relationship

$$\frac{\text{Efflux time of sample (t)} - \text{Efflux time of Blank } (t_o)}{\text{Efflux time of blank } (t_o)} = \text{Specific viscosity } (\eta_{sp})$$

$$\frac{\text{Specific Viscosity } (\eta_{sp})}{\text{Concentration of sample}} = \text{Reduced Viscosity } (\eta_r)$$

The molecular weight (M) of the sample is calculated from the following relation [27], where $\eta_{in}$ (intrinsic viscosity) is the intercept of the graph, and it is estimated by extrapolating the line to the Y axis.

$$[\eta_{in}]=K M^{a}$$

$K= 7.9\times 10^{-3}$ and $a = 0.78$

**Estimation of $K_La$**

$K_{La}$ was determined by the static gassing out method [30]. The medium was first degassed with nitrogen followed by aeration (0.5, 1.0, 1.5 and 2.0 vvm) and agitation (400, 500, 600 and 700 rpm) rates. At every condition of aeration and agitation, percentage dissolved oxygen concentration in the medium was measured using DO probe as a function of time. The value of $K_{La}$ is the slope of the semi-logarthmic plot of $(1-pO_2)$ against time by the following equation:

$$\ln (1-pO_2) = -K_{La} * t$$

**Results and Discussion**

**Kinetics of curdlan production in shake flask**

Figure 1 shows the kinetics of cell growth, curdlan production, sucrose and ammonium consumption as a function of time in the shake flask (500 mL) at a batch size of 100 mL and for a batch time of 120 h. The initial pH of the medium was adjusted to 6.5 and the pH was neither measured nor controlled during the

fermentation. At 72$^{nd}$ h, the medium was depleted of nitrogen, while the cell growth reached the maximum value. Production of curdlan was noticed at the 50$^{th}$ h and it reached to 48 g $L^{-1}$ in 120 h. Lawford *et al.* [5] also observed a similar trend in a 2.0 L baffled stirred tank reactor.

**Effect of initial pH on curdlan production in shake flask for 120 h**

pH of the culture is one of the most important factors because it significantly influences the cell growth and hence the curdlan production [6]. The effect of initial pH on the production was studied in the shake flask by varying it between 5.5 and 7.5. It was found that maximum cell growth and curdlan production were observed when the initial pH was 6.5 for a batch time of 120 h (Figure 2). As the reaction proceeds, the pH of the medium at the end of 120h decreases to a value of about 5.2. No cell growth or curdlan production was observed when the initial pH was 5.5. High viscosity of the culture broth is often a critical problem in polysaccharide production and this can be overcome by operating the fermentation at a slightly acidic pH because curdlan is insoluble under such conditions and will settle down as precipitate. The curdlan production is divided into two phases- the cell growth phase and the curdlan production phase [2]. The latter happens after the depletion of nitrogen. Lee et al [6] sought an optimal pH profile to maximize curdlan production in a batch fermentation of *Agrobacterium* species and observed that the cell growth was maximum at pH 7.0 while curdlan production was maximal at pH 5.5. Gummadi and Kislay Kumar [19] studied the effect of initial pH and found that maximum curdlan production was obtained when the initial pH was 7.5. The current result suggests that higher yield of curdlan can be achieved by high cell density by growing *Agrobacterium sp*. at pH 6.5.

**Effect of DAHP and uracil concentration on curdlan production in shake flask for 120 h**

It was observed that curdlan production started only when ammonium (Di Ammonium Hydrogen Phosphate, DAHP) concentration reached a low value (under nitrogen limited conditions). It has been reported that isoprenoid lipid which plays a crucial role in transporting cellular oligosaccharides would be available for the synthesis of cellular exopolysaccharides under nitrogen- limiting conditions [17]. The effect of initial ammonium concentration on curdlan production was studied with sucrose as carbon source (100 g $L^{-1}$) at different initial ammonium concentrations (0.50, 1.0, 1.4 and 1.8 g $L^{-1}$). The maximum production (52 g $L^{-1}$) was observed when the initial ammonium concentration was 1.4 g $L^{-1}$, even though the glucose consumption was the same in all the cases

