0273-2289/04/118/0021/$25.00

Fermentative Production of Curdlan

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Received March 24, 2003; Revised August 27, 2003; Accepted August 28, 2003

Abstract

Curdlan was produced by pure culture fermentation using *Agrobacterium radiobacter* NCIM 2443. Three different carbon sources (glucose, sucrose, maltose) were selected for study. Sucrose was found to be the most efficient. Utilization of sugar during the course of fermentation was studied, and the data were correlated to the production of curdlan. Curdlan mimics a secondary metabolite, in that its synthesis is associated with the poststationary growth phase of nitrogen-depleted batch culture. This was inferred from the results obtained from utilization of nitrogen. Regulation of pH at 6.1 ± 0.3 resulted in an increased yield of curdlan from 2.48 to 4.8 g/L, and the corresponding increase in succinoglucan production was from 1.78 to 2.8 g/L. An attempt was made to increase curdlan production by the addition of the uridine nucleotides UMP and UDP-glucose to the fermentation broth. It was found that UDP-glucose at 0.8 µg/mL and UMP at 0.6 µg/mL served as precursors for curdlan and succinoglucan production when added after 18 h of nitrogen depletion in the fermentation broth.

Index Entries: Curdlan; succinoglucan; fermentation; UDP-glucose; UMP.

Introduction

Curdlan, a high molecular weight polymer of glucose, β-(1→3)-glucan, is produced by pure culture fermentation from a nonpathogenic and nontoxicogenic strain of *Agrobacterium biobar1* (identified as *Alcaligenes faecalis* var. *myxogenes*) or *Agrobacterium radiobacter (1)*. This water-insoluble polysaccharide has an unusual property of forming an elastic gel on heating its aqueous suspension. Harada *(2)* and Harada et al. *(3)* have shown that curdlan forms two types of heat-induced gels. First is the low-set gel, which is obtained when the aqueous suspension is heated to 55–60°C, and then subsequently cooled to below 40°C. The second type, high-set gel,

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requires heating the aqueous suspension to 80°C or above. The functional properties of the two gels are demonstrated to be quite different *(4)*. The low-set gel is thermoreversible, whereas the high-set gel is thermoirreversible. It is available as a white powder with high flow ability and can be stored for a long period of time without affecting its gelling properties. It is described as a nutritionally inert dietary fiber *(5)*.

The successful design of the fermentation process relies on producing a product according to set specifications (in terms of purity, solubility, gel strength, pH of solution, loss on drying, sulfated ash), while achieving yield and productivity economically as per new specifications prepared at the 53rd JECFA (1999). These goals can best be reached by optimizing media composition, fermentation conditions, and fermentor design as well by developing superior strains by mutation *(6)*. In addition to carbon and nitrogen sources, the medium for curdlan production also requires inorganic salts, trace elements, growth factors and precursor of fermentation product, dissolved oxygen, and other gases *(7)*.

The fermentation is sometimes partially diverted to succinoglucan by the producing organism *(8)*. This can, however, be minimized by regulating the fermentation conditions. Succinoglucan itself is an important metabolite of industrial use and has application in oil production and gravel packing, as a thickening, suspending, emulsion-stabilizing, and precipitation agent. It has also been employed in the cosmetic industry in the manufacture of shampoos, creams, emulsions, and hair dye compositions. Its use in the production of detergents and ink is also documented *(9,10)*.

Curdlan production is carried out in two stages. In the first stage, cells are grown in the seed culture, and in the second, seed culture is inoculated into the fermentation medium for curdlan production. The seed flask cultures represent an important microbial technique, providing a convenient method for growing the microorganisms in submerged culture.

The objectives of the present work were to study curdlan production using *A. radiobacter* NCIM 2443 and *A. faecalis* NCIM 2959 with respect to medium optimization and important fermentation parameters affecting curdlan production. For the synthesis of most polysaccharides, sugar nucleotides serve as glycosyl donors. UDP-glucose and UMP are among such precursors. However, very little is known about their effect on curdlan production. No data are reported on the exact concentration at which these nucleotides stimulate curdlan production. An attempt was made to increase the curdlan production by the addition of the uridine nucleotides UMP and UDP-glucose to the fermentation broth.

