J Ind Microbiol Biotechnol (2009) 36:433–438
DOI 10.1007/s10295-008-0514-7

ORIGINAL PAPER

Lactose mother liquor as an alternative nutrient source for microbial concrete production by *Sporosarcina pasteurii*

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Received: 14 October 2008 / Accepted: 4 December 2008 / Published online: 24 December 2008
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Abstract Microbiologically induced calcite precipitation by the bacterium *Sporosarcina pasteurii* (NCIM 2477) using the industrial effluent of the dairy industry, lactose mother liquor (LML) as growth medium was demonstrated for the first time in this study. The urease activity and the calcite precipitation by the bacterium was tested in LML and compared with the standard media like nutrient media and yeast extract media. Calcite constituted 24.0% of the total weight of the sand samples plugged by *S. pasteurii* and urease production was found to be 353 U/ml in LML medium. The compressive strength of cement mortar was increased by *S. pasteurii* in all the media used compared to control. No significant difference in the growth, urease production and compressive strength of mortar among the media suggesting LML as an alternative source for standard media. This study demonstrates that microbial calcite acts as a sealing agent for filling the gaps or cracks and fissures in constructed facilities and natural formations alike.

Keywords *Sporosarcina pasteurii* · *Bacillus pasteurii* · Calcite · Urease · Lactose mother liquor · Compressive strength

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Introduction

Microbial metabolic activities often contribute to selective cementation by producing relatively insoluble organic and inorganic compounds intra- or extracellularly. Considerable research on carbonate precipitation by bacteria has been done by using ureolytic bacteria. These bacteria are able to influence the precipitation of calcium carbonate by the production of urease enzyme. This enzyme catalyzes the hydrolysis of urea to CO_2 and ammonia, resulting in an increase of the pH and carbonate concentration in the bacterial environment [26].

Precipitation of calcium carbonate crystals occur by heterogenous nucleation on bacterial cell walls once supersaturation is achieved. Microbial calcite has potential technical and industrial applications for the preservation and restoration of calcareous stone statuaries and historic monuments. Microbial mineral precipitation (biodeposition) technologies have already been used for consolidation of sand columns [9, 21, 26], for repair of limestone monuments [8, 24, 27], and to a small extent for remediation of cracks in concrete [2, 3, 22].

The efficiency of microbial plugging is affected by the porosity of the medium, the number of cells present and the total volume of nutrients added [22]. The total cost of a biotechnology-based product includes labor, medium, cultivation operating costs, waste treatment and any downstream or up stream processing. In most processes, the medium ingredients are a major cost factor, ranging between 10 and 60% of the total operating costs [15]. Researchers have hitherto used standard nutrient media like nutrient medium or yeast extract medium to grow the bacteria. Use of these media is expensive and prohibits field trial of the technology. An alternative inexpensive nutrient source is imperative for wide use of

the technique. Moreover, there are many industrial effluents that are rich in protein. If released in the altered form they are hazardous for the atmosphere and such effluents can be used as nutrients. Thus dual benefits of cost reduction and environmental protection can be achieved.

Species of the *Bacillus* group are able to precipitate calcite on their cell constituents and in their micro-environment by conversion of urea into ammonia and carbon dioxide. *Bacillus pasteurii*, a soil bacterium, produces intracellular urease constituting close to 1% of the cell weight [2]. *B. pasteurii* induced calcium carbonate precipitation played an important role as a microbial sealent to remediate cracks and fissures in structural formations of granite and concrete [11, 22].

In the present investigation, lactose mother liquor (LML), an industrial effluent of the dairy industry is used as the sole source of nutrients to grow the bacterium *Sporosarcina pasteurii* (*B. pasteurii* NCIM 2477). Its efficiency with other standard media has been compared. Studies were also performed to evaluate microbiological calcite precipitation, urease production and compressive strength of mortar in LML and compared with the standard growth media.

