Solid-State Fermentation for the Production of Meroparamycin by *Streptomyces* sp. strain MAR01

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The antibiotic meroparamycin was produced in the free culture system of *Streptomyces* sp. strain MAR01. Five solid substrates (rice, wheat bran, Quaker, bread, and ground corn) were screened for their ability to support meroparamycin production in solid-state fermentation. In batch culture, wheat bran recorded the highest antibacterial activity with the lowest residual substrate values. The highest residual substrate values were recorded for both ground corn and Quaker. On the other hand, no antibacterial activity was detected for rice as a solid substrate. The use of the original strength of starch-nitrate medium in the solid-state fermentation gave a lower antibacterial activity compared with the free culture system. Doubling the strength of this medium resulted in the increase in the activity to be equivalent to the free culture. The initial pH (7.0) of the culture medium and 2 ml of spore suspension (1 ml contains 5×10^9 spores/ml) were the optima for antibiotic production. The water was the best eluent for the extraction of the antibiotic from the solid-state culture. Ten min was enough time to extract the antibiotic using a mixer, whereas, 60 min was required when shaking was applied. Semicontinuous production of meroparamycin using a percolation method demonstrated a more or less constant antibacterial activity over 4 runs (450--480 μg/ml). The semicontinuous production of the antibiotic was monitored in a fixed-bed bioreactor and the maximum activity was attained after the fourth run (510 μg/ml) and the overall process continued for 85 days.

Keywords: *Streptomyces* sp. strain MAR01, solid-state fermentation, meroparamycin, wheat bran

Solid-state fermentation (SSF) refers to the growth of microorganisms on solid substrates without the presence of free liquid between substrate particles [21]. There are two types of solid matrices: (a) Natural solid materials such as

foodstuffs and agricultural by-products. These include crops such as soybean, sugar beet, sweet potato, potato, and crop residues such as bran and straw of wheat and rice as well as of fruit processing residues such as wastes of apple, grape, pineapple, and carrot [15, 21]. These materials act as the main source of nutrients and the organism releases enzymes for breaking down and modifying these solid materials. (b) An inert solid support supplemented with nutrients for microbial growth. These solid materials act only as a physical support for growth through which the enzymes can be released by the organism and the nutrients can diffuse [20, 21]. A typical traditional example of SSF is waste-based composting, which has been practiced worldwide. *Koji* (starter) making is one of the most common applications of SSF in the Orient. Products are hand-made, and the technology is simple and appropriate as the resources are locally available as described by Josephson [12]. Although scientific understanding of much of the basic principles of SSF is still lacking and this has urged the development of these technologies [25, 29], the physiology of microorganisms in SSF shows similarity to its physiology in a liquid medium, so a similar strategy could be adapted for efficient physiological processes to proceed [1].

Genus *Streptomyces* is a heterotrophic feeder that can utilize both simple and complex molecules as nutrient and energy sources [16]. Since the early discovery of this genus's ability to produce clinically useful antibiotics [27, 30], the genus has received much more attention [4, 6, 8–11, 18, 22]. SSF has been applied for secondary metabolites production such as antibiotics [1, 2, 28] and enriched foodstuffs [1, 6]. *S. clavuligerus* was grown under optimum solid-state fermentation conditions for maximum cephalosporin C production. Solid-state fermentation was also applied for oxytetracycline production after the protoplast fusion of *S. rimosus* [32].

Streptomyces sp. strain MAR01 was isolated from the Egyptian soil and found to produce one major bioactive substance [10] identified as meroparamycin (Fig. 1). The objective of this study was to produce meroparamycin using a simple and low-cost effective solid-state fermentation.

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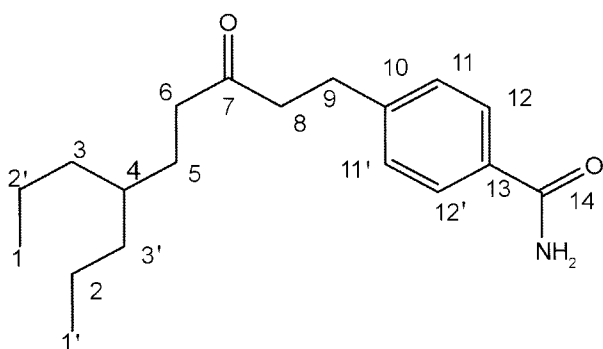Phone:+20-30101479849 ; Fax: +20-30101479849;
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Fig. 1. The molecular structure of the antibiotic meroparamycin produced by *Streptomyces* sp. MAR01.