(Table 1). At high ammonium concentration (1.8 g $L^{-1}$) it was found that it did not get completely consumed, which in turn reflected in low curdlan production (42.8 g $L^{-1}$) although the cell dry weight was the highest (7.6 g $L^{-1}$). As mentioned before, curdlan production is higher during nitrogen limited conditions. At a higher concentration of ammonium in the medium, glucose was utilized for cell growth rather than for curdlan production. However, even at lower concentration of ammonium in the medium (0.5 g$L^{-1}$), curdlan production was lower (38.9 g $L^{-1}$) than that produced at an initial concentration of 1.4 g $L^{-1}$ (52.8 g $L^{-1}$). This is probably due to the fact that at lower concentrations of nitrogen, cell growth was low which in turn affected curdlan production. Hence maintaining high cell growth as well as driving the process to nitrogen limitation is crucial for maximum curdlan production. Kim *et al*. (1997) and Gummadi and Kumar [19] also arrived at the same conclusions based on their experimental findings.

It has been shown that when curdlan synthesis was initiated, the intracellular levels of UMP and AMP were highly elevated [18]. As nitrogen was depleted, levels of UMP rose and addition of uracil to the culture medium increased the curdlan yield [18]. Hence the effect of various concentrations of uracil (0.5, 1.0 and 1.5 g $L^{-1}$) added at the 48$^{th}$ h on curdlan production was studied here. The maximum production (57 g $L^{-1}$) was observed when 1.0 g $L^{-1}$ of uracil was added at the 48$^{th}$ h and at an initial nitrogen concentration of 1.4 g $L^{-1}$ (Table 1). The time of uracil addition was very important to improve curdlan production. When uracil was added along with ammonium to the fermentation medium, it was used as a nitrogen source for the cell growth and hence though the cell amount increased, there was a decrease in curdlan produced. When uracil was added after 48 h when nitrogen was depleted in the medium, it was degraded slowly and enhanced the curdlan produced.

With the addition of both, sucrose (50 g $L^{-1}$) (150 g $L^{-1}$ of sucrose was added initially) and uracil (1 g $L^{-1}$) at the 48$^{th}$ h, curdlan yield reached 57 g $L^{-1}$ at 120 h of fermentation (Table 1). It was reported that the addition of uracil, a precursor of UDPGlc after 48h of cell growth resulted in an elevation in curdlan production [22]. It was shown that uracil addition at 46$^{th}$ h to a sucrose containing medium increased curdlan production compared to an unsupplemented medium after 160 h of growth [23]. The current results are consistent with the reported findings.

**Effect of sucrose concentrations on curdlan production in shake flask**

The effect of various concentrations of sucrose (100,150 and 180 g $L^{-1}$) on curdlan production indicated that a maximum of 50 g $L^{-1}$ curdlan was produced at a sucrose concentration of 150 g $L^{-1}$ (Table 2). When 200 g $L^{-1}$ of sucrose was added in two installments, namely 150 g $L^{-1}$ at the initial stage and the remaining

at the 48$^{th}$ h, there was an increase in curdlan production (55 g $L^{-1}$). This yield was much higher than when using 180 g $L^{-1}$ of sucrose. Using maltose, glucose and sucrose as carbon source Lee *et al* [3] obtained a yield of 48, 40 and 47 g $L^{-1}$ respectively. Sucrose is a cheaper carbon substrate than glucose and maltose, and hence it was used in the current study.

**Effect of trace elements concentrations on curdlan production in shake flask**

The effect of trace element ions such as $Fe^{2+}$, $Mn^{2+}$, $Zn^{2+}$ and $Co^{2+}$ on curdlan production was studied by varying the quantity of the trace element solution in the production medium (10, 13 and 15 ml/L). The ratio of the trace elements was maintained constant. When the quantity of the trace element solution was 13 ml/L, the curdlan production was maximum (50 g $L^{-1}$) (Table 3).The results suggest that trace elements are essential in increasing the biomass, and curdlan production. Trace elements are also called micro nutrients and they are essential because they play an important role in the metabolism. $Fe^{2+}$ is a component of cytochromes and certain non-heme proteins and it is also an important cofactor of various enzymes. $Mn^{2+}$, $Zn^{2+}$ and $Co^{2+}$ are the main cellular inorganic cations and cofactors for various enzymes and therefore they too play a vital role in the biosynthesis of curdlan [8, 17, 19].