Materials and Methods

Microbial Culture Strains

Strains of *A. radiobacter* NCIM 2443 were procured from NCIM (Pune, India).

Maintenance of Culture and Production of Seed Culture

A. radiobacter NCIM 2443 was maintained on a medium containing 0.5 g of beef extract, 0.1 g of yeast extract, 0.5 g of peptone, 0.5 g of sucrose, 30 mg of $MgSO_4 \cdot 7H_2O$, and 2% agar in 100 mL of distilled water at 4°C and subcultured every 2 wk. The cells grown at 28°C for 48 h were used for inoculation into seed culture medium *(7)*.

The cell suspension was transferred to a 500-mL conical flask containing 100 mL of sterile seed culture medium. The flasks were incubated at 28°C for 24 h on a rotary shaker at 230 rpm. The medium was optimized by varying carbon source and nitrogen source and by the addition of stimulators for maximum curdlan production.

Optimization of Carbon Source

The basic medium contained 150 g/L of sucrose, 2.4 g/L of NH_4Cl, 1.0 g/L of KH_2PO_4, 0.5 g/L of $MgSO_4 \cdot 7H_2O$, and 10 mL of trace elements (5 g of $FeSO_4 \cdot 7H_2O$ + 2 g of $MnSO_4$ + 1 g of $CoCl_2 \cdot H_2O$ + 1 g of $ZnCl_2$ in 1 L of 0.1 *M* HCl) *(7)*. Sucrose was substituted by other sugars such as glucose and maltose at 50–250 g/L and studied for curdlan production in a shake flask. One hundred milliliters of autoclaved medium (with an initial pH of 7.0) was inoculated with 10 mL of seed culture of *A. radiobacter* NCIM 2443 and incubated for 72 h on a rotary shaker at 230 rpm at 28°C.

Optimization of Nitrogen Source

Nitrogen sources such as ammonium acetate, ammonium citrate, ammonium chloride, ammonium sulfate, potassium nitrate, and sodium nitrate were selected. These were added at 2.5 g/L and studied for curdlan production in shake flask. One hundred milliliters of autoclaved medium (with an initial pH of 7.0) was inoculated with 10 mL of seed culture of *A. radiobacter* NCIM 2443 and incubated for 72 h on a rotary shaker at 230 rpm at 28°C. Further, NH_4Cl was substituted at 1.5–3.0 g/L and studied for curdlan production in shake flask.

Effect of pH

Two different fermentation runs were carried out. One was without pH control and the other at a regulated pH of 6.1 ± 0.3. The pH in the shake flask was regulated every 6 h using 0.1*N* NaOH. The effect of pH regulation on growth curve, carbon utilization, nitrogen utilization, and curdlan production was studied.

Growth Curve for *A. radiobacter* NCIM 2443

One milliliter of broth was withdrawn every 8 h during the course of a 72-h fermentation. The broth was then diluted to 15 mL with 1*N* NaOH and vortexed to selectively dissolve curdlan. The suspension was centrifuged at 6000*g* for 10 min at 4°C. The supernatant was separated, and the cell pellet obtained was washed twice with distilled water to remove the

other polysaccharide and again centrifuged as just described. The cell pellet obtained was dried to constant weight at 60°C *(11)*.

Sugar Utilization During Fermentation

The phenol sulfuric acid method *(12)* was used for detection of total sugars in the fermentation broth during the entire course of a 72-h fermentation. One milliliter of broth was removed every 6 h of the 72-h fermentation. Each sample was clarified by centrifuging at 4000*g* for 15 min at 4°C to remove the cells and then suitably diluted. A quantity of 0.1 mL of each sample was analyzed by the phenol sulfuric acid method as follows: To 0.1 mL of diluted sample, 1 mL of 5% (w/v) phenol solution and 5 mL of 95% sulfuric acid were added. The tubes were mixed by shaking after 10 min, and cooled at 25°C for 30 min. The extinction was read at 490 nm. A standard curve was plotted using glucose in the concentration range of 10–100 µg/mL.