MATERIALS AND METHODS

Microorganisms

The producer strain was isolated from the Egyptian soil and identified as *Streptomyces* sp. strain MAR01 [10] according to its cultural and physiological characteristics and the nucleotide sequence of the 16S rRNA gene (1,509 pb). *Staphylococcus aureus* ATCC 29737 was used as a test organism for the bioassay of the meroparamycin produced by *Streptomyces* sp. strain MAR01 [10].

Maintenance of the Stock Cultures

Streptomyces sp. strain MAR01 cultures were maintained on starch-nitrate agar slants. The inoculated agar medium was incubated for 7 days at 30°C and then stored at 4°C. A test organism stock culture was maintained on tryptic soy agar slants. The inoculated agar medium was incubated for 24 h at 37°C and then stored at 4°C until further use.

Culture Media

Starch-nitrate agar medium This medium was used for maintenance of *Streptomyces* sp. strain MAR01. It was composed of (g/l) starch, 10; $NaNO_3$, 2; K_2HPO_4, 5.65; KH_2PO_4, 2.35; $MgSO_4 \cdot 7H_2O$, 1; $CaCl_2$, 0.1; microelement, 1 ml; and agar 18. The pH was adjusted to 7.2 before sterilization using 1 N NaOH or 1 N HCl. Stock solution was composed of (g/500 ml) $FeSO_4 \cdot 7H_2O$, 0.5; $MnCl_2 \cdot 4H_2O$, 0.5; and $ZnSO_4 \cdot 7H_2O$, 0.5 [24].

Starch-nitrate broth This medium contained the same ingredients as mentioned above for starch-nitrate agar with the omission of agar. It was used as the antibiotic production medium throughout the work and was further optimized.

Antibiotic Bioassay

The antibacterial potential of the culture filtrate was assayed using 50 μl filtrate to fill the agar-well (6 mm i.d.) punched in the Mueller-Hinton agar plates freshly seeded with 0.3 ml of *S. aureus* (3×10^6 CFU/ml) as the test organism (MIC of the purified antibiotic=6.25 μg/ml). The inhibition zone diameter was measured in millimeters after incubation at 37°C for 24 h. Care was taken to confirm that no agonistic or antagonistic substances were produced during the production of the antibiotic in the bioreactor, so HPLC assay was also carried out. The assay was performed on a Hewlett-Packard 1090A liquid chromatograph equipped with a scanning diode-array detector. The latter facilitated peak identification due to the characteristic UV chromophore (230 nm) of the antibiotic. The column used was a

Spherisorb C18, 5 μm (25 cm by 4.6 mm), eluted with n-hexane:ethyl acetate (2:8 v/v) at a flow rate of 1 ml/min. Spectral scans were compared with the data obtained from the authentic sample of the purified antibiotic. Antibiotic concentration was determined using a standard curve for the purified sample.

Effect of Solid Substrates on the Antibacterial Activity

The effect of the commercial solid substrates (rice, 0.4–0.5 cm; wheat bran, 0.3–0.4 cm; Quaker, 0.5–0.6 cm; bread, 0.5–0.6 cm; and ground corn, 0.2–0.3 cm) on the antibacterial production was performed in 250-ml Erlenmeyer flasks. Each flask contained 10 g of solid substrate and 15 ml of starch-nitrate medium. For rice preparation, 10 g of rice was washed and soaked in 15 ml starch-nitrate medium at room temperature overnight. The medium was decanted; rice was spread uniformly in the Erlenmeyer flask. Flasks were sterilized and inoculated with 2 ml of spore suspension (5×10^9 spores/ml) and incubated at 30°C for 7 days. The antibacterial activity as inhibition zone diameter, the residual substrate (gm), and the final pH of the medium were all determined. Care was taken to keep the original moisture content for all solid substrates at 40%.

Effect of Initial Moisture Content

The influence of initial moisture content of the solid substrate was investigated at various moisture levels (40, 50, 60, 70, 80, 90 and 100%) of wheat bran, using distilled water before autoclaving and the initial moisture percent was calculated.

Solid-State Culture (Percolation Method)

A cylindrical percolation vessel with a conical bottom was used. The end of the percolator was filled with groups of marbles. The wheat bran substrate (0.3–0.4 cm) was mixed with the sintered glass beads (Siran Carriers produced by Schött, Glassweke, Mainz, Germany) and wetted with starch-nitrate broth (60% moisture level) and then added to the percolator. After sterilization, the distilled water was added manually and the extract was allowed to continuously discharge from an outlet controlled by a gate valve at the bottom of the percolator. At the end of the first run (7 days), the extract was drained from the percolator and fresh starch-nitrate medium was replaced in a semicontinuous pattern. The antibiotic concentration was determined at the end of each run.