Materials and methods

Microorganism and media

Sporosarcina pasteurii (previously known as *B. pasteurii*) NCIM 2477, procured from National Chemical Laboratory, Pune, India was used in this study. The culture was routinely maintained on Nutrient agar (pH 8.0) medium. Lactose mother liquor was collected from the dairy industry (Cepham Milk Specialties Pvt. Ltd, Derrabassi, Punjab, India) and analyzed for its physico-chemical properties (Table 1). Microbiological urease production, calcite precipitation and compressive strength tests were carried out in the following three media (per liter); LML-urea medium (10% LML, 5 g NaCl, 2% urea and 25 mM $CaCl_2$), NB-urea medium [8 g nutrient broth (Himedia, Mumbai, India), 5 g NaCl, 2% urea and 25 mM $CaCl_2$] and YE-urea medium [1 g yeast extract (Himedia, Mumbai, India), 5 g NaCl, 2% urea and 25 mM $CaCl_2$]. The pH of the media was adjusted to 6.5 with 1 N HCl prior to autoclaving without urea and $CaCl_2$. Filter-sterilized urea and $CaCl_2$ was added later. The growth profile of *S. pasteurii* in three media was tested by taking the absorbance (OD_{600}) at regular time intervals and corresponding cfu/ml were counted after overnight incubation at 37°C.

Table 1 Physico-chemical characteristics of the lactose mother liquor (LML)

Component	Quantity
pH	6.20
Solid (%)	5.50
Lactose (%)	15.40
Proteins (%)	8.00
Fats (%)	2.00
Ash (%)	0.53
Calcium (mg/l)	353
Phosphorus (mg/l)	35
Potassium (mg/l)	186
Sodium (mg/l)	44
Chloride (mg/l)	90
sulfur (mg/l)	15

Urease activity

The urease activity was determined in all three media according to the phenol–hypochlorite assay method [20]. Ammonium chloride (50–1,000 μM) was used as the standard. The culture filtrates (250 μl) were added to a mixture containing 1 ml of 0.1 M potassium phosphate buffer (pH 8.0) and 2.5 ml of urea (0.1 M). The mixture was incubated at 37°C for 5 min followed by addition of phenol nitroprusside and alkaline hypochlorite, 1 ml each and incubated at 37°C for 25 min. Optical density was measured at 626 nm and one unit of urease is defined as the amount of enzyme hydrolyzing 1 μmole urea/min.

Microbiological sand plugging

Microbiological sand plugging was performed to study calcite precipitation. Fifty milliliters of grown culture ($OD_{600} = 1.0$) was mixed with 100 g sterilized river sand and was packed into a plastic column (height = 15 in.; diameter = 3 in.) and bottom side of column was blocked using Whatman filter paper. A control reaction was packed in column in which sterile sand was mixed with different media only (without bacteria). All columns were fed continuously with three specific media separately at room temperature to mimic the natural environmental conditions. The experiments in all the sand columns were terminated after 10 days. Microbial sand column was divided into three layers (upper, middle and lower layer) and each layer was individually ground and sieved through a 45 μm diameter mesh prior to calcite estimation. Precipitated calcite from each layer was measured by EDTA titration method [1]. One gram of sand sample from each layer was dissolved with 3 N HCl and 4 ml of 5 N NaOH was added

to the precipitate and the final volume was made up to 50 ml using distilled water. Few drops of hydroxyl napthol blue were added as an indicator and the mixture was finally titrated against 0.05 M EDTA. End point was noted from pink to blue, and the amount of $CaCO_3$ formed was calculated by the volume of EDTA used × 0.005004 × 1,000/ml of sample used.

Compressive strength test

Locally available clean, dry, well graded, natural river sand was mixed with cement (3:1 w/w). A cube mold of 70.6 mm was prepared, as per IS 4031–1988. Sand and cement were thoroughly mixed, adding along with grown culture of *S. pasteurii* correspondence to OD_{600} of 1.0. Cubes were cast and compacted in a vibration machine. After de-molding all specimens were cured in corresponding medium at room temperature until compression testing at the intervals of 3, 7 and 28 days. Compression testing was performed using automatic compression testing machine, COMPTEST 3000.

All the experiments were performed in triplicate. The data was analyzed by Analysis of Variance (ANOVA) and the means were compared using Tukey's test. All the analyses were performed using GraphPad Prism (4.1) software.

Results and discussion

Growth and pH profile

The growth profile studies up to 30 h in different media showed that *S. pasteurii* has the ability to grow well and utilize the nutrients in LML along with other standard media. The growth profile was similar in all media and there was no significant difference observed among the media in relation to their cfu/ml (Fig. 1). The pH of the medium significantly increased as the growth increased in all the media. The maximum pH of 11.0 was observed after 30 h of incubation. The pH profile did not differ significantly among the media (Fig. 2). Some bacterial species may be able to use more complex biological polymers such as lipids, polysaccharides and nucleic acids because these organisms produce a range of extracellular hydrolytic enzymes that can degrade large organic molecules. We, however, did not find any literature reports directly documenting an instance of *Sporosarcina* living on medium containing LML. The growth of *S. pasteurii* appeared to be correlated with pH. The rise in pH causes a precipitation of calcium carbonate, which occurs during urea degradation [8]. In medium containing urea and $CaCl_2$, that supports microbial growth, NH_4^+ and Cl^- react with OH^- and H^+.