**Effect of hydrogen peroxide concentrations on curdlan production in shake flask**

The effect of addition of $H_2O_2$ at 48$^{th}$ h on curdlan production was studied by varying its concentration in the production medium (2, 5, 8 and 20 mM). The results indicate that a maximum of 53 g $L^{-1}$ curdlan was produced at a $H_2O_2$ concentration of 8 mM (Figure 3). At higher concentration of $H_2O_2$ (20 mM), yield was low since it was lethal to the cells. Treatment of microbial cultures with sub-lethal doses induced the production of Reactive Oxygen Species (ROS) in the culture, which increased the cell growth and subsequently the yield of curdlan. Oxidative stress results when the rate of production of ROS exceeds the capacity of the cells to dispose it. Under physiological conditions, ROS are continuously generated in aerobic cells from metabolic processes such as respiration, fatty acid biosynthesis or due to environmental factors such as near UV or ionizing radiations [ref]. ROS can be induced in cultures by treatment with chemicals such as $H_2O_2$, hypochlorous acid (HOCl), menadione, paraquat, etc. Surabhi *et al* [24] observed a maximum cell growth and high specific activities of enzymes such as α- amylase and protease when *B. subtilis* was treated with ROS inducing agents such as $H_2O_2$ and HOCl.

**Scale up of curdlan production**

The production of curdlan was scaled up in a 5.0 L fermentor with a working volume of 2.4 L. Figure 4 shows the ammonium and sucrose consumption, biomass and curdlan yield as a function of time during the fermentation process. Nitrogen which was added initially was completely consumed in 14 h and the cell concentration increased to 6.7 g $L^{-1}$. The pH was set initially at 6.5 which was optimal for maximum cell growth and it was reset to a value of 5.5 after 15 h, since this was optimal for the production of curdlan under the nitrogen limited conditions. pH of the culture is one of the most important factors because it significantly influences the rate of cell growth and product formation. Sucrose consumption and cell growth leads to acidic byproducts and hence alkali is dosed continuously into the fermentor to maintain the set pH. The DO (dissolved oxygen) level was continuously monitored during the fermentation process. From the changes in the DO level, the physiological state of the cells could be indirectly accessed. After the consumption of ammonium, the physiological state of cell culture changes from growth to production phase. When the cells are in the growth stage, they need plenty of oxygen and hence the DO level in the fermentor is low. However, the oxygen demand of the cell during curdlan production stage is relatively less since there is no cell growth. Hence the DO increases after the consumption of ammonium. This observation can be a signal to decrease the pH from 6.5 to 5.5, namely from optimal value for cell growth to optimal for curdlan production respectively [6]. *Agrobacterium sp.* ATCC 31750 is highly aerobic and therefore an adequate supply of oxygen is important for its maximum growth. Since at acidic pH, curdlan is insoluble in water, the fermentation broth is of relatively low viscosity and there is little resistance to oxygen transfer from gas to the solid cells. It is difficult to maintain the pH in the shake flask whereas; in a fermentor it can be controlled accurately. Curdlan production started by 24$^{th}$ h in the fermentor but in the shake flask it commenced only after the 72$^{nd}$ h. The amount in the fermentor reached a value of 57.6 g $L^{-1}$ in 120 h, which was much higher than what was obtained in the shake flask under same conditions.

A relationship between the gas to liquid mass transfer ($K_La$) in a shake flask and the operating parameters such as shaker speed (N) and the ratio of volume of liquid ($V_L$) to the flask volume ($V_o$) is given by the following relationship [25].

$$K_L a = 0.141 N^{0.88} \left( \frac{V_L}{V_o} \right)^{-0.8}$$

For the current operating conditions ($V_L$=100 mL, $V_o$=500 mL, N=180 rpm), $K_La$ is equal to 0.0137 $s^{-1}$. The oxygen transfer rate (OTR) is given by the relation:

$OTR = k_La\ (C^*-C_L)$

where C*=7 mg/L and if $C_L = 0$, then OTR=0.0959 mg $L^{-1}$ $s^{-1}$
(C* = Equilibrium concentration and $C_L$ = concentration of oxygen dissolved in the liquid)

Figure 5 shows the estimated $K_La$ as a function of agitator rpm and air flow rate in the fermentor using static gassing out method. $K_La$ increases when these operating parameters are increased. For the current operating conditions (N=600 rpm, and 0.5 vvm), $K_La = 0.0275\ s^{-1}$ and the OTR is 0.192 mg $L^{-1}$ $s^{-1}$

The OTR in the fermentor is about twice than that in the shaker and in addition, the pH is controlled in the fermentor through a feed back loop, while it is not controlled in the shaker and, it is left to reach its own value. The amount of biomass and curdlan produced in the fermentor was higher than what was obtained in the shake flask (biomass of 5.86 and 6.56 g $L^{-1}$ and curdlan of 48.69 and 57.6 g $L^{-1}$ in the shaker and in the fermentor respectively).