Semicontinuous Culture in Fixed-Bed Bioreactor

Semicontinuous production for the antibiotic produced by *Streptomyces* sp. strain MAR01 in a wheat bran solid-state culture in a fixed-bed bioreactor was experimented. For this purpose, a glass column (30× 7 cm) containing 30 g of wheat bran (particle size 0.3–0.4 cm) moistened with starch-nitrate medium (60% moisture content) was used. After autoclaving, the column was inoculated with 6 ml of spore suspension and incubated statically at 30°C for 5 days for each run. At the end of each run, the culture was washed with water to extract the antibiotic and a new run was started by adding fresh medium. The antibiotic concentration was determined at the end of each run.

Electron Microscopy

Scanning-electron micrographs of adsorbed *S. violatus* mycelia on the glass rings in a fixed-bed bioreactor were produced using a JEOL JSM-5300 scanning electron microscope (JEOL Technics Ltd., Tokyo, 196–8558, Japan) at the Electron Microscope Unit, Faculty of Science, Alexandria University, Egypt.

RESULTS AND DISCUSSION

Among the factors important and most crucial for microbial growth and activity in a solid-state culture are substrate type, particle size, and moisture level/water activity. Generally, smaller substrate particles would provide a larger surface area for microbial attack. However, too small substrate particles may interfere with microbial respiration, and thus result in poor growth [19]. At the same time, larger particles provide better respiration/aeration efficiency because of increased interparticle space, but provide limited surface for microbial attack. In the present investigation, wheat bran (0.3–0.4 cm) from the local market provided the highest antibacterial activity and the lowest residual substrate. Quaker (0.5–0.6 cm), bread (0.5–0.6 cm), and ground corn (0.2–0.3 cm) gave the lowest antibacterial activities in a descending order. The highest residual substrate was recorded for both ground corn and Quaker. On the other hand, although rice (0.4–0.5 cm) provides a nutritionally complete medium for microbial growth, *Streptomyces* sp. strain MAR01 failed to produce any antibacterial activity (Table 1). This could be attributed to the fact that rice at low moisture content becomes agglomerated, and it might be possible that at these lower moisture levels, transport of nutrients or supply during idiophase becomes difficult. Tetracycline production by *S. aureofaciens* with sweet potato residues showed a 270% increase when the medium was enriched with 10% soluble starch [31], but the addition of 10% glucose caused a 60% decrease in the antibiotic titer.

High antibiotic titer (450 μg/g) was achieved when the initial moisture level was 60% when compared with lower or higher moisture contents (Fig. 2). The importance of the moisture level in solid-state fermentation media and its effect on the secondary metabolites formation could be attributed
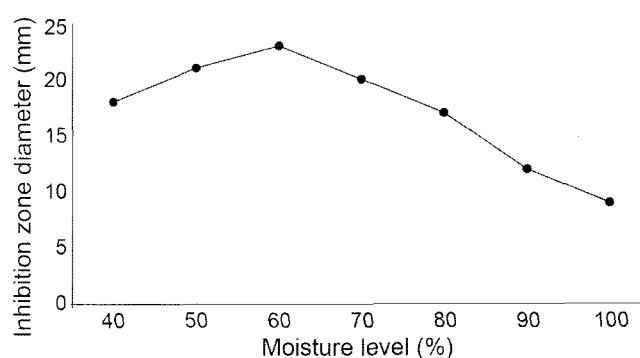

Fig. 2. Effect of the moisture level (%) on the production of meroparamycin.

to the interference with physical properties of the solid particles. Increasing the moisture may reduce the porosity of the wheat bran, and thus limit oxygen and mass transfer. The lower moisture content may cause a reduction in the nutrient solubility and cause a low degree of swelling [25]. Moreover, the reduction of the interparticle space under higher moisture contents may cause inhibition to the sporulation process. Since sporulation is associated with antibiotic production, it is possible those effects may be related to the lower antibiotic production.

The morphology of actinomycetes mycelium is well suited to invasive growth on solid medium [1]. This morphology is responsible for considerable difficulties in large-submerged culture. These include sheer forces, increasing viscosity due to the metabolic secretion, and the reduction in the metabolic stability. This results in very high requirements for mixing and oxygen transfer efficiency and can lead to problems during product recovery [14]. Therefore, SSF technology can be exploited as an alternative, allowing better oxygen circulation [7, 26], especially when large quantities of secondary metabolites are required in short fermentation periods with minimal expenditure of media and downstream processing.

In the present investigation, the original strength of starch-nitrate medium in flask fermentation gave a lower antibacterial activity (16.0 mm) compared with the free culture (21.00 mm). Hence, the effect of medium strength on the antibacterial activity in a solid-state culture was investigated. The doubling of each of the ingredients in this medium resulted in the