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Production of Curdlan by some Bacteria Isolated from Egyptian Soils

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ABSTRACT

Thirty two curdlan producing bacterial isolates were obtained from eight different Governorates in Arab Republic of Egypt. Four isolates namely K10, K17, K20 and K74 were selected as highly efficient of curdlan production. The effect of some nutritional and environmental factors on curdlan production by those selected isolates was investigated using shake flask as a batch culture. The optimum concentration of carbon and nitrogen sources was also studied. Incubation period and shaking rate at different levels were tested for the optimization of fermentation process to yield the highest curdlan amount. The bacterial isolate K10 was identified as *Rhizobium radiobacter* and gave the maximum yield of curdlan being 22g/L on the modified (YP) fermentation medium containing (g/L): 100 g sucrose, 9.5 g yeast extract (as a carbon and nitrogen sources, respectively), 0.5 g $CaCo_3$, 1 g K_2HPO_4, 0.6 g $MgSO_4$ with initial pH level 7 after 72 h of fermentation period at 30 ºC on a rotary shaker at the shaking rate of 100 rpm with 50 mL of modified fermentation medium.

Key words: Bacterial exopolysaccharide – *Agrobacterium* - Curdlan production -Nutritional and environmental factors.

Introduction

Microbial exopolysaccharides were produced by various genera of bacteria and yeasts. Polysaccharides are natural, non-toxic, and biodegradable polymers that cover the surface of most cells and play important roles in various biological mechanisms such as immune response, adhesion, infection, and signal transduction (Kumar *et al*., 2007). Besides the interest on their applications in the health and bionanotechnology sectors, polysaccharides are also used as thickeners, bioadhesives, stabilizers, probiotic, and gelling agents in food and cosmetic industries (Nicolaus *et al*., 2010 and Donot *et al*., 2012).

Many studies have focused on the optimization of culture conditions for the production of polysaccharides. Microbial polysaccharide production was greatly influenced by fermentation conditions such as pH, temperature, oxygen concentration and agitation as well as by the composition of the culture medium (Sutherland, 2007 and Nicolaus *et al*., 2010).

Curdlan is an insoluble polysaccharide composed exclusively of *β*-1,3-linked glucose residues and is synthesized by *Agrobacterium* species and *Alcaligenes faecalis* under the nitrogen-limiting conditions (Harada, 1977). After Takeda Chemical Industries Ltd. (Haze *et al*., 1994) introduced curdlan to the market, as a biopolysaccharide, its usage rapidly increased. This gum was approved by the Food and Drug Administration (FDA) in 1996, being used in food industry owing to its ability of producing an excellent, hard, and resistant gel. The production of curdlan has drawn considerable interest because of its unique rheological and thermal gelling properties. Curdlan's linear structure makes it resistant to heat and other external forces, including pH. Researchers studied curdlan sulfate as an antiviral agent for the inhibition of human immunodeficiency virus (HIV)-1 infection (Lee & Park 2001 and Zhang *et al.*, 2012). It was also used as a drug delivery polymer to sustain drug and control drug diffusion (Kim *et al*., 2000a). Curdlan has also been used as an admixture of concrete to enhance its fluidity. It has been commercialized by Takeda Chemical Industries Ltd., Japan. There are other polysaccharides used as an admixture of concrete, and therefore their competitive price must be minimized to have an economic feasibility. Therefore, it is important to increase curdlan productivity by manipulating the environmental conditions and its production cost should be minimized to compete with other polysaccharides (Khayat & Yahia, 1997). The increase of curdlan production rate decreases the production cost and enhances the profits. Therefore, the optimization of the curdlan fermentation process for maximal production has been of much interest to industry. There are many factors involved in optimizing curdlan production. Temperature, pH, nutrients, agitation, and aeration are the most typical factors.

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In this study, the Curdlan production by locally isolated strains was grown at different nutritional and environmental factors to optimize the cultivation condition for obtain a high production of Curdlan.

Material and Methods

Isolation medium

Yeast Peptone (YP) medium was used according to (Vijayendra *et al*., 2003) slightly modifications were made on the previous medium. Firstly the aniline blue dye was added because it specifically binds with curdlan producing bacteria these show as blue colonies (Kima *et al*., 2003). Secondly sucrose was substituted by glucose because it is used for the commercial production of curdlan, the most commonly used carbohydrate in media and easily metabolized by variety of bacteria (Stasinopoulos *et al*., 1999; Yotsuzuka, 2001 and Portilho *et al*., 2006).

It has the following composition (g/L): 20g glucose, 5g peptone, 5g yeast extract, 0.05g aniline blue dye, 20g agar, at pH 7.0.

Maintenance medium

Nutrient agar (NA) medium was used for maintenance of the isolated bacteria and subcultured monthly according to (Palombo *et al*., 1989 and Triveni *et al*., 2001).

Soil rhizosphere samples

The samples were collected from the rhizosphere of three plants (bean; clover and peach) and placed in clean plastic container.

Isolation and purification of curdlan producing bacteria

Isolation of curdlan producing bacteria was carried out using serial dilution agar plate technique on modified YP medium in triplicate. Plates were incubated at 28±2°C for 48 h. The curdlan producing bacteria form colonies that stain dark blue on modified YP agar medium containing aniline blue whereas curdlan non producers form non staining colonies (Kima *et al*., 2003). The blue colonies obtained after incubation were picked up and subcultured several times (by pouring plates technique) under the same previous condition of isolation for purity.

Screening of efficient bacterial isolates for curdlan production and fermentation process

The selected curdlan producing bacteria were grown on 50mL of the modified (YP) medium which was used as standard medium for batch studies without agar and aniline blue in 250mL Erlenmeyer flask. Each flask was inoculated with 5% (v/v) and incubated at 28±2°C for 96 h on a rotary shaker set at 150 rpm (Shivakumar & Vijayendra, 2006). The preculture nutrient broth was used as seed medium for batch fermentation studies.

Determination of concentration of cells and curdlan yield

The quantity of cell biomass and curdlan was determined by measuring the dry weight. The fermented broth was separated by centrifugation at 8000g for 30min. The pellet consisting of cells and curdlan was washed with 0.01M HCl, and harvested by recentrifugation. The curdlan was kept soluble by the addition of 0.5M NaOH over 1 h period. Cells were separated by centrifugation at 8000 g for 30 min. The curdlan present in the supernatant phase was precipitated under acidic conditions by the addition of an appropriate volume of 2.0M HCl. Both cells and curdlan were washed twice and dried in a hot air oven at 80°C until constant weight was obtained (Lee & Park, 2001; Kima *et al*., 2003; Saudagar & Singhal, 2004 and Shivakumar & Vijayendra, 2006).

Identification of the most efficient bacterial isolate up to species

The most efficient bacterial isolate in curdlan production was completely identified according to its cultural, morphological and biochemical characteristics based on Bergey's Manual of Systematic Bacteriology (Krieg, 1984) and selected Biolog Automated System was used as a technique for identification of microorganisms. This part was carried out at Microbiological Resources Center (Cairo MIRCEN), Faculty of Agriculture, Ain Shams University, Egypt.

Optimization of carbon source

Ten sugars (fructose, mannose, galactose, maltose, xylose, glycerol, glucose, mannitol, lactose and sucrose) were used as carbon sources instead of glucose in the modified (YP) medium. Each of these sugars was added into the medium as the same carbon weight of glucose until to save the C/N ratio of the medium. After choose the best carbon source optimum concentration will be studied (Lee *et al.*, 1997; Saudagar & Singhal, 2004 and Portilho *et al*., 2006).

Optimization of nitrogen source

Twelve nitrogen sources were used instead of peptone and yeast extract in the modified (YP) medium. Two types of nitrogen sources were used: Organic sources (yeast extract urea, tryptone, meat extract, peptone and corn steep liquor (CSL)) and non-organic sources (potassium nitrate, ammonium chloride, ammonium carbonate, ammonium dihydrogen phosphate, ammonium nitrate and ammonium sulfate). Each of these nitrogen sources was added into the medium as the same nitrogen weight of peptone and yeast extract until to save the C/N ratio of the medium**.** After choose the best nitrogen source optimum concentration will be studied (Saudagar & Singhal, 2004).

Effect of initial pH

The initial pH of the modified YP medium is varied from 5 to 9, separately, using either 1M HCl or 1M NaOH to select the optimum pH (Shivakumar & Vijayendra, 2006). The Erlenmeyer flasks containing 50 mL of these media were inoculated with 5% (v/v) preculture seed medium using nutrient broth (NB) in each case and incubated at 28±2°C for 96 h on a rotary shaker set at 150 rpm. The fermented broth was analyzed for cell biomass, curdlan content and final pH.

Effect of concentration of calcium carbonate

The initial pH of modified YP medium was adjusted at 7.0 and different concentrations of $CaCO_3$ ranging from 0.05 to 0.3 % were tested. The fermented broth was analyzed for cell biomass, curdlan content and final pH.

Effect of concentration of potassium phosphate

To assess the effect of K_2HPO_4 concentrations on both biomass and curdlan production, various concentrations of K_2HPO_4 ranging from 0 to 2.5 g/L were added to the modified standard medium. Take into consideration the suitable carbon and nitrogen source and the best concentration of both with the selected four bacterial isolates.

Effect of concentration of magnesium sulfate

To assess the effect of $MgSO_4.7H_2O$ concentrations on both biomass and curdlan production, various concentrations of $MgSO_4.7H_2O$ ranging from 0 to 0.8 g/L were added to the modified standard medium with the addition of the best concentration of C, N, and P sources for each bacterial isolate.