Lee et al [21] have reported approximately 64 g $L^{-1}$ of curdlan production in 5 and 300 L fermentors both operated at 0.5 vvm. These reactors have turbine agitators with agitator to vessel diameter as 0.48 and 0.34 respectively, and it is equal to 0.38 for the current fermentor.

## Characterization of curdlan

The FTIR spectra of curdlan produced in the fermentor is shown in Figure 6. The spectrum is dominated by a broad band at about 3370 $cm^{-1}$, which is assigned to the stretching vibration modes of OH groups. The peak at 1644 $cm^{-1}$ in the spectrum is attributed to the existence of water molecule which is not completely removed from the sample. The peaks at 1160, 1234, 1261, 1080, 1373, 1317& 2917 in the spectrum correspond to $C_1$-O-$C_3$, C-O, C-OH, C-O, CH, $CH_2$ and $CHCH_2$ groups respectively present in the samples. The absorption near 890 $cm^{-1}$ and 1160 $cm^{-1}$ are indicative of β – linked glycosidic bonds and $C_1$-O-$C_3$ linkage respectively and these are present in the products produced in the shaker and the fermentor.

The viscosity average molecular weight of curdlan produced in the fermentor is $1.4 \times 10^5$and that of Sigma Aldrich Curdlan was $7.4 \times 10^5$. The literature reported viscosity average molecular weight of curdlan is between $5.3 \times 10^4$ and $2.0 \times 10^6$ [27-29]. Under alkaline conditions curdlan remains in solution and hence can undergo further polymerization, while if it precipitates out in the acidic conditions, the extent of polymerization may be curtailed. So this could be

one of the reasons for the differences in the molecular weight reported in the literature.

## Conclusions

The current study indicates that curdlan production in shake flask can be enhanced by suitably varying the concentrations of sucrose, ammonium, uracil, trace elements and pH. A two-step fed-batch operation in the shake flask led to a maximum biomass production followed by maximum curdlan production. The solubility of curdlan in the medium increases with culture pH and under alkaline conditions a viscous broth is formed. Efficient transfer of oxygen from the gas phase to cells is a crucial step in the process. Hence operating the reactor under acidic pH during the production stage could lead to facile operation of the fermentor. Under best conditions of nutrients and pH (at a pH 6.5, ammonium of 1.4 g $L^{-1}$ uracil of 1.0 g $L^{-1}$ and sucrose of 150 g $L^{-1}$ and addition of one-third of sucrose and all of uracil at $48^{th}$ h), a high concentration of curdlan (57 g $L^{-1}$) was obtained in 120 h of cultivation using *Agrobacterium sp*. ATCC 31750 in shake flask. Addition of ROS inducing agent such as $H_2O_2$ also enhanced curdlan production (53 g $L^{-1}$ from 46 g $L^{-1}$). A trouble free scale up of curdlan production can be attained by controlling pH in growth and curdlan production phase and maintaining high gas to liquid mass transfer (with high air flow rate and agitator rpm). This study indicates that it is possible to obtain very high yields of curdlan even in shake flask. Further studies need to be carried out by optimizing the culture conditions in the fermentor to enhance the curdlan production.

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**Table 1 Effect of DAHP uracil concentration on curdlan production in shake flask for 120 h.** Fermentation was carried out at for 120 h at 180 rpm and 30 $^{o}$C on a rotatory shaker. Uracil was added at 48$^{th}$ h along with 50 g $L^{-1}$ glucose. Experiments were performed in triplicates under identical conditions and the values reported are mean.