Nitrogen Utilization During Fermentation *(8)*

The fermentation broth was clarified by centrifuging at 4000*g* for 15 min at 4°C. The broth was suitably diluted and analyzed for nitrogen content during the entire course of fermentation using Nessler's reagent as follows: To each tube containing clarified broth, 2 mL of distilled water and 2 mL of Nesseler's reagent were added and mixed. Three milliliters of 2 *N* NaOH was added after 2 min and mixed. The absorbance was read at 420 nm.

Curdlan Assay

Samples were withdrawn every 8 h during the entire course of a 72-h fermentation. A suitably diluted sample was centrifuged at 8000*g* for 30 min at 4°C. The pellet consisting of cells and curdlan was washed with 0.1*M* HCl and harvested by centrifugation. NaOH (0.5*M*) was then added to selectively dissolve curdlan over 1 h. Cells were separated by centrifuging at 5000*g* for 30 min. The curdlan present in the supernatant was precipitated at pH 6.5 by the addition of an appropriate volume of 2*M* HCl. Curdlan thus separated was washed and dried to constant weight.

Succinoglucan Assay

Samples were withdrawn at an interval of 8 h during the entire course of a 72-h fermentation. Suitably diluted samples were centrifuged at 8000*g* for 30 min at 4°C. The succinoglucan was then precipitated from the supernatant by the addition of 2 vol of isopropyl alcohol. The precipitated succinoglucan was separated by centrifugation as just described. The succinoglucan was washed twice with isopropyl alcohol and dried to constant weight.

Effect of UDP-Glucose and UMP as Stimulating Agents on Curdlan Production by A. radiobacter *NCIM 2443*

Initially, the point of addition of both the stimulators to the fermentation medium was optimized. For this, three different fermentation runs

Table 1
Optimization of Carbon Source
for Curdlan Production Using *A. radiobacter* 2443[a]

Carbon source	Concentration (g/L)	Curdlan yield (g/L)
Glucose	50	0.39 ± 0.2
	75	1.2 ± 0.3
	100	1.8 ± 0.33
	125	2.10 ± 0.35
	150	3.16 ± 0.2
	200	4.0 ± 0.31
	250	3.8 ± 0.20
Sucrose	50	0.5 ± 0.10
	75	1.5 ± 0.25
	100	2.3 ± 0.22
	125	2.7 ± 0.31
	150	4.8 ± 0.40
	200	5.0 ± 0.1
	250	4.73 ± 0.2
Maltose	50	0.30 ± 0.16
	75	1.0 ± 0.12
	100	2.5 ± 0.20
	125	2.9 ± 0.28
	150	3.10 ± 0.26
	200	3.40 ± 0.20
	250	3.55 ± 0.34

[a]Results are the mean ± SD of three determinations.

were carried out. UDP-glucose and UMP were added at (1) the beginning of the fermentation, (2) after 32 h, and (3) after 50 h of fermentation. The yield of curdlan was monitored in each case.

Optimization was carried out by supplementing the fermentation media with different concentrations of both the stimulators (1, 10, 100, and 1000 µg/100 mL) at the optimized time interval (after 50 h of fermentation), and determining the yield of curdlan. The fermentation was carried out at 28°C at pH 6.1 ± 0.3 on a rotary shaker at 200 rpm. Further, using the stimulators in the range of 10–100 µg/100 mL and determining the yield of curdlan determined the exact or accurate concentration of UDP-glucose and UMP stimulating the curdlan synthesis.

Results and Discussion

High productivity using inexpensive carbon sources is important for industrial production of curdlan. Lee et al. *(13)* reported maltose and sucrose to be efficient carbon sources for the production of curdlan by *A. radiobacter* species. They also reported that molasses is a useful substrate for production of curdlan. Table 1 documents the effect of carbon sources

on curdlan production. Sucrose at 15% gave a maximum yield of 4.8 g/L, whereas glucose at 20% gave a maximum yield of 4.0 g/L. Maltose was least effective and gave a yield of 3.55 g/L at a 20% concentration. It was also seen that increasing the concentrations of sucrose and glucose to 25% decreased the yield of curdlan. An abrupt increase in sucrose concentration could have stressed the cells owing to the associated changes in osmotic pressure and thereby decreased curdlan production.