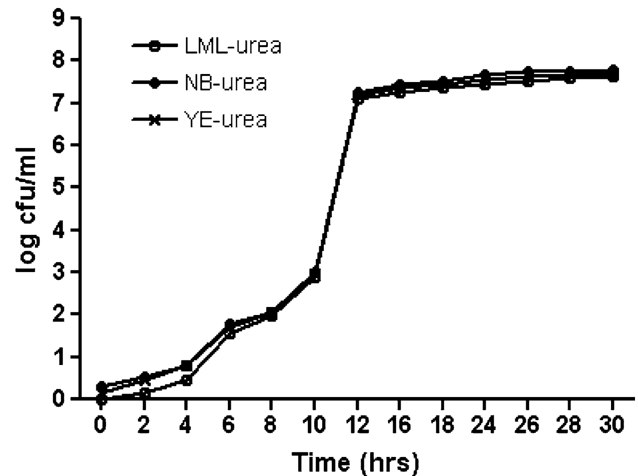


Fig. 1 Growth profile of *S. pasteurii* in LML, nutrient and yeast extract media supplemented with urea

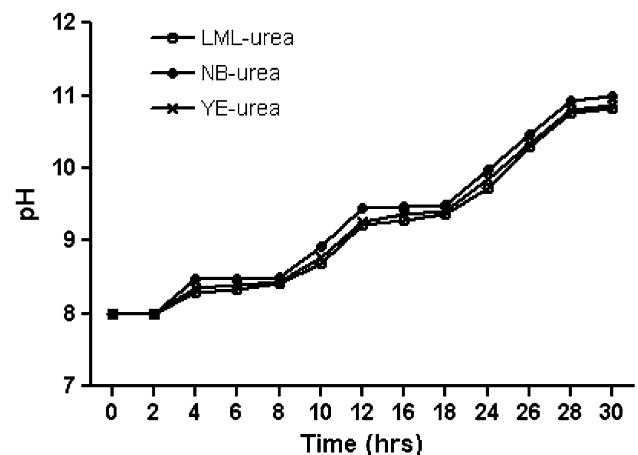


Fig. 2 pH profile of *S. pasteurii* in LML, nutrient and Yeast extract media supplemented with urea

Microbiologically induced $CaCO_3$ precipitation occurs via far more complicated processes than chemically induced precipitation.

Urease activity

Sporosarcina pasteurii showed maximum urease production in NB-urea medium (412 U/ml) followed by YE-urea medium (366 U/ml) and LML-urea medium (353 U/ml) (Fig. 3). The highest productivities in all the media were obtained at 120 h. Urease production in NB-urea medium and YE-urea medium was 0.17- and 0.04-fold higher than those in LML-urea medium, which was not statistically significant. After 120 h, urease production was decreased in all the media. Bacteria are known to hydrolyze urea by urease for the purposes of: (1) increasing the ambient pH [6], (2) utilizing it as a nitrogen source [5], and (3) using it

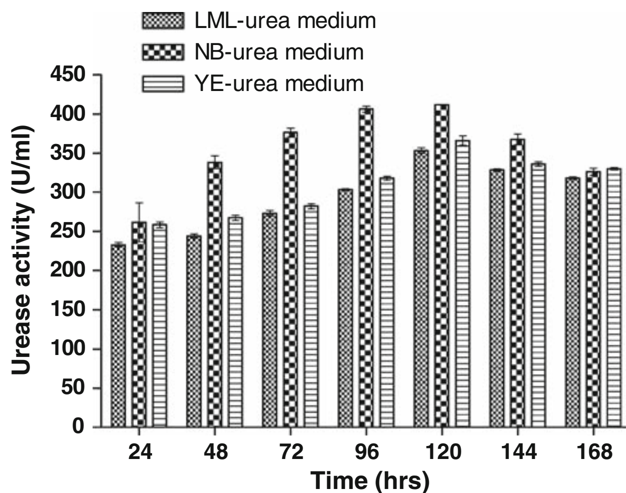


Fig. 3 Urease activity of *S. pasteurii* in LML-urea, NB-Urea and YE-Urea media. *Error bars* show SD ($n = 3$)