Effect of incubation temperatures

The inoculated flasks containing the modified YP medium were incubated at different temperatures ranged from 20 to 40 °C for 96 h to detect the optimum temperature for curdlan production (Shivakumar & Vijayendra, 2006). The fermented broth was analyzed for cell biomass, curdlan content and final pH.

Effect of shaking and aeration rate

To study the effect of aeration rate on curdlan production against static batch culture, the each selected bacterial isolates were cultivated in 250-mL Erlenmeyer flasks containing various volumes of the modified (YP) medium 25, 50, 75 and 100mL to give different levels of dissolved oxygen (DO). The flasks were agitated on a rotary shaker at different shaking speeds 0, 50, 100, 150 and 200 rpm. The biomass and curdlan production was determined in each case to know the optimum shaking rate with the optimum working volume of medium that gives the maximum production of curdlan (Lee *et al*., 1999b).

Effect of fermentation time- course on curdlan production

To study the effect of the time-course of fermentation on cell biomass and curdlan production, the fermented broth was withdrawn every 8-h during the course of a 120-h fermentation, and analyzed for cell biomass and curdlan production (Saudagar and Singhal, 2004).

Statistical analysis

The collected data were statistically analyzed using SAS Computer Analysis Programs (SAS, 2006).

Results and Discussion

Isolation of curdlan producing bacteria

As showed in Table (1), Different numbers of rhizosphere samples were taken from each plant (100 samples). Also, different numbers of curdlan producing bacteria were obtained from each rhizosphere (32 isolates). The maximum number of curdlan producing bacteria (16 isolates) were obtained from the rhizosphere of clover, this followed by (14 isolates) from the rhizosphere of bean; while the lowest number (2 isolates) were obtained from the rhizosphere of peach. The maximum number of curdlan producing bacteria (7 isolates) was obtained from El-Dakhahelya Governorate, this is followed by (6 isolates) from Kafer El-Sheikh and (6 isolates) from El-Monofaya Governorates, while no curdlan producing bacteria were obtained from both El-Kalyoubia and El-Giza Governorates.

Table 1: Explain the isolate locations, number of rhizosphere samples and isolates producing curdlan.

Ser. No.	Governorates	Villages	Number of Rhizosphere samples			Number of isolates producing Curdlan		
			Bean	Clover	Peach	Bean	Clover	Peach
1	Kafer El-Sheikh	Mehalet Deii	3	3	-	4	2	-
2	El-Monofaya	EL-Maey	2	2	-	3	3	-
3	El-Kalyoubia	Tokh	5	5	-	-	-	-
4	El-Giza	Abou-Raoash	5	5	-	-	-	-
5	El-Gharbia	Meet EL-Rakha	5	5	15	3	1	1
6	El-Dakhahelya	Aga	6	6	15	1	5	1
7	Port Said	EL-Selah	5	5	-	2	4	-
8	Banisweef	Bany Adey	4	4	-	1	1	-

Selection of the highest efficiency of curdlan producing bacteria

Data in Table (2) indicate that the maximum dry cell weight (2.4 g/L) was obtained from the isolate No.K10. This is followed by the isolates No.K20, K17 and K74. The dry cell weights were 2.2, 2.0 and 1.8 g/L respectively. The maximum curdlan production (3.6 g/L) was obtained from the isolate No.K10. This is followed by the isolates No.K20, K17 and K74. The curdlan production were 3.4 3.2 and 3.0 g/L respectively. The lowest production of dry cell weight (1.1 g/L) was obtained from the isolates No.K18 and K54 while the lowest production of curdlan (1.8 g/L) was obtained from both isolates No.K14 and K65.

Effect of different carbon sources on the biomass and curdlan production

Data in Table (3) reveal that the maximum biomass and curdlan production which obtained from the bacterial isolate No.K10 was obtained from sucrose. It was 2.7 and 5.0 g/L respectively. The maximum biomass and curdlan production which obtained from the bacterial isolate No.K17 was obtained from lactose. It was 2.0 and 4.0 g/L respectively.

The maximum biomass and curdlan production which obtained from the bacterial isolate No.K20 was obtained from fructose. It was 2.5 and 4.6 g/L respectively. But that produced by the bacterial isolate No.K74 was obtained from maltose. It was 1.8 and 3.7 g/L respectively.

As showed from Table (3), high levels of biomass and curdlan production were observed when sucrose, fructose, lactose and maltose were used as a sole carbon source. It might be expected that, curdlan-producing bacteria generally metabolize the previous carbon sources and produce acids, that to do the culture pH during the fermentation course was significantly drop for inducing the curdlan production.

The work obtained by (Lee *et al*., 1997) showed that maltose and sucrose were found to be the best carbon sources for curdlan production by *Agrobacterium* sp. ATTC 31750. Similar results were obtained by (Saudagar and Singhal, 2004). They found that sucrose was the best carbon source for the production of curdlan by a strain of *Agrobacterium radiobacter* NCIM 2443 it was 4.8 g/L. Thus the results were obtained from the

bacterial isolates No.K10 and K74 to be accepted with the previous reported. It is noteworthy to state that there was obvious relationship between the production of curdlan and biomass (Nakanishi *et al*., 1992).

Table 2: Quantity of dry cell weight and curdlan production produced by the different curdlan producing isolates.

Serial No.	Bacterial isolates	Dry cell weight (g/L)	Curdlan production (g/L)
1	K21	1.5^{DE}	2.5^{de}
2	K15	1.2^{GHIJK}	2.4^{defg}
3	K1	1.4^{EFGH}	2.0^{kl}
4	K14	1.2^{GHIJK}	1.8^{l}
5	**K10**	$\mathbf{2.4^{A}}$	$\mathbf{3.6^{a}}$
6	K4	1.3^{EFGHIJ}	2.2^{ghijk}
7	**K17**	$\mathbf{2.0^{BC}}$	$\mathbf{3.2^{c}}$
8	K11	1.4^{EFGHI}	2.2^{ghijk}
9	**K20**	$\mathbf{2.2^{AB}}$	$\mathbf{3.4^{b}}$
10	K5	1.4^{EFGHIJ}	$2.4^{defghij}$
11	K18	1.1^{JK}	2.6^{d}
12	K2	$1.3^{EFGHIJK}$	2.5^{def}
13	K3	1.2^{FGHIJK}	2.2^{hijk}
14	K22	1.5^{EFG}	2.5^{defg}
15	K9	1.2^{IJK}	2.3^{efghij}
16	K7	1.2^{GHIJK}	2.2^{fghij}
17	K12	$1.3^{EFGHIJK}$	2.5^{de}
18	**K74**	$\mathbf{1.8^{CD}}$	$\mathbf{3.0^{c}}$
19	K31	1.1^{IJK}	2.0^{kl}
20	K65	1.0^{K}	1.8^{l}
21	K76	1.2^{GHIJK}	2.5^{def}
22	K52	1.2^{GHIJK}	2.2^{ghijk}
23	K30	1.1^{IJK}	2.5^{defg}
24	K38	1.4^{EFGHI}	2.2^{ghijk}
25	K63	1.5^{EFG}	2.1^{jk}
26	K50	1.5^{EF}	$2.4^{defghij}$
27	K54	1.1^{JK}	2.6^{d}
28	K37	1.2^{HIJK}	2.4^{defgh}
29	K61	1.2^{FGHIJK}	2.2^{hijk}
30	K32	1.4^{EFGHIJ}	2.3^{efghij}
31	K36	1.2^{IJK}	2.4^{defghi}
32	K62	1.2^{GHIJK}	2.1^{ijk}

Values in the same column followed by the same letter do not significantly differ from each other, according to Duncan's at 5 % level.

Table 3. Effect of different carbon sources on the biomass and curdlan production with each selected four bacterial isolates.

Variance	Bacterial isolates							
	K10		K17		K20		K74	
Carbon sources	Dry cell weight (g/L)	Curdlan production (g/L)	Dry cell weight (g/L)	Curdlan production (g/L)	Dry cell weight (g/L)	Curdlan production (g/L)	Dry cell weight (gL^{1})	Curdlan production (g/L)
Fructose	2.3^{BC}	3.8^{de}	1.3^{KLMNO}	3.4^{fghi}	$\mathbf{2.5^{AB}}$	$\mathbf{4.6^{b}}$	1.3^{KLMNO}	2.7^{jklm}
Galactose	2.2^{DC}	3.4^{ghi}	1.5^{IJKL}	3.3^{hi}	1.8^{FGH}	3.6^{efg}	1.4^{JKLM}	2.7^{jklm}
Glucose(control)	2.2^{DC}	3.5^{fghi}	1.6^{GHIJ}	3.2i	2.1^{DC}	3.2^{hi}	1.5^{IJKL}	3.2^{i}
Glycerol	1.5^{HIJKL}	2.1^{p}	1.4^{JKLM}	2.8^{jkl}	2.1^{DC}	3.3^{hi}	1.2^{MNO}	2.6^{klm}
Lactose	2.2^{DC}	3.2^{i}	$\mathbf{2.0^{EF}}$	$\mathbf{4.0^{d}}$	0.8^{Q}	1.1^{q}	1.3^{MNO}	2.8^{jk}
Maltose	1.9^{EFG}	4.4^{bc}	1.4^{JKLM}	2.7^{jklm}	0.9^{PQ}	1.1^{q}	$\mathbf{1.8^{FGH}}$	$\mathbf{3.7^{ef}}$
Mannitol	1.8^{FGH}	3.3^{hi}	1.4^{JKLMN}	3.4^{fghi}	1.8^{FGH}	3.5^{fgh}	1.1^{OP}	2.2^{op}
Mannose	1.7^{GHI}	3.3^{hi}	1.5^{IJKL}	3.3^{hi}	2.1^{CDE}	4.3^{c}	1.1^{NO}	2.5^{mno}
Sucrose	$\mathbf{2.7^{A}}$	$\mathbf{5.0^{a}}$	1.5^{IJKL}	3.2^{i}	0.8^{Q}	1.1^{q}	1.5^{IJKL}	2.5^{klmn}
Xylose	1.3^{KLMNO}	2.5^{lmn}	1.1^{NOP}	2.9^{j}	1.6^{HIJK}	2.3^{nop}	0.8^{Q}	2.2^{op}

Values in the same column followed by the same letter do not significantly differ from each other, according to Duncan's at 5 % level.