| Initial ammonium (g $L^{-1}$) | % consumption of ammonium at 120 h | % sucorse consumption at 120 h | Biomass (g $L^{-1}$) | Curdlan (g $L^{-1}$) | g curdlan per g biomass |
|---|---|---|---|---|---|
| 0.5 | 0.023 | 5.92 | 4.5±0.14 | 38.92±1.1 | 8.65 |
| 1.0 | 0.025 | 6.85 | 5.12±0.1 | 48.69±0.9 | 9.51 |
| 1.4 | 0.018 | 5.96 | 6.03±0.2 | 52.82±2.1 | 8.76 |
| 1.8 | 0.14 | 5.73 | 7.58±0.4 | 42.75±1.9 | 5.64 |
| 1.4 + 0.5 g $L^{-1}$ uracil + 50 g $L^{-1}$ sucrose at 48 h | 0.025 | 5.85 | 6.05±0.1 | 53.01±0.8 | 8.76 |
| 1.4 + 1.0 g $L^{-1}$ uracil + 50 g $L^{-1}$ sucrose at 48 h | 0.012 | 5.91 | 6.12±0.12 | 57.46±1.1 | 9.4 |
| 1.4 + 1.5 g $L^{-1}$ uracil + 50 g $L^{-1}$ sucrose at 48 h | 0.068 | 5.77 | 6.24±0.18 | 52.05±1.5 | 8.34 |

**Table 2 Effect of sucrose concentrations on curdlan production in shake flasks at 120 h.** Fermentation was carried out at for 120 h at 180 rpm and 30 $^{o}$C on a rotatory shaker. Experiments were performed in triplicates under identical conditions and the values reported are mean.

| Initial sucrose concentration (g $L^{-1}$) | Biomass (g $L^{-1}$) | % sucrose consumption | Curdlan (g/L) | g curdlan per g biomass |
|---|---|---|---|---|
| 100 | 5.71±0.11 | 6.52 | 48.3±0.9 | 7.41 |
| 150 | 6.82±0.27 | 4.56 | 50.23±2 | 11.02 |
| 180 | 4.53±0.04 | 18.91 | 44.51±0.5 | 9.83 |
| 150 +50* | 6.00±0.09 | 5.8 | 55.1±1.1 | 9.53 |

* Initial sucrose concentration was 150 g $L^{-1}$ and 50 g $L^{-1}$ of sucrose was added at 48$^{th}$ h of fermentation

**Table 3 Effect of trace elements concentrations on curdlan production in shake flask.** Fermentation was carried out at for 120 h at 180 rpm and 30 $^{o}$C on a rotatory shaker. Initial concentration of glucose and ammonium was 150 and 1.4 g $L^{-1}$ respectively. Experiments were performed in triplicates under identical conditions and the values reported are mean.

| Trace element concentration ml $L^{-1}$ | Biomass g $L^{-1}$ | Curdlan g $L^{-1}$ | g curdlan per g biomass |
|---|---|---|---|
| 10 | 5.71±0.11 | 48.69±0.5 | 8.53 |
| 13 | 6.34±0.13 | 50.05±1.0 | 7.94 |
| 15 | 6.02±0.3 | 47.51±2.3 | 7.92 |