Harada et al. *(14)* studied the effect of various inorganic nitrogen compounds as sole nitrogen sources on curdlan production. They reported that $NaNH_4HPO_4$ gave a maximum yield of 1.760 mg/100 mL. In the present work, the effect of different nitrogen sources on curdlan production was studied. Of the various sources selected, ammonium chloride at 2.5 g/L gave the maximum yield of 4.8 g/L, whereas ammonium acetate, ammonium citrate, and ammonium sulfate at 2.5 g/L gave yields of 4.1, 4.7, and 3.2 g/L, respectively. Sodium nitrate and potassium nitrate did not produce a significant effect on curdlan production and gave a yield of 3.6 g/L. Variation of ammonium chloride from 1.5 to 3.0 g/L again indicated 2.5 g/L as its optimum concentration and, hence, was selected for further study.

pH plays a very important role in the production of curdlan by *Agrobacterium* species, because it significantly influences both cell growth and product formation. Various studies show curdlan production to be divided into two phases: cell growth phase and curdlan production phase. Lee et al. *(15)* reported that curdlan was maximal at pH 5.5, whereas Railton et al. *(8)* optimized the pH for maximal production of curdlan to pH 6.2. Phillips et al. *(16)* reported curdlan production to be maximal at pH 5.9 ± 0.2. Considering these results, in the present study the pH for curdlan production phase was regulated at pH 6.1 ± 0.3 and compared with the unregulated control. The growth curve, sugar utilization, nitrogen utilization, curdlan production, and succinoglucan production were monitored in the 72-h fermentation. Curdlan producers are also known to synthesize succinoglucan, a water-soluble polymer in their metabolic pathway *(8)*. Hence, the organism selected in the present work, *A. radiobacter* NCIM 2443 was also checked for the production of succinoglucan.

Figure 1 shows the effect of pH regulation on the growth curve of *A. radiobacter* NCIM 2443 and production of curdlan. As observed, unregulated pH caused a lower cell mass, a decreased stationary phase, and an abrupt change to death phase compared with the regulated pH. The cells are in the lag phase up to 16 h, in log phase between 16 and 32 h, then into stationary phase from 32 to 64 h of fermentation, and finally slowly enter the death phase after 64 h. Under these conditions, the production of curdlan was also found to be affected by pH regulation. After 72 h of fermentation with unregulated pH, the yield was 2.48 g/L of curdlan. The corresponding value under regulated pH was 4.8 g/L.

The results of sugar utilization during the fermentation and succinoglucan production are shown in Fig. 2. Under pH regulation, 110 g/L of the 150 g/L of added sucrose was utilized at the end of the fermentation.

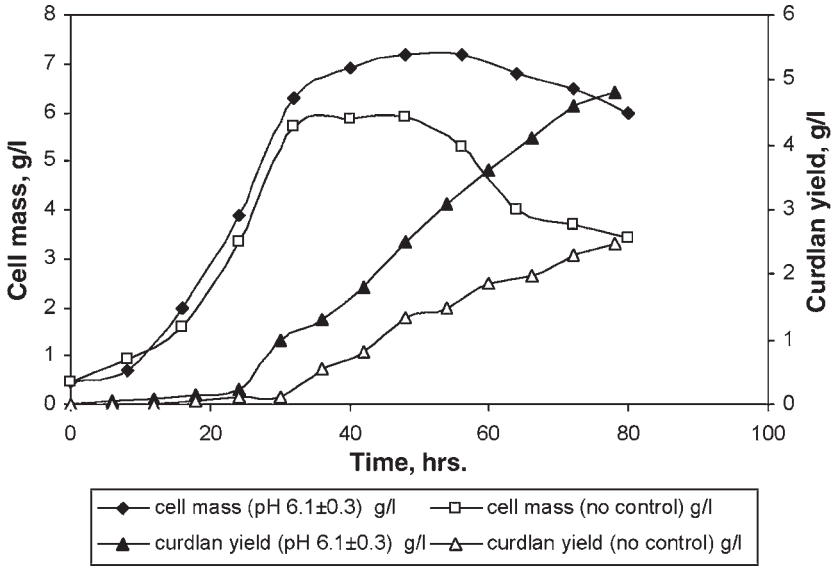

Fig. 1. Effect of pH regulation on growth curve and curdlan production by *A. radiobacter* NCIM 2443.