Effect of different concentrations of the best carbon source on the biomass and curdlan production

Data presented in Table (4) and illustrated by Fig. (1 & 2) show that sucrose was the best carbon source for the isolate No.K10. The optimum concentration of sucrose 10% showed the maximum biomass and curdlan production. It was 4.2 and 6.7 g/L respectively. Lactose was the best carbon source for the isolate No.K17. The optimum concentration of lactose 10% showed the maximum biomass and curdlan production. It was 3.4 and 5.2 g/L respectively. Fructose was the best carbon source for the isolate No.K20. The optimum concentration of fructose 8% showed the maximum biomass and curdlan production being 3.9 and 7.0 g/L respectively. Maltose

was the best carbon source for the isolate No.K74. The optimum concentration of maltose 12% showed the maximum biomass and curdlan production being 3.1 and 5.5 g/L respectively.

The previous results showed that the maximum biomass production 4.2 g/L was obtained from the bacterial isolate NoK10. This is followed by the bacterial isolates No. K20, K17 and K74. It was 3.9, 3.4 and 3.1 g/L respectively. While the maximum curdlan production 7.0 g/L was obtained from the bacterial isolate No.K20. This is followed by the bacterial isolate No.K10, K74 and K17. It was 6.7, 5.5 and 5.2 g/L respectively.

The amount of both curdlan and biomass increased with increases in carbon source concentration up to the optimum concentration and decreased slightly beyond this value. The abrupt increase in carbon source concentration could have stressed the cells owing to the associated changes in osmotic pressure and thereby decreased biomass and curdlan production.

Table 4. Effect of different concentrations of the best carbon source on the biomass and curdlan production with each selected four bacterial isolates.

Variance	Bacterial isolates							
Carbon source concentrations (%w/v)	K10		K17		K20		K74	
	Dry cell weight (g/L)	Curdlan production (g/L)	Dry cell weight (g/L)	Curdlan production (g/L)	Dry cell weight (g/L)	Curdlan production (g/L)	Dry cell weight (g/L)	Curdlan production (g/L)
2(control)	2.7^{E}	4.7^{e}	1.7^{E}	3.6^{c}	2.2^{D}	4.5^{e}	1.5^{F}	3.5^{f}
4	3.2^{D}	4.9^{e}	2.2^{D}	3.8^{c}	2.6^{C}	5.2^{d}	1.8^{E}	4.0^{e}
6	3.4^{CD}	5.1^{d}	2.6^{C}	4.4^{b}	3.3^{B}	6.3^{b}	2.1^{D}	4.4^{d}
8	3.8^{BC}	5.6^{c}	2.9^{B}	4.7^{b}	**3.9^{A}**	**7.0^{a}**	2.4^{C}	4.9^{c}
10	**4.2^{A}**	**6.7^{a}**	**3.4^{A}**	**5.2^{a}**	3.1^{B}	5.7^{c}	2.8^{B}	5.2^{b}
12	3.8^{B}	6.1^{b}	3.0^{B}	4.7^{b}	2.1^{D}	3.9^{f}	**3.1^{A}**	**5.5^{a}**
14	3.5^{BCD}	5.7^{c}	2.9^{B}	4.5b	1.6^{E}	3.7^{g}	2.7^{B}	5.2^{b}
16	3.3^{D}	5.7^{c}	2.7^{C}	4.6^{b}	1.4^{F}	3.4^{h}	2.4^{CD}	5.0^{bc}

Values in the same column followed by the same letter do not significantly differ from each other, according to Duncan's at 5 % level.

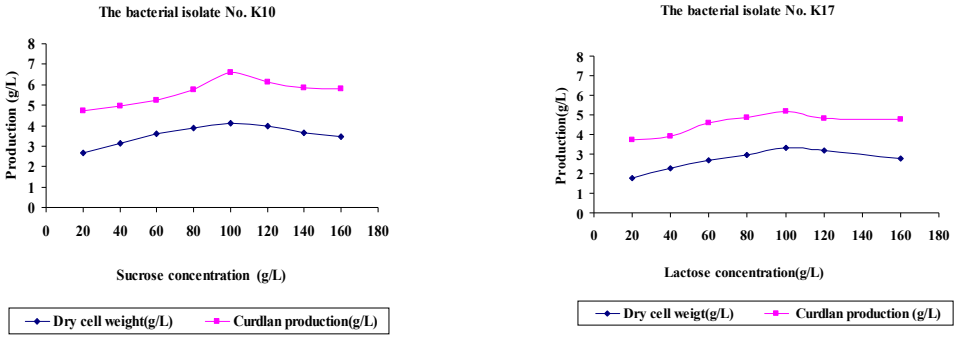

Fig. 1: Effect of sucrose and lactose concentrations on the biomass and curdlan production by the bacterial isolates No. K10 and K17.

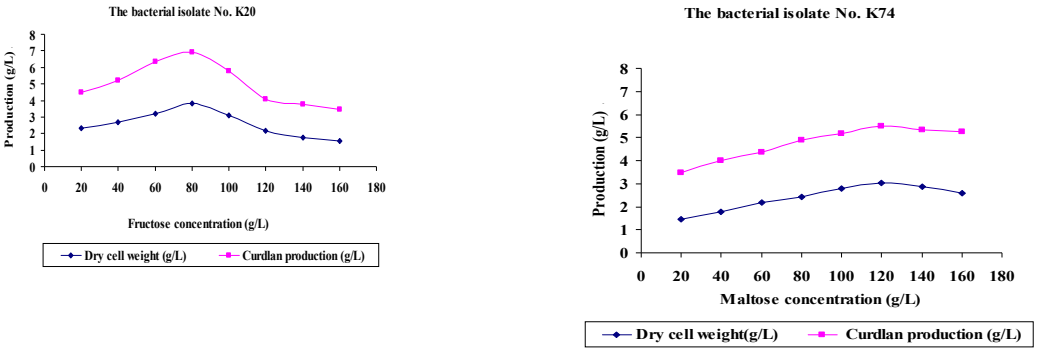

Fig. 2: Effect of fructose and maltose concentrations on the biomass and curdlan production by the bacterial isolates No. K20 and K74.

Effect of different nitrogen sources on the biomass and curdlan production

Results presented in Table (5) indicate that the maximum biomass production yielded from both the bacterial isolate No.K10 and K20 was obtained from Peptone. It was 7.5 g/L, while the maximum curdlan production was obtained from Yeast extract. It was 8.4 and 8.3 g/L respectively. The maximum biomass and curdlan produced by the bacterial isolate No.K17 was obtained from Meat extract. It was 5.6 and 7.3 g/L respectively. While the maximum biomass and curdlan produced by the bacterial isolate No. K74 was obtained from Tryptone. It was 5.5 and 7.6 g/L respectively.

Current data from this experiment show that the maximum biomass production 7.5 gL^1 was obtained from both the bacterial isolate No.K10 and K20 grown on Peptone as a nitrogen source. This was followed by 5.6 and 5.5 g/L which produced from the bacterial isolates No.K17 and K74 which were grown on Meat extract and Tryptone respectively.

The maximum curdlan production 8.4 g/L was obtained from the bacterial isolate No.K10 grown on Yeast extract as a nitrogen source that probably owing to its abundant nutrients as well as several growth factors responsible for substrate uptake and/or metabolism. Some pathways may be regulated by the concentration of Yeast extract in the medium (Fava *et al*., 1995). This was followed by 8.3, 7.6 and 7.3 g/L which obtained from the bacterial isolates No. K20, K74 and K17. They was grown on Yeast extract, Tryptone and Meat extract respectively.

The organic nitrogen sources considerably stimulated curdlan production, probably owing to its abundant nutrients but the inorganic nitrogen sources resulted in poor curdlan production.

Effect of different concentrations of the best nitrogen source on biomass and curdlan production

The effect of different total nitrogen concentrations ranging from 0.025 to 0.17 % was tested on the biomass and curdlan production. The obtained results recorded in Table (6) and illustrated by Fig. (3 & 4) show that the maximum curdlan production from the bacterial isolate No.K10 was 8.6 g/L. It was obtained at total nitrogen concentration 0.1% that corresponding to 9.5 g/L of Yeast extract. The biomass related with the same optimum total nitrogen concentration was 6.4 g/L. The maximum curdlan production from the bacterial isolate No.K17 was 9.0 g/L. It was obtained at total nitrogen concentration 0.05% that corresponding to 4.1 g/L of Meat extract. The biomass related with the same optimum total nitrogen concentration was 3.4g/L. The maximum curdlan production from the bacterial isolate No.K20 was 8.5g/L. It was obtained at total nitrogen concentration 0.1% that corresponding to 9.5g/L of Yeast extract. The biomass related with the same optimum total nitrogen concentration was 6.3g/L. The maximum curdlan production from the bacterial isolate No.K74 was 9.0g/L. It was obtained at total nitrogen concentration 0.05% that corresponding to 3.9g/L of Tryptone. The biomass related with the same optimum total nitrogen concentration was 3.1g/L.