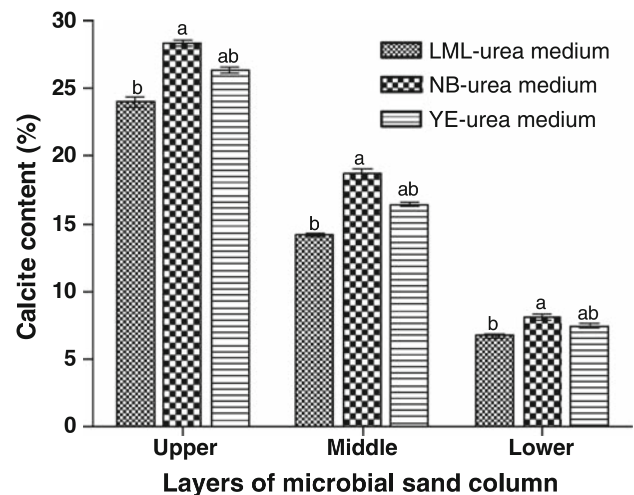
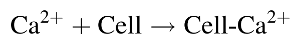


Fig. 4 Calcite content in different layers of sand columns consolidated with *S. pasteurii* in LML-urea, NB-urea and YE-urea media. *Error bars* show SD ($n = 3$)

as a source of energy [17]. In biological systems, many calcareous organisms couple calcification to their metabolic assimilation processes to scavenge protons [16]. The subsequent increase of pH in surrounding medium due to the presence of ammonia ions and the additional release of CO_2 from the enzymatic urea hydrolysis further accelerate the rate of the urease induced calcite precipitation. Thus, an active participation of urease is of essence in biochemical calcite precipitation.

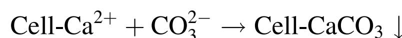
Calcite precipitation in sand plugging

Bacillus species are known to produce a large amount of urease in soil environments [2, 4, 7, 18]. The urease identified from these bacteria is found to be extracellular, so it can be directly applied to consolidate sand column for calcite precipitation rather than whole bacterial cells [13]. All sand columns prepared with *S. pasteurii*, using three media were found to be tightly packed except control sand column (after removing the plastic, it lost its form and collapsed). Calcite content was found to be maximum in case of upper layer of microbial sand column prepared with all the three media, as compared to other two layers. Calcite constituted 28.4, 26.3 and 24.0% of the total weight of the sand samples plugged by *S. pasteurii* in NB-urea medium, YE-urea medium and LML-urea medium, respectively (Fig. 4). Calcite precipitation occurred predominantly in the areas close to the surface of the sand column. It is mainly due to the fact that facultatively anaerobic *S. pasteurii* grows at a higher rate in the presence of oxygen and consequently induces active precipitation of $CaCO_3$ around the surface area. There was not much significant difference in calcite content produced by *S. pasteurii* grown in LML medium as compared to other media. LML provides variety of ions in the medium in the form of Ca, Na, K, Mg and other elements. Due to which bacterial cell surface could nonspecifically induce mineral deposition by providing a nucleation site. Especially, Ca^{2+} is not likely to be utilized by microbial metabolic processes; it rather accumulates outside the cell [25]. Possible biochemical reactions in medium containing urea and $CaCl_2$ to precipitate $CaCO_3$ at the cell surface can be summarized as follows [26]:



$$Ca^{2+} + Cell \rightarrow Cell\text{-}Ca^{2+}$$

$$Cl^{-} + HCO_3^{-} + NH_3 \rightarrow NH_4Cl + CO_3^{2-}$$



$$Cell\text{-}Ca^{2+} + CO_3^{2-} \rightarrow Cell\text{-}CaCO_3 \downarrow$$

Muynck et al. [19] indicated that the type of bacterial culture and media composition have a profound impact on calcite crystal morphology. Crystal growth can be inhibited or altered by the adsorption of proteins, organic matter or inorganic components to specify crystallographic planes of the growing crystals [12, 14, 19]. Differences in size and morphology between the different types of calcium source and different types of bacterial culture can also be attributed to the presence of organic matter. Hammes et al. [12] suggested that differences in crystal morphology, which are obtained with different bacterial cultures, could be due to the level of the actual urease activity. In the present study, the $CaCO_3$ precipitation and urease activity was similar in all the three media tested with *S. pasteurii*. This indicates that LML can serve as an alternative medium for precipitation of calcite in place of nutrient medium or yeast medium, which is costlier. In addition, LML offers a greener option by recycling industrial effluent.