## List of Figures

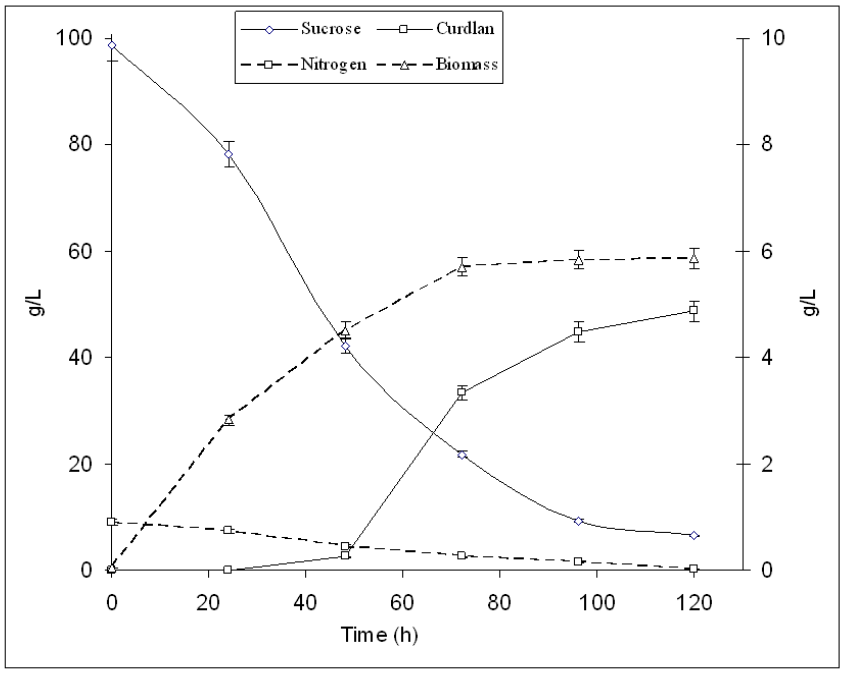


**Figure 1** Kinetics of curdlan production in shake flask at pH 7.0. Fermentation was carried out at for 120 h at 180 rpm and 30 $^{o}$C on a rotatory shaker. Experiments were performed in triplicates under identical conditions and the values reported are mean.

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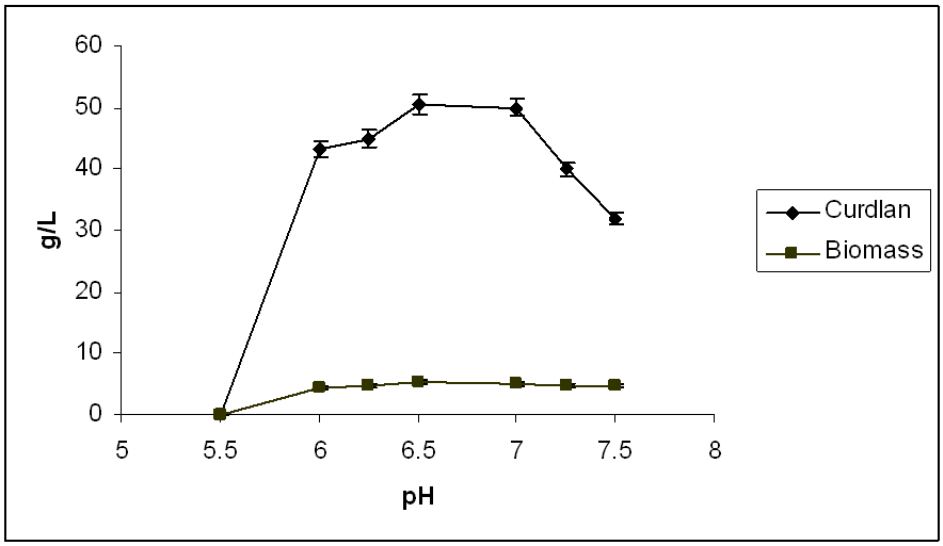


**Figure 2** Effect of initial pH on curdlan production in shake flask for a batch time of 120 h. Fermentation was carried out at for 120 h at 180 rpm and 30 $^{o}$C on a rotatory shaker. Experiments were performed in triplicates under identical conditions and the values reported are mean.