As a conclusion of this experiment, the maximum biomass production is increased as the total nitrogen oncentrations increased. It reached to 10.9 g/L with both the bacterial isolates No.K10 and K20. This is followed by the bacterial isolates No.K74 and K17. It was 9.4 and 8.8 g/L respectively

Table 5. Effect of different nitrogen sources on the biomass and curdlan production with each selected four bacterial isolates

Variance	Bacterial isolates							
	K10		K17		K20		K74	
Nitrogen sources	Dry cell weight (g/L)	Curdlan production (g/L)	Dry cell weight (g/L)	Curdlan production (g/L)	Dry cell weight (g/L)	Curdlan production (g/L)	Dry cell weight (g/L)	Curdlan production (g/L)
Peptone	**7.5^{A}**	6.4^{ghi}	5.1^{E}	6.9^{de}	**7.5^{A}**	6.2^{i}	5.1^{E}	6.7^{efg}
Tryptone	5.2^{E}	7.7^{b}	5.1^{E}	6.4^{fghi}	7.2^{B}	6.6^{efgh}	**5.5^{D}**	**7.6^{b}**
Yeast extract	5.8^{C}	**8.4^{a}**	4.9^{F}	6.6^{efgh}	6.0^{C}	**8.3^{a}**	4.7^{FGH}	7.2^{c}
Meat extract	7.2^{B}	6.3^{hi}	**5.6^{D}**	**7.3^{c}**	5.2^{E}	6.7^{ef}	5.2^{E}	6.3^{ghi}
CSL	5.5^{D}	7.1^{cd}	4.5^{IJ}	5.6^{j}	5.9^{C}	7.9^{b}	4.6^{HIJ}	6.1^{i}
NH_4 Cl	4.8^{FG}	5.6^{j}	3.3^{QR}	4.7^{mn}	4.2^{L}	5.6^{j}	3.4^{PQ}	5.1^{kl}
K NO_3	3.3^{QR}	4.7^{mn}	4.3^{KL}	4.6^{mn}	3.0^{T}	5.3^{k}	3.0^{T}	4.2^{o}
$(NH4)_2$ SO_4	3.8^{N}	4.2^{o}	4.0^{M}	3.5^{p}	4.4^{JK}	4.5^{no}	3.3^{QR}	4.6^{mn}
NH_4 NO_3	3.6^{OP}	4.5^{mn}	3.3^{QRS}	3.7^{p}	3.1ST	4.2^{o}	2.4^{U}	3.7^{p}
NH_4 H_2PO_4	5.2^{E}	5.8^{j}	3.8^{NO}	4.7^{mn}	4.1^{LM}	5.2^{k}	3.6^{NO}	5.1^{k}
Urea	4.2^{L}	5.2^{k}	3.6^{OP}	4.2^{o}	4.7^{GHI}	4.8^{lm}	3.2^{RST}	4.4^{no}
Peptone+yeast extract (control)	4.1^{L}	6.5^{ghi}	3.3^{QRS}	5.1^{kl}	3.8^{M}	6.9^{e}	3.0^{T}	5.4^{l}

Values in the same column followed by the same letter do not significantly differ from each other, according to Duncan's at 5 % level.

Table 6. Effect of different total nitrogen concentrations of the best nitrogen source on the biomass and curdlan production with each selected four bacterial isolates.

Variance	Bacterial isolates							
Total nitrogen concentrations (%w/v)	K10		K17		K20		K74	
	Dry cell weight (g/L)	Curdlan production (g/L)	Dry cell weight (g/L)	Curdlan production (g/L)	Dry cell weight (g/L)	Curdlan production (g/L)	Dry cell weight (g/L)	Curdlan production (g/L)
0.025	2.3^{E}	4.8^{c}	2.0^{E}	5.6^{c}	2.1^{E}	4.8^{d}	1.9^{E}	3.4^{d}
0.05	3.2^{D}	6.3^{b}	3.4^{D}	**9.0^{a}**	3.6^{D}	6.9^{b}	3.1^{D}	**9.0^{a}**
0.1(control)	6.4^{C}	**8.6^{a}**	5.7^{C}	7.3^{b}	6.3^{C}	**8.5^{a}**	5.5^{C}	7.5^{b}
0.15	10.2^{B}	4.8^{c}	7.6^{B}	5.5^{c}	9.4^{B}	5.6^{c}	8.1^{B}	4.8^{c}
0.17	**10.9^{A}**	2.8^{d}	**8.8^{A}**	4.7^{d}	**10.0^{A}**	3.4^{e}	**9.4^{A}**	3.2^{d}

Values in the same column followed by the same letter do not significantly differ from each other, according to Duncan's at 5 % level.

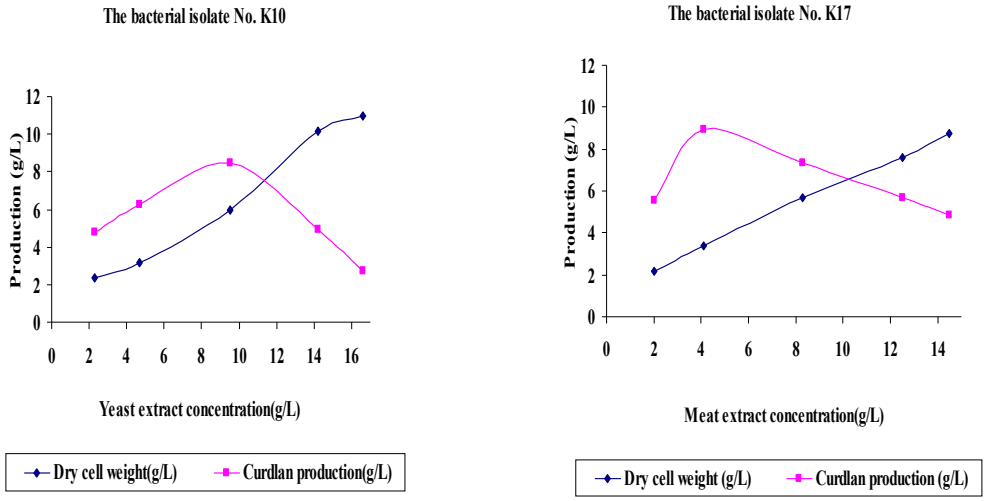

Fig. 3: Effect of yeast and meat extract concentrations on the biomass and curdlan production by the bacterial isolates No. K10 and K17.

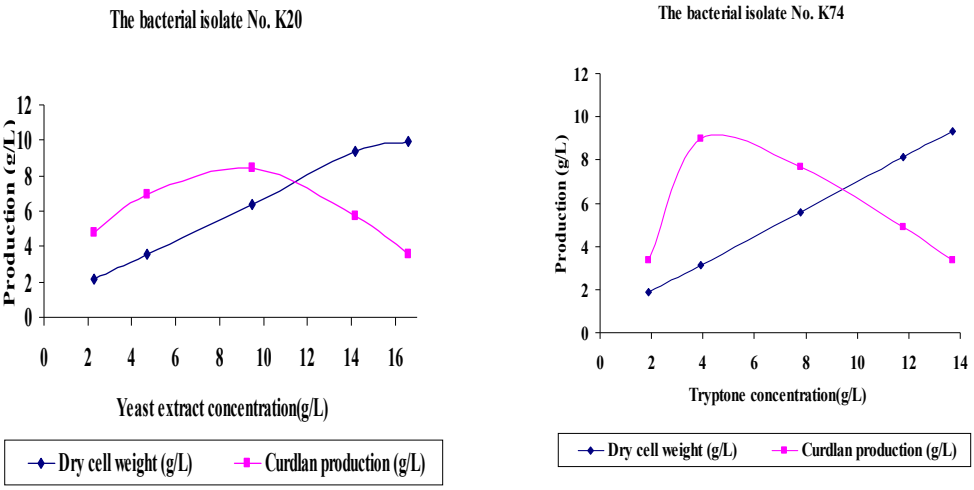

Fig. 4: Effect of yeast extract and tryptone concentrations on the biomass and curdlan production by the bacterial isolates No. K20 and K74.

While the maximum curdlan production 9.0 g/L was obtained from both the bacterial isolates No.K17 and K74. This was followed by the bacterial isolates No.K10 and K20. It was 8.6 and 8.5 g/L respectively. The amount of curdlan produced increased with increasing in total nitrogen up to the optimum concentration, and sharply decreased beyond this value. This is due to the fact that curdlan production is started biosynthesis just after nitrogen depletion (Phillips & Lawford, 1983; Nakanishi *et al*., 1992 and Kim *et al*., 2000 b). Therefore, the curdlan production is retarded at higher nitrogen concentration. The Yield of curdlan improved in shake flask as batch fermentation which the culture medium was initiated with a high carbon concentration and low nitrogen concentration (high C/N ratio) that resulted is clearly seen with both the bacterial isolates No. K17 and K74.

Effect of different concentrations of potassium phosphate on the biomass and curdlan production

Data in Fig. (5&6) show that the bacterial isolate No.K10 gave the maximum biomass production 8.0 g/L at the concentration of KH_2PO_4 2 g/L while the maximum curdlan production was 9.3 g/L at KH_2PO_4 concentration 1 g/L. The bacterial isolate No.K17 showed the maximum biomass production 5.6 g/L at the concentration of KH_2PO_4 2 g/L while the maximum curdlan production was 9.8 g/L at KH_2PO_4 concentration 1.5g/L. The bacterial isolate No.K20 showed the maximum biomass production 8.0 g/L at the concentration of KH_2PO_4 1.5g/L while the maximum curdlan production was 9.8 g/L at KH_2PO_4 concentration 1 g/L. The bacterial isolate No.K74 showed the maximum biomass and curdulan production were 6.9 and 9.6 g/L at the concentration of KH_2PO_4 1.5 and 0.5 respectively.