Compressive strength

The compressive strength had increased for the mortar cubes that contained microbial cells irrespective of the media used to grow the cells compared to control (Fig. 5). The highest compressive strength was obtained with mortar cubes that were incubated for 28 days. Mixing of *S. pasteurii* in mortar cubes with LML-urea medium showed around 17% improvement in compressive strength at 28 days (26.3 MPa) with respect to control (23.2 MPa); whereas in case of NB-urea and YE-urea media, it was 27.9 and 27.2 MPa, respectively. However, there was no significant difference observed in compressive strength among the media. This improvement in compressive strength is probably due to deposition of $CaCO_3$ on the bacterial cell surfaces and within the pores of cement–sand matrix, which plug the pores within the mortar [22, 23]. The compressive strength was similar in all the treatments at 3 and 7 days of curing. Ghosh et al. [10] also showed the improvement of compressive strength of cement mortar by the addition of *Shewanella* species.

The overall trend of an increase in compressive strength up to 28 days might be attributed to the behavior of microbial cells within the cement mortar matrix. During the initial curing period, microbial cells obtained good nourishment, because the cement mortar was still porous; but growth might not be proper due to the completely new environment for microbes. It may also be possible that as the pH of the cement remained high, cells were in inactive condition and as curing period was increased, it started growing slowly. Upon cell growth, calcite would have precipitated on the cell surface as well as within the cement mortar matrix. Once many of the pores in the matrix were plugged, the flow of the nutrients and oxygen to the bacterial cells stopped, eventually the cells either died or turned into endospores and acted as an organic fiber, increasing the compressive strength of the mortar cubes. This explains the behavior of the increased compressive strength at the age of 28 days in cement mortar cubes prepared with microbial cells. There was a measurable increase in compressive strength of cement mortar cubes prepared with *S. pasteurii*, supported by previous studies [3, 22]. Thus, it was concluded that the increase in compressive strengths is mainly due to consolidation of the pores inside the cement mortar cubes with microbiologically induced calcium carbonate precipitation.

The performance of *S. pasteurii* in urease production, calcite precipitation and improvement in compressive strength appears equally effective whether they are grown in media containing 10% LML or other nutrient media. Addition of *S. pasteurii* has a positive effect on the compressive strength of cement mortar. Undoubtedly, *S. pasteurii* not only provides a nucleation site for calcite precipitation but also creates an alkaline environment inducing further growth of calcite.

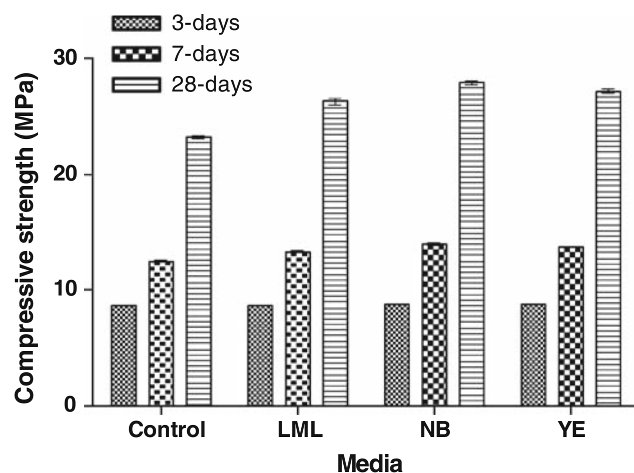


Fig. 5 Effect of *S. pasteurii* grown in LML-urea, NB-Urea and YE-Urea media on the compressive strength of cement mortar cubes at 3, 7 and 28 days. *Error bars* show SD ($n = 3$)

Conclusions

LML is a good source of nutrients that can support growth and urease activity of this bacterium. The present study results suggest that LML can serve as a better nutrient source for the growth of the bacteria and also for calcite precipitation. Use of LML in place of standard media not only to reduce the cost in remediation of cracks in structures and fissures by bacteria, but also serve as eco-friendly technology to prevent environmental pollution.

Acknowledgments The financial assistance to this study received from Atomic Energy Research Board, Department of Atomic Energy and Department of Science & Technology, India is gratefully acknowledged. The authors are thankful to TIFAC-CORE for the experimental facilities.

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