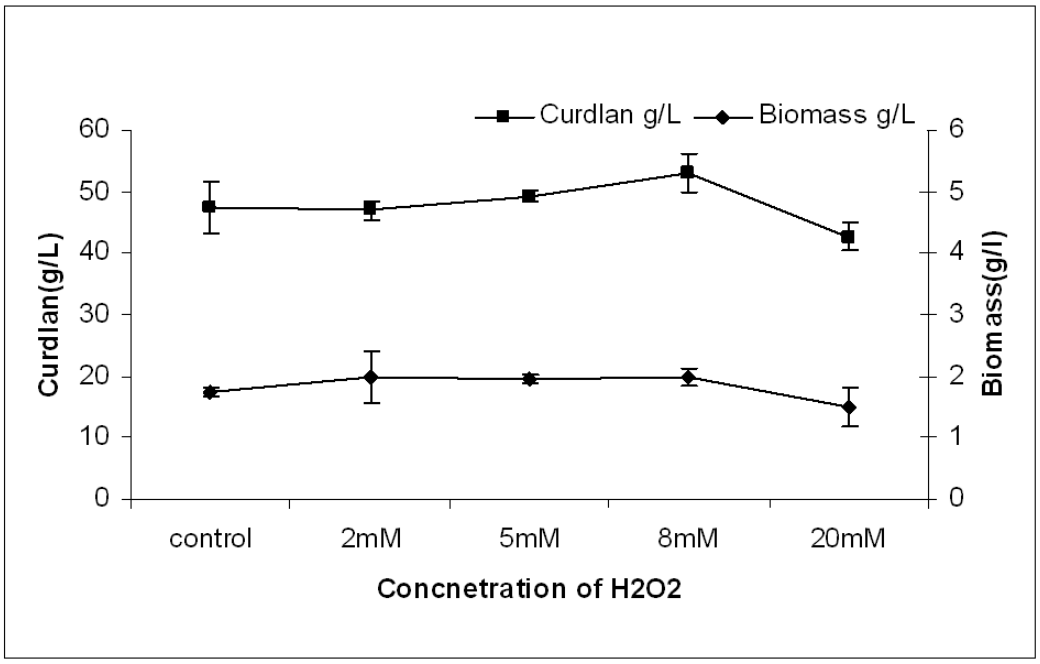


**Figure 3** Effect of hydrogen peroxide concentrations on curdlan production in shake flask. Fermentation was carried out at for 120 h at 180 rpm and 30 $^{o}$C on a rotatory shaker. Various concentrations of $H_2O_2$ are added to flasks at 36 h of growth. Experiments were performed in triplicates under identical conditions and the values reported are mean.

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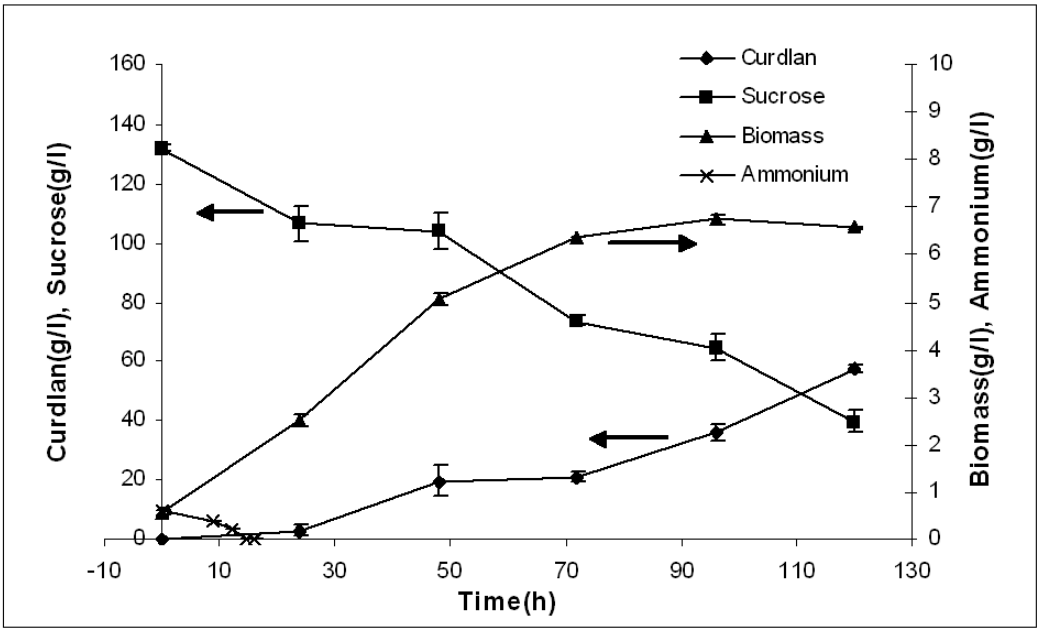


**Figure 4** Kinetics of curdlan production in a Fermentor. Fermentation was carried out at for 120 h in 5.0 L bioreactor with 2.4 L working volume. Conditions are temperature: 30 $^{o}$C, pH; initially maintained at 6.5 till nitrogen limitation and changed to 5.5 and controlled till 120 h, agitation speed: 600 rpm and aeration rate: 0.5 vvm.