The increasing of biomass related with the increasing of curdlan production, the highest biomass 6.2 g/L was obtained from both the bacterial isolates No.K10 and K20. This was followed by 4.5 and 3.7 g/L those obtained from the bacterial isolates No.K17 and K74, respectively. These values were higher than that obtained in absence of potassium phosphate. It was 5.5, 5.2 and 3.1 obtained from the bacterial isolates No.K10, K20, K17 and K74, respectively. The highest curdlan production was 9.8g/L. It was obtained from both the bacterial isolates No.K17 and K20. This was followed by 9.6 and 9.3 g/L which were obtained from the bacterial isolates No.K74 and K10, respectively. These values were higher than in absence of potassium phosphate. It was 8.5, 8.7, 8.9 and 8.3 that obtained by the bacterial isolates No.K20, K17, K74 and K10, respectively. Therefore, potassium phosphate addition enhanced both biomass and curdlan production, but the production of curdlan decreased sharply beyond the optimum value of potassium phosphate concentration.

Effect of different concentrations of magnesium sulfate on the biomass and curdlan production

Data presented in Fig. (7&8) show that the bacterial isolate No.K10 gave the maximum biomass and curdlan production, being 6.8 and 9.8 g/L, respectively at the magnesium sulfate concentration of 0.6 g/L, while the maximum biomass and curdlan production, being 4.6 and 10.2 g/L, respectively at the magnesium sulfate concentration of 0.2 g/L obtained by isolate No.K17 .The bacterial isolate No.K20 gave the maximum biomass and curdlan production, being 6.6 and 10.2 g/L, respectively at the magnesium sulfate concentration of 0.4 g/L.The bacterial isolate No.K74 gave the maximum biomass and curdlan production, being 4.4 and 10.2 g/L, respectively at the magnesium sulfate concentration of 0.4g/L.

The highest biomass production was 6.8g/L. It was obtained from the bacterial isolate No.K10. This was followed by 6.6, 4.6 and 4.4 g/L which were obtained from the bacterial isolates No.K20, K17 and K74, respectively. While the highest curdlan production was 10.2g/L, it was obtained from the bacterial isolates No.K17, K20 and K74. This was followed by 9.8g/L which was obtained from the bacterial isolate No.K10. These values were higher than in the absence of magnesium sulfate. It was 9.7, 9.6, 9.5 and 9.1 g/L which were obtained from the bacterial isolates No.K17, K20, K74 and K10, respectively.

It is well known that metabolic ions play an important role in curdlan production under nitrogen limitation that is further dependent on an optimum concentration of phosphate (Kim *et al.,* 2000b) and sulfate (Phillips & Lawford, 1983) and on the cation composition of the medium (Nakanishi *et al.,* 1992).

Effect of initial pH on the biomass and curdlan production

It is clear from the data in Fig. (9 & 10) that the maximum biomass and curdlan was obtained with all selected four bacterial isolates when pH was initially adjusted at pH 8. The maximum biomass production was obtained from the bacterial isolate No.K10. It was 8.8g/L. This was followed by the bacterial isolates No.K20, K17 and K74. It was 8.6, 7.7 and 6.9 g/L, respectively. The maximum curdlan production was obtained from the bacterial isolate No.K10. It was 16.3 g/L. This was followed by the bacterial isolates No.K74, K20 and K17. It was 15.8, 15.7 and 15.4g/L, respectively.

The drop in final pH was observed with all selected four bacterial isolates when pH was initially adjusted at pH 8. It seemed suitable for growth and curdlan production because the highest production of curdlan is

depending on the concentration of cells in the pre-stationary phase and on the biosynthetic capability of cells in the post-stationary phase (Nakanishi *et al.,* 1992 McIntosh *et al.,* 2005).

The sharply drop in final pH may be inhibiting an important enzymes which responsible on the biosynthesis of curdlan. Hence the addition of $CaCO_3$ at different concentrations will be studied to improve the curdlan production by slowed the drop in final pH. pH plays a very important role in the production of curdlan by *Agrobacterium* species, because it influences both cell growth and curdlan production. Various studies show that the maximum curdlan production can be obtained by divided into two phases, cell growth phase and curdlan production phase (Lee *et al*., 1999a). Lee & Park, 2001 reported that the optimum curdlan production (60 g/L in 120 h) was obtained at pH 5.5 and specific cell growth at pH 7.0, with feedback inferential control of pH. Recently, Wang *et al.,* 2002 also observed an improvement of 20.4% in curdlan production by *A. faecalis* WXC12, when the pH was maintained constantly at 5.6, than in the pH uncontrolled batch. Whereas, Phillips *et al.,* 1983 and Saudagar & Singhal, 2004 optimized the pH for maximal production phase of curdlan to pH 5.9 ±0.2. The differences in the results may be attributed to variations in the experimental design, the cultures used and the type of strain. Considering these results in the present study the optimum initial pH for biomass and curdlan production was investigated in one stage batch fermentation by varying the initial pH of the modified standard medium to 5, 6, 7, 8 and 9 with each selected four bacterial isolates.

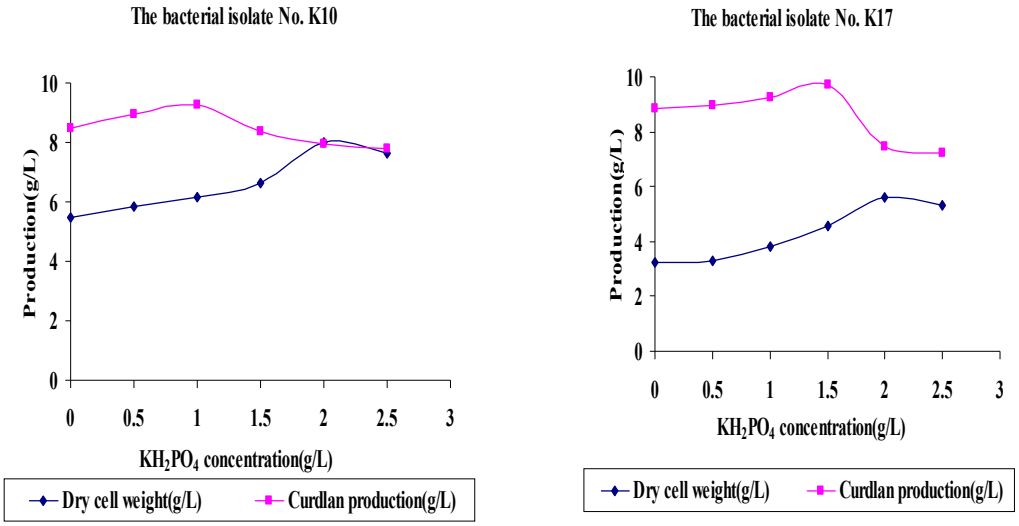

Fig. 5: Effect of KH_2PO_4 concentrations on the biomass and curdlan production by the bacterial isolates No. K10 and K17.

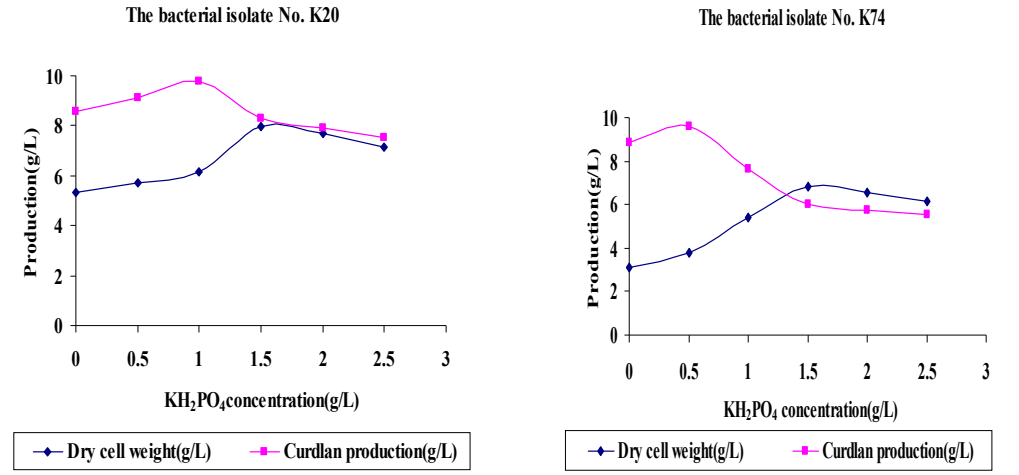

Fig. 6: Effect of KH_2PO_4 concentrations on the biomass and curdlan production by the bacterial isolates No. K20 and K74.