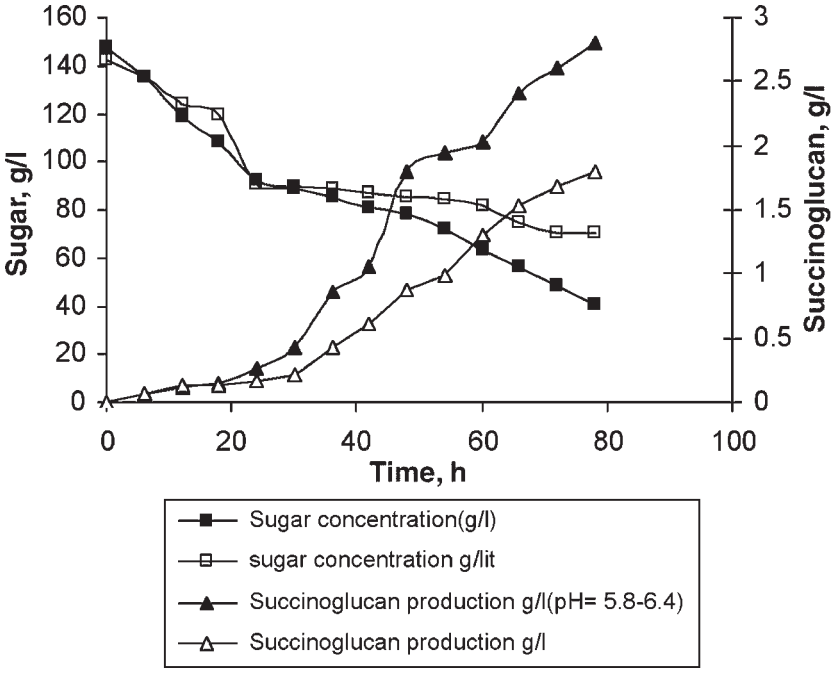

Fig. 2. Effect of pH regulation on sugar utilization and succinoglucan production by *A. radiobacter* NCIM 2443.

From this the production of curdlan can be expressed as 43 mg/g of sugar. For unregulated pH, only 79.5 g/L of sugar was utilized. The production of curdlan in unregulated control can be expressed as 31 mg/g of sugar.

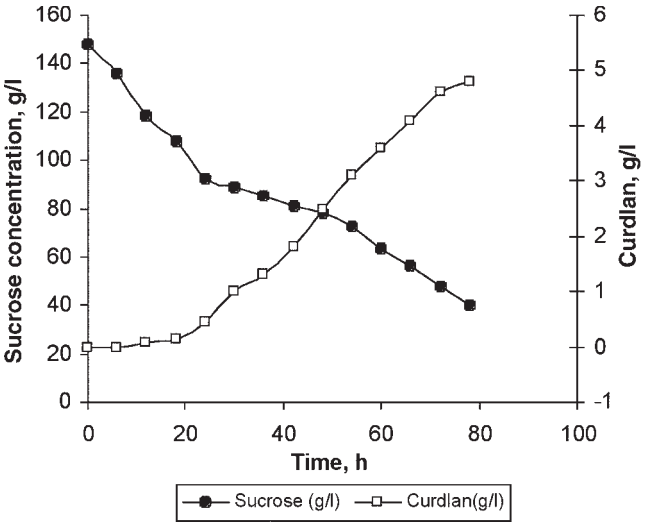

Fig. 3. Relation between curdlan production and sugar utilization under pH-regulated conditions.

After 72 h of fermentation with unregulated pH, the yield was 1.78 g/L of succinoglucan. The corresponding value under the regulated pH conditions was 2.8 g/L. Utilization of nitrogen for curdlan production with and without pH regulation was also checked. In the case of unregulated pH, 1.26 g/L of the 2.5 g/L of nitrogen source that was added was utilized at the end of fermentation. In addition, the nitrogen utilization stopped after about 20 h. This decreased the cell mass and also the curdlan production. Under regulated pH conditions, nearly 2 g/L of the 2.5 g/L nitrogen source that was added was utilized at the end of the fermentation. Nitrogen utilization stopped after 32 h, when the cells reached the poststationary phase of the growth cycle. No utilization of nitrogen source was observed when the cells reached the stationary phase. This is clearly seen in Fig. 4. It is important to know the point of nitrogen depletion, since it provides the limiting factor for cell growth.