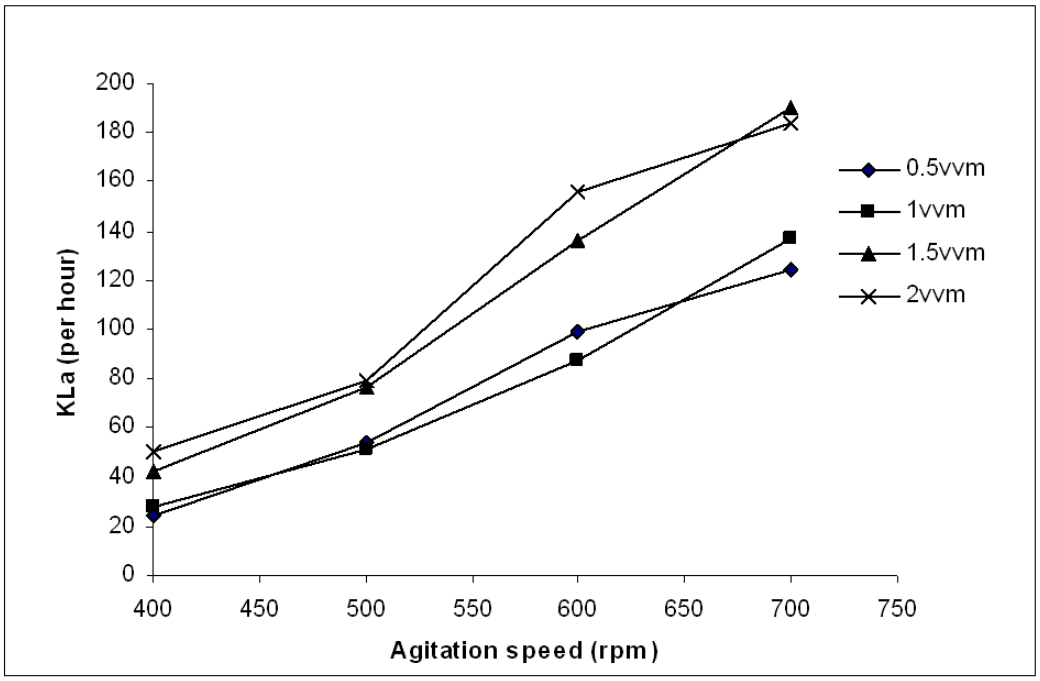


**Figure 5** Estimation of $K_La$ in 5L fermentor. $K_{La}$ was determined using static gassing out method as described in materials and methods. In case of shake flasks, $K_{La}$ was estimated using correlation [25].

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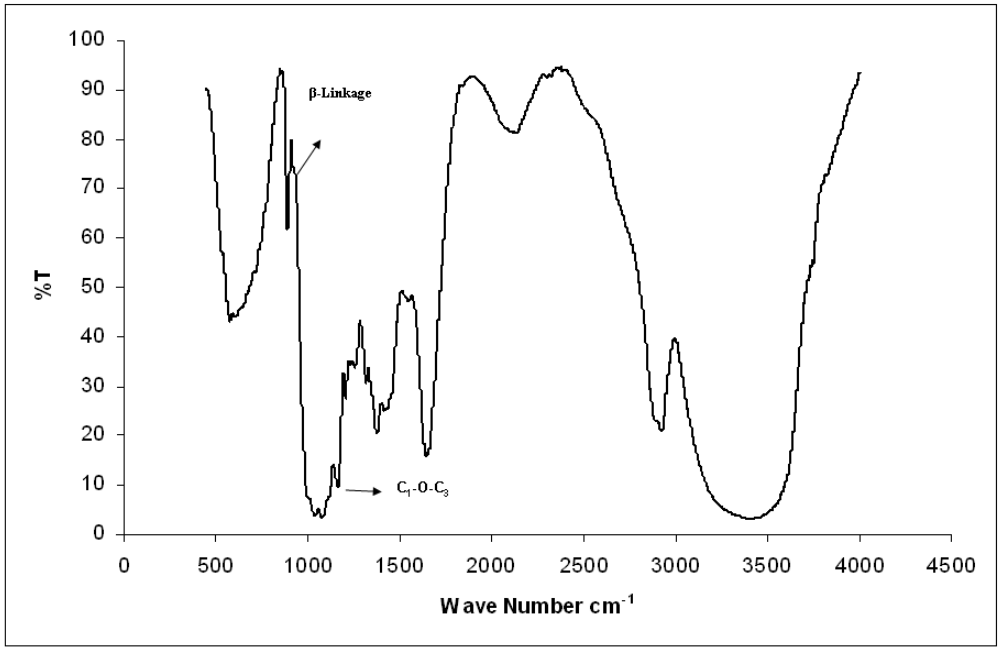


**Figure 6** FTIR spectra of curdlan produced by *Agrobacaterium*sp. ATCC 31750

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