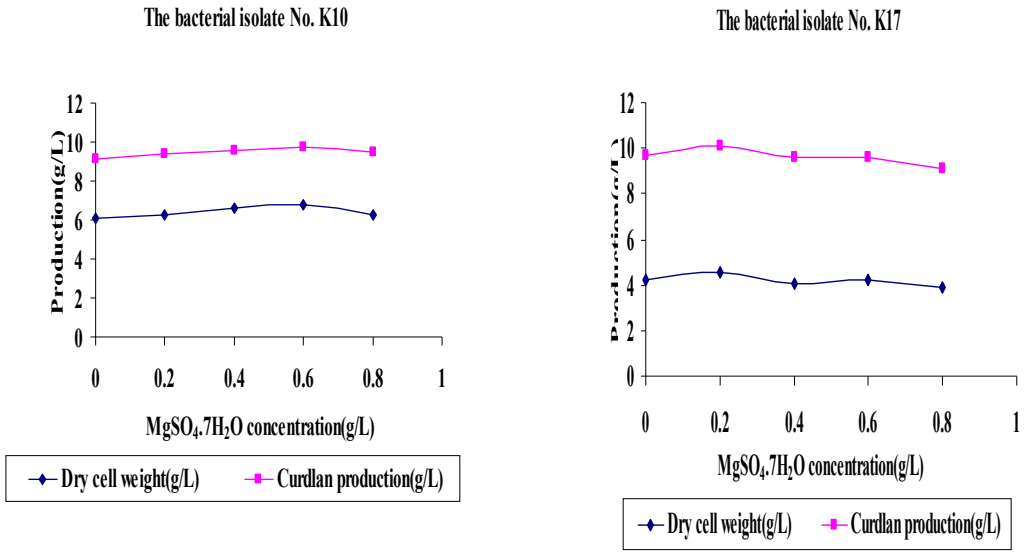

Fig. 7: Effect of $MgSO_4.7H_2O$ concentrations on the biomass and curdlan production by the bacterial isolates No. K10 and K17.

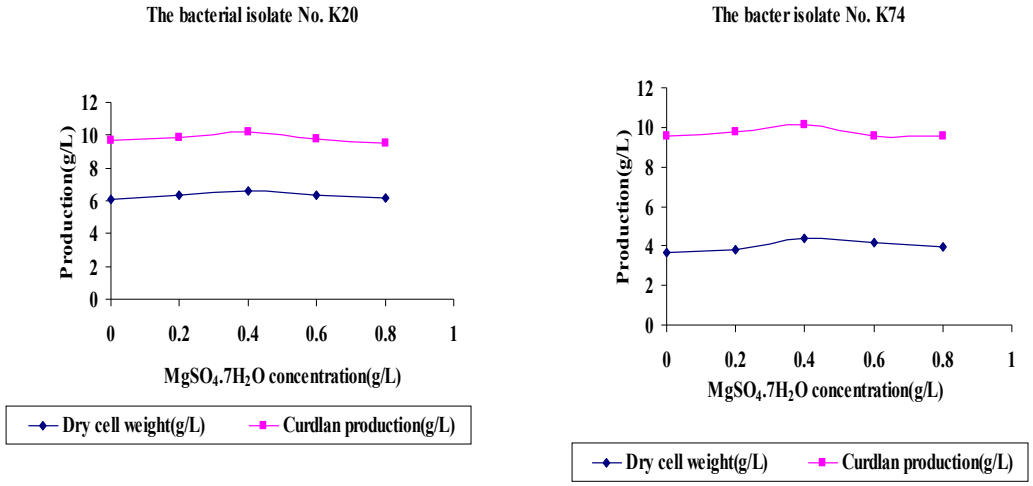

Fig. 8: Effect of $MgSO_4.7H_2O$ concentrations on the biomass and curdlan production by the bacterial isolates No. K20 and K74.

Effect of different concentrations of calcium carbonate on the biomass and curdlan production

The initial pH was adjusted at 7.0 and different concentrations 0.05, 0.1, 0.2 and 0.3 % of $CaCO_3$ was added to neutralize the acidity produced during curdlan production.

Data presented in Fig. (11&12) reveal that, the addition of $CaCO_3$ prevented the sharply drop in final pH with all selected bacterial isolates. The final pH was reaching to 5.57 and 5.72 with bacterial isolates No.K10 and K17 at 0.05 % $CaCO_3$ (w/v) while the final pH was reaching 5.83 and 5.86 with bacterial isolates No.K20 and K74 at 0.1 % $CaCO_3$ (w/v). Curdlan production increased by the addition of calcium carbonate with adjusted the culture medium at pH 7.0 more than the adjusted culture medium at pH 7.0 only with all selected four bacterial isolates. The maximum biomass production was obtained from the bacterial isolate No.K10. It was 8.8g/L. This was followed by the bacterial isolate No.K20, K17 and K74. It was 8.8, 7.6 and 7.3g/L, respectively. The maximum curdlan production was obtained from the bacterial isolate No.K10. It was 17.0g/L. This was followed by the bacterial isolate No.K20, K74 and K17. It was 16.1, 16.0 and 15.7g/L, respectively.

Finally, it is recommended to add $CaCO_3$ at ranging from 0.05 to 0.1% in the production medium to increase the curdlan productivity, through prevent sharp shift in final pH due to increase of cells and keep the biosynthesis process at favored pH for high curdlan production.

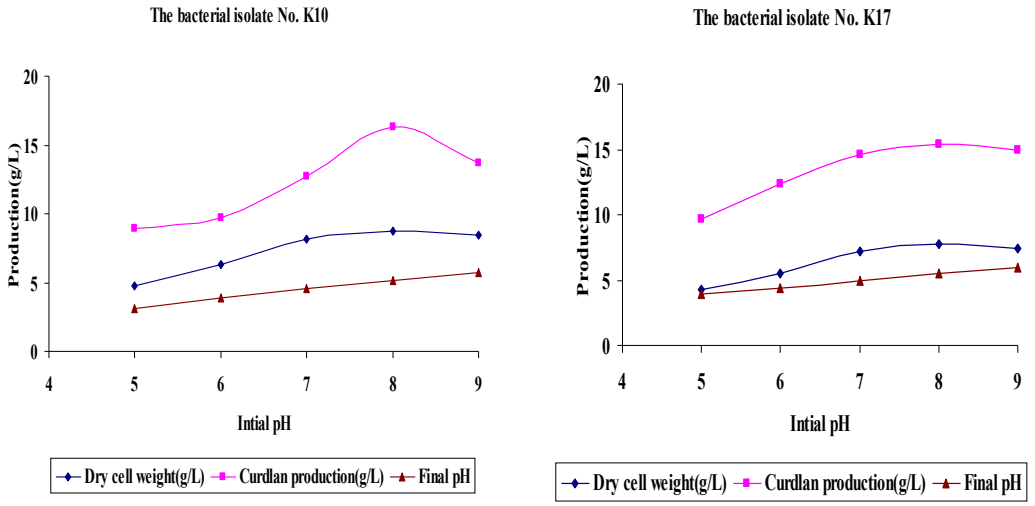

Fig. 9: Effect of different initial pH on the biomass and curdlan production by the bacterial isolates No. K10 and K17.

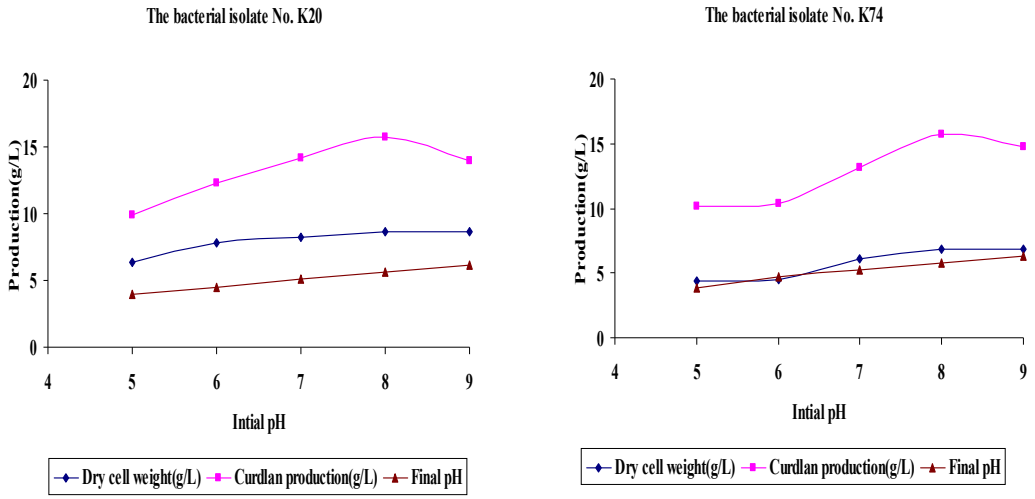

Fig. 10: Effect of different initial pH on the biomass and curdlan production by the bacterial isolates No. K20 and K74.

Effect of incubation temperatures on the biomass and curdlan production

The modified standard medium was inoculated with actively growing culture of each selected four bacterial isolates and incubated at different temperatures 20, 25, 30, 35 and 40°C for 96-h on a rotary shaker at 150 rpm.

Data presented in Fig. (13&14) indicate that, the favor temperature ranging from 25 to 30°C which give high production of biomass and curdlan. The maximum production of biomass and curdlan was found to be at 30°C with all selected bacterial isolates. Incubation temperature at 20, 25 and 35°C resulted in low quantity of curdlan with all selected bacterial isolates. Whereas at 40°C resulted in very low quantity of curdlan with the bacterial isolates No.K10 and K17. Also there was no curdlan production noticed with the bacterial isolates No.K17 and K20. The previous results were accepted with the reported by Zhan *et al.,* 2001. Although the reason is not known, probably the high temperature would have inhibited the enzymes involved in the biosynthesis of curdlan. Finally optimum temperature for the growth of *Agrobacterium* is around 30°C, which is the most favorable temperature for the production of curdlan (Shivakumar & Vijayendra, 2006). Thus the obtained results were in line with the previous reports.