The biosynthetic pathway of curdlan shows that UMP converts into UDP, which then acts as a precursor for curdlan biosynthesis *(17)*. Hence, the effect of UDP-glucose and UMP on curdlan production was studied. It was essential to optimize the time of addition of these stimulators. They were added at 1 µg/mL at the start of the fermentation, after 32 h of fermentation, and after 50 h of fermentation. The yield of curdlan and dry cell mass was measured. When the stimulators were added to the medium at the beginning and after 32 h of fermentation, the cells used them as the nitrogen source and showed an increase in cell mass with a small increase in curdlan production. When the stimulators were added to the medium after 50 h of fermentation, i.e., after 18 h of nitrogen depletion, the cells were already in the stationary phase and the stimulators added at this point were used as a precursor for curdlan production. Under these conditions, the yield of

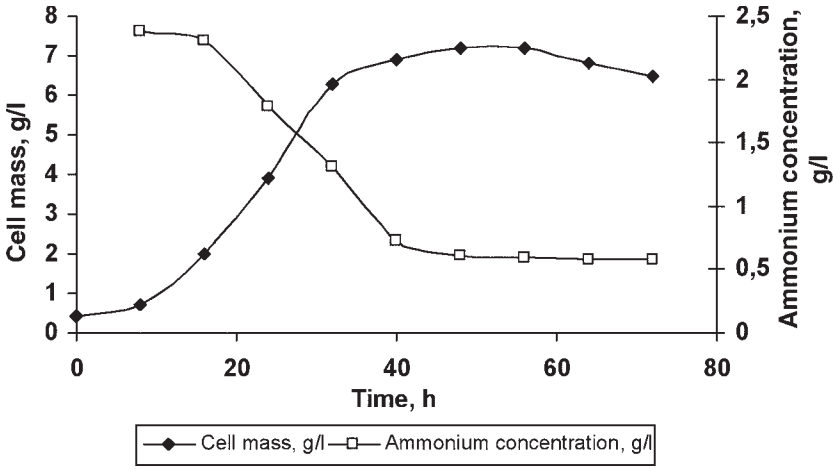

Fig. 4. Relation between growth curve for *A. radiobacter* NCIM 2443 and nitrogen utilization.

Table 2
Determination of Exact Amount of Stimulators
for Curdlan Production by *A. radiobacter* NCIM 2443[a]

Stimulator (µg/100 mL)	Curdlan (g/L)	
	UDP-glucose	UMP
10	4.8 ± 0.24	4.8 ± 0.35
20	5.25 ± 0.3	5.31 ± 0.16
40	5.42 ± 0.3	5.8 ± 0.12
60	5.61 ± 0.2	6.74 ± 0.21
80	6.29 ± 0.3	6.714 ± 0.23
100	6.30 ± 0.1	6.73 ± 0.20

[a]Results are the mean ± SD of three determinations.

curdlan increased to 6.28 and 6.60 g/L on addition of 1 µg/mL of UDP-glucose and UMP, respectively. This level of 1 µg/mL was chosen arbitrarily, and in order to arrive at the proper concentration, stimulators were added at 0.01, 0.1, 1, and 10 µg/mL after 50 h of fermentation and curdlan yield was determined. The best results appeared to be in the range of 0.01 to 0.1 µg/mL for both stimulators; hence, this was further optimized by taking smaller intervals of 0.01–0.1 µg/mL (Table 2). The addition of 0.8 µg/mL of UDP-glucose and 0.6 µg/mL of UMP gave the best yield of curdlan. The effect of UDP-glucose (at 0.8 µg/mL) on sugar utilization, curdlan production, and succinoglucan production is shown in Fig. 5, and that for UMP at 0.6 µg/mL is shown in Fig. 6. The results show that the addition of UDP-glucose to the fermentation media after 50 h of fermentation increased the curdlan production from 4.8 to 6.3 g/L, and the sugar utilization increased from 110 to 115 g/L. It was found that the addition of

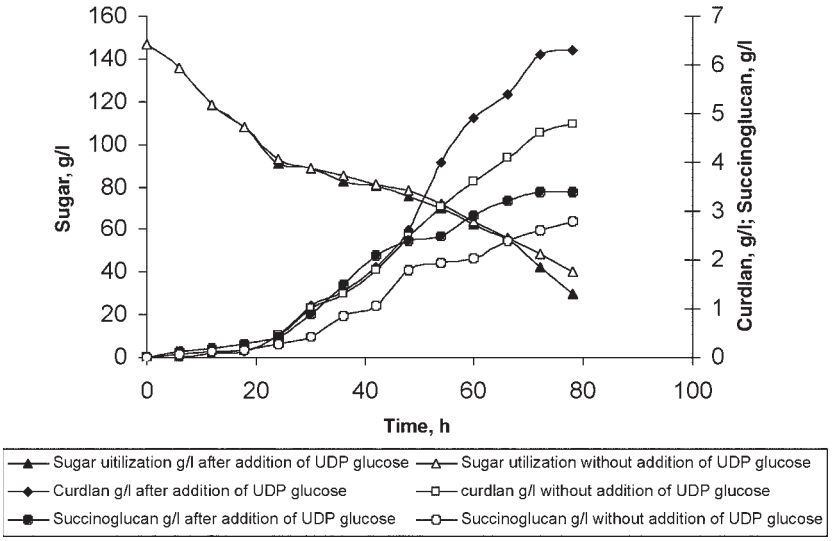

Fig. 5. Effect of UDP-glucose on sugar utilization and curdlan and succinoglucan production by *A. radiobacter* NCIM 2443.

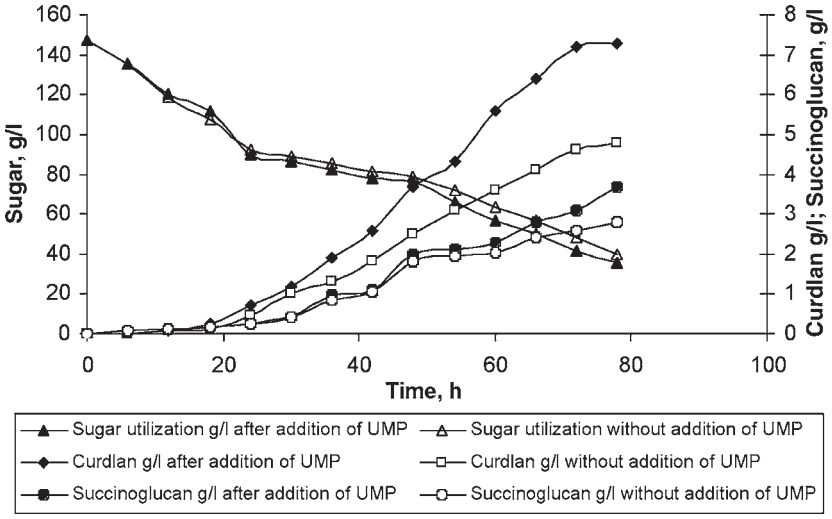

Fig. 6. Effect of UMP addition on curdlan production by *A. radiobacter* NCIM 2443.

UDP-glucose also stimulated the production of succinoglucan since the yield of succinoglucan was also increased from 1.8 to 3.4 g/L. The addition of UMP at 0.6 µg/mL to the fermentation media after 50 h of fermentation increased the curdlan production from 4.8 to 6.78 g/L. It also increased the sugar utilization from 110 to 115 g/L and stimulated the production of succinoglucan, increasing its yield from 1.8 to 3.7 g/L.

Conclusion

Based on all of our studies, the final protocol for maximum production of curdlan can be summarized as follows: a medium composition with a high carbon and limiting nitrogen concentration, pH-regulated conditions with pH 7.0 for efficient cell growth and pH 6.1 ± 0.3 for curdlan production. UDP-glucose and UMP serve as precursors for curdlan and succinoglucan production when added to the medium in concentrations of 0.8 and 0.6 µg/mL, respectively, after 18 h of nitrogen depletion in the fermentation broth.

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