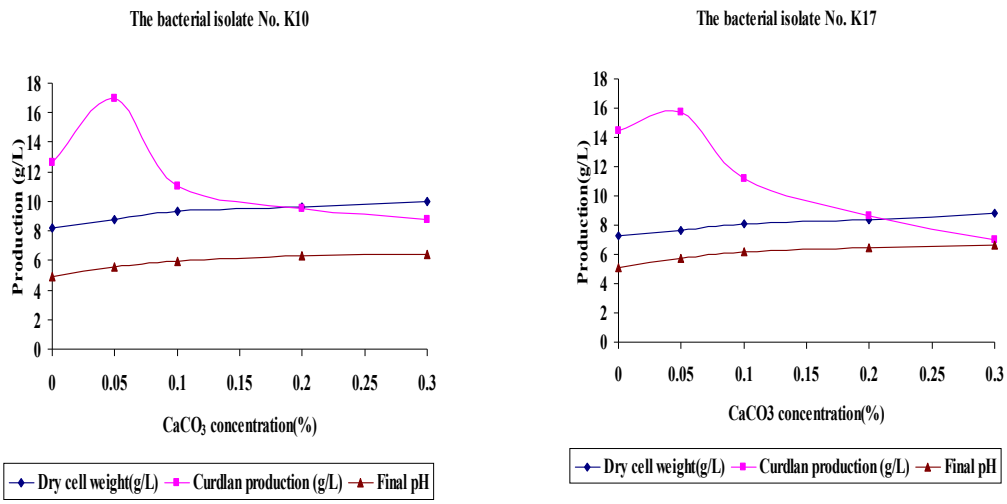

Fig. 11: Effect of different $CaCO_3$ concentrations on the biomass, curdlan production and final pH by the bacterial isolates No. K10 and K17.

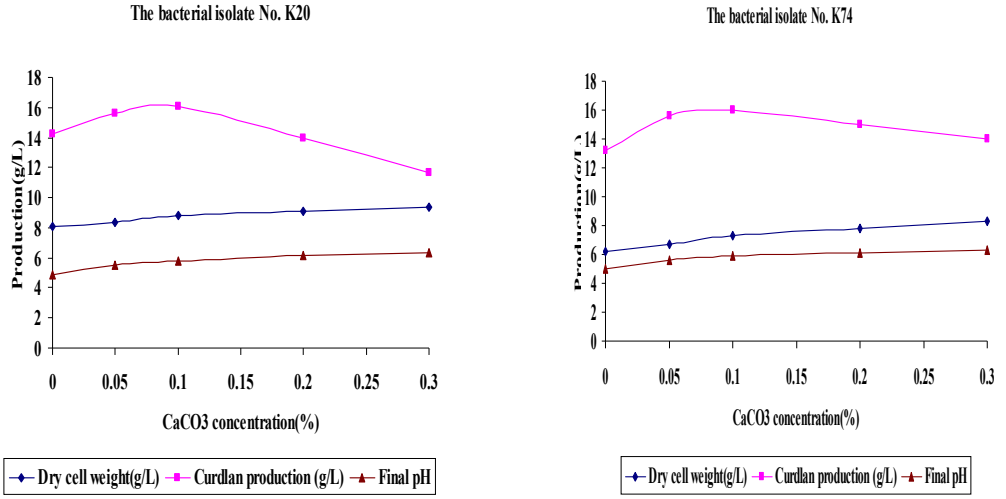

Fig. 12: Effect of different $CaCO_3$ concentrations on the biomass, curdlan production and final pH by the bacterial isolates No. K20 and K74.

Effect of shaking and aeration rate on the biomass and curdlan production

The each selected four bacterial isolates were cultivated in 250-mL Erlenmeyer flasks containing various volumes of the modified standared medium and agitated on a rotary shaker at different shaking rate. Both biomass and curdlan production were determined in each case to choose the optimum working volume with shaking rate that gives the maximum biomass and curdlan production.

Tables (7) and (8) showed the concentrations of cell mass and curdlan production at various volumes with different shaking rate. The maximum curdlan production was obtained when the cells were cultivated at shaking rate 100 rpm in flasks containing 25 mL of the modified standard medium by the bacterial isolates No.K10. It was 18.6 g/L. This was followed by the bacterial isolates No.K20, K74 and K17. It was 17.6 and 16.9 g/L respectively. The optimum shaking rate was used for curdlan production by the bacterial isolates No.K10, K20 and K74 was 100 rpm while it was 150 rpm for the bacterial isolate No.K17. The optimum working volume was used for curdlan production by the bacterial isolates No.K10, K17 and K74 were 25 mL while it was 50 for the bacterial isolate No.K20. As culture volumes were increased from 25 to 100mL with different shaking rate the curdlan production decreased with agitated and static cultivation. These are cleary seen with the bacterial isolates No.K10, K17 and K74. On contrast the curdlan production was increased as well as culture volumes were increased from 25 to 50 mL with agitated and static cultivation by the bacterial isolate

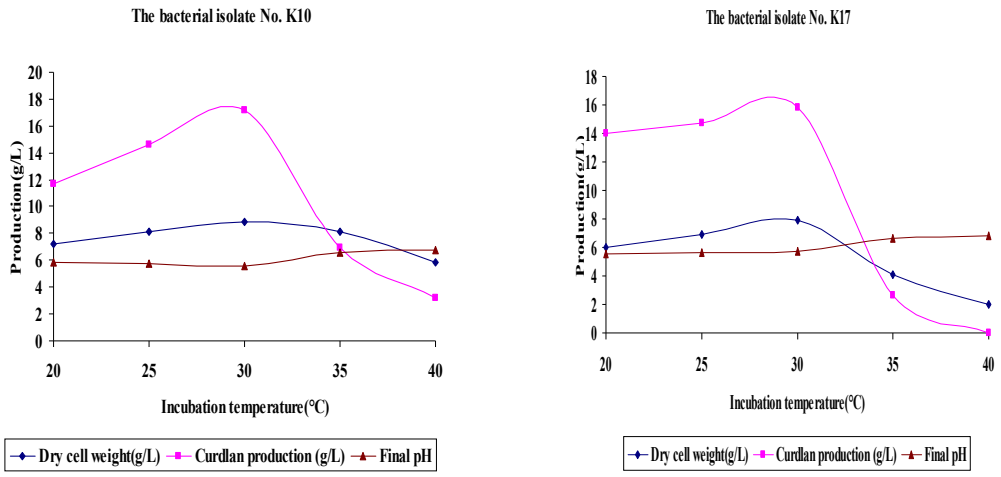

Fig. 13: Effect of different incubation temperatures on the biomass, curdlan production and final pH by the bacterial isolates No. K10 and K17.

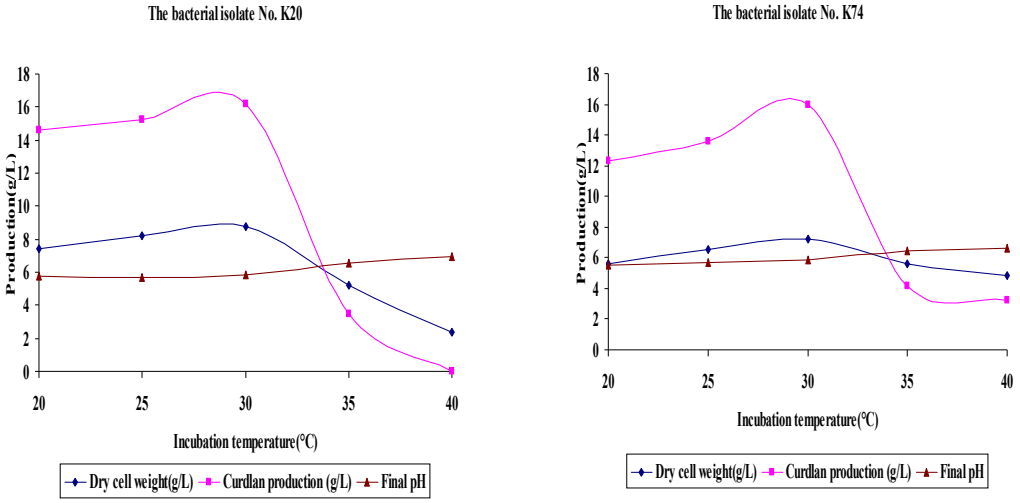

Fig. 14: Effect of different incubation temperatures on the biomass, curdlan production and final pH by the bacterial isolates No. K20 and K74.

No.K20. However, it is uncertain, that curdlan production itself was affected by aeration rate since curdlan synthesis is commenced only after nitrogen exhaustion (Lee *et al* 1999).

In the static culture condition, the amount of curdlan produced gradually increased, with decreased working volumes of the fermentation medium except bacterial isolate No.K20. The maximum amount of curdlan produced by static cultivation was obtained from the bacterial isolate No.K10. It was 14.1g/L. This was followed by the bacterial isolates No.K20, K74 and K17. It was 13.8, 11.6 and 9.6 g/L respectively. It seemed that the curdlan-synthesizing mechanism, yielded more curdlan in agitated culture condition than the static culture, probably as a result of better aeration and the condition has more shear stress, this might cause the difference in production. Moreover, in the static culture condition, the mobility of the microorganism decreases compared to those in the agitated culture condition. Therefore, the uptake of carbon source may be dependent on diffusion permitting more sugar to go to curdlan-synthesizing enzyme. However, for the production of curdlan, the agitated culture condition was better than the static in this study.

The maximum biomass production was obtained when the cells were cultivated at shaking rate 200 rpm in flasks containing 25 mL of the modified standard medium by all selected 4 bacterial isolates. The bacterial isolate No.K10 showed 10.4 g/L. This was followed by the bacterial isolates No.K20, K17and K74. It was 10.3, 9.1 and 8.5g/L respectively. These results are indicating to a higher dissolved oxygen (DO) level is beneficial for cell growth and may be further slowed by the presence of curdlan on the cells surface (Lee *et al* 1999).

Table 7. Effect of different shaking rate with various volumes on the biomass production with each selected four bacterial isolates.

Shaking rate(rpm)	Biomass production (g/L)															
	Bacterial isolates															
	K10				K17				K20				K74			
	25mL	50mL (control)	75mL	100mL	25mL	50mL (control)	75mL	100mL	25mL	50mL (control)	75mL	100mL	25mL	50mL (control)	75mL	100mL
0	6.6^{UVW}	5.6^{CCD}	4.8^{IIJ}	4.2^{MM}	3.7^{OO}	3.3^{PP}	2.5^{RRS}	2.0^{TT}	6.3^{WXY}	5.1^{GGH}	3.9^{NN}	2.4^{SS}	5.2^{GGH}	4.0^{NN}	3.2^{PP}	2.7^{RR}
50	7.0^{RS}	6.1^{YZ}	5.4^{EEF}	5.1^{HH}	5.2^{FFGH}	4.7^{IIJ}	3.7^{OO}	2.9^{QQ}	7.8^{KL}	7.3^{PQ}	4.5^{KKL}	3.9^{NN}	5.9^{BBA}	4.8^{IIJ}	4.2^{MM}	3.3^{PP}
100	7.8^{KL}	7.4^{P}	6.7^{TU}	5.5^{CCDE}	7.7^{LMN}	6.6^{UV}	5.3^{EEFG}	4.3^{LLM}	8.9^{EF}	8.4^{IJ}	5.7^{BBC}	5.2^{FFGH}	6.7^{TU}	6.2^{XYZ}	5.4^{DDEF}	4.9^{II}
150(control)	9.5^{C}	8.6^{GH}	7.6^{MNO}	6.8^{TU}	8.9^{FG}	7.7^{LMN}	6.2^{YZ}	4.6^{IJK}	9.7^{B}	8.8^{FG}	7.0^{RS}	6.3^{XY}	7.8^{LM}	7.1^{QR}	6.0^{AAZ}	5.3^{FFGH}
200	**10.4^{A}**	9.2^{D}	8.3^{IJ}	7.8^{LM}	**9.1^{DE}**	8.2^{J}	7.4^{OP}	6.7^{TU}	**10.3^{A}**	9.2^{D}	8.0^{K}	7.5^{NOP}	**8.5^{HI}**	7.7^{LM}	6.8^{ST}	6.4^{VWX}

Values in the same column followed by the same letter do not significantly differ from each other, according to Duncan's at 5 % level.

Table 8. Effect of different shaking rate with various volumes on the curdlan production with each selected four bacterial isolates.

Shaking rate(rpm)	Curdlan production (g/L)															
	Bacterial isolates															
	K10				K17				K20				K74			
	25mL	50 mL (control)	75mL	100mL	25mL	50mL (control)	75mL	100mL	25mL	50mL (control)	75mL	100mL	25mL	50mL (control)	75mL	100mL
0	14.1^{kl}	12.6^{tu}	10.3^{eef}	8.2^{lm}	9.6^{ggh}	8.6^{kk}	8.0^{mm}	6.0^{pp}	9.6^{ggh}	13.8^{mno}	13.1^{s}	12.6^{tu}	11.6^{aayz}	10.8^{cc}	9.4^{hhi}	6.6^{oo}
50	16.3^{f}	14.1^{kl}	12.7^{t}	10.1^{ff}	11.2^{bb}	10.1^{ff}	9.2^{iij}	7.3^{nn}	10.4^{dde}	15.6^{i}	13.8^{mno}	12.2^{vw}	13.7^{mnop}	12.5^{tu}	10.5^{dde}	8.3^{ll}
100	**18.6^{a}**	17.8^{b}	13.3^{qr}	11.3^{bb}	13.5^{pq}	12.0^{wx}	9.6^{ggh}	7.3^{nn}	11.4^{aab}	**17.6^{b}**	15.6^{hi}	13.4^{pq}	**17.6^{b}**	16.0^{g}	13.9^{lmno}	12.1^{w}
150(control)	17.2^{c}	16.6^{e}	14.3^{k}	13.6^{opq}	**16.9^{d}**	15.7^{hi}	13.1^{rs}	10.6^{ccd}	9.8^{gg}	15.9^{gh}	14.0^{klm}	12.5^{tu}	16.8^{de}	15.9^{ghi}	13.6^{nopq}	11.7^{xyz}
200	14.8^{ij}	13.5^{pq}	13.1^{s}	12.6^{tu}	15.8^{ghi}	14.6^{j}	11.8^{xy}	9.6^{ggh}	9.0^{ijj}	14.0^{klm}	12.4^{uv}	11.4^{aabz}	14.7^{j}	13.9^{lmn}	11.5^{aabyz}	9.7^{ggh}

Values in the same column followed by the same letter do not significantly differ from each other, according to Duncan's at 5 % level

Effect of time course of fermentation on curdlan production

Data in Table (9) reveal that the optimum time course of fermentation for the bacterial isolates No.K10 and K17 were required about 72-h to produce the maximum quantity of curdlan, it was 22.0 and 19.4 g/Lrespectively. This is result was accepted with the report of (Shivakumar and Vijayendra, 2006). The report mentioned the maximum curdlan production was 11.50 g/L after 72-h cultivation in a batch process. The bacterial isolates No.K20 and K74 were required about 96-h to produce the maximum quantity of curdlan, it was 17.8 and 17.7g/L respectively. This is result was accepted with the report of (Lee *et al*., 1997). The report mentioned the maximum curdlan production was 32 g/L after 95-h cultivation in a batch process. The initiation of curdlan production with each selected four bacterial isolates was noticed after 16-h. (Lee & Lee, 2001 and Lee & Park, 2001) have also reported about the initiation of curdlan production which noticed only after 20 h. As observed by Pham *et al.,* 2000 the cause of reduction in the curdlan production beyond 96-h of fermentation might be due to the production of hydrolyzing enzymes. A marked reduction in the curdlan yield with prolonged fermentation seems to be dependent on the strain (Gancel and Novel 1994) and culture conditions used, such as pH (Mozzi *et al*., 1994), temperature (Mozzi *et al*., 1995) and carbohydrate source (Gassem *et al*., 1997).

Table 9. Effect of time course of fermentation on the biomass and curdlan production with each selected four bacterial isolates.

Variance	Bacterial isolates							
Time course of fermentation (h)	K10		K17		K20		K74	
	Dry cell weight (g/L)	Curdlan production (g/L)	Dry cell weight (g/L)	Curdlan production (g/L)	Dry cell weight (g/L)	Curdlan production (g/L)	Dry cell weight (g/L)	Curdlan production (g/L)
0	0.2FF	ND	0.2FF	ND	0.2FF	ND	0.2FF	ND
8	0.6EE	ND	0.7EE	ND	0.6EE	ND	0.7EE	ND
16	1.4CC	3.4ff	1.6BB	2.9hh	1.1DD	2.0ii	1.7BB	1.6jj
24	3.2Y	6.4aa	2.7Z	5.6bb	2.3AA	4.0ee	2.3AA	3.4ff
32	4.8U	9.5u	3.8X	8.5w	3.3Y	6.2aa	3.3Y	4.8dd
40	6.7MN	12.2p	5.4S	11.4q	4.5V	8.4wx	4.3VW	6.3aa
48	8.1GH	14.7kl	6.9L	14.9k	5.7QR	10.5t	5.1T	8.2x
56	8.2FG	17.7fg	9.0C	18.3e	6.7M	12.4o	5.8Q	11.0r
64	8.4EF	20.0b	9.3B	18.7d	7.9HI	14.2m	6.2P	13.4n
72	8.8D	22.0a	9.6A	19.4c	8.1GH	15.9j	6.2P	16.0j
80	7.8IJ	20.1b	8.7D	17.6fg	8.2FG	17.0h	6.4OP	16.7i
88	7.6JK	19.2c	8.6D	17.0h	8.3FG	17.7fg	6.5NO	17.5g
96(control)	7.5K	18.4e	8.6DE	16.7i	8.2FG	17.8f	6.6MN	17.7fg
104	6.2P	14.6l	5.6R	12.3op	7.6JK	13.3n	4.8U	10.8s
112	4.4V	11.0r	4.2W	8.7v	6.7LM	8.3wx	2.7Z	7.8y
120	2.3AA	7.5z	2.6Z	6.2aa	5.8QR	3.2gg	1.1DD	5.3cc

***ND** not detected*

Values in the same column followed by the same letter do not significantly differ from each other, according to Duncan's at 5 % level

Identification of the highest efficient bacterial isolate

The bacterial isolate No.K10 that gave the maximum curdlan production was identified by the Bio-log automated system. This method of identification is depending on the effect of use different types of carbon sources on the growth of isolate.

Finally the bacterial isolate No. K10 was identified as *Rhizobium radiobacter* (formerly *Agrobacterium radiobacter*) (Young *et al* 2001). This is result was accepted with similar reported by (Phillips *et al*., 1983; Lee *et al*., 1997 and Saudagar & Singhal, 2004).

Conclusion and recommendations for future work

In conclusion, the results obtained in this study revealed that the optimum nutritional requirements and environmental factors which were needed with the local four bacterial isolates to produce curdlan at a high yield in shake flasks as batch fermentation. Many researchers have investigated the bacterial production of curdlan the highest productivity reported being 0.2 g/L/h after 120h, which was obtained from the batch culture of *Agrobacterium* sp. under optimal conditions (Lee *et al* 1997). Using the local strain of bacteria *Rhizobium radiobacter* developed in this study, we increased the productivity to 0.3 g/L/h after 72h under the same conditions. Further research will focus on this polymer can be economically produced in large quantities in fermenter by different methods and show biological activity as an immune stimulator, it has a potentially large market in both the nutraceutical and the pharmaceutical industries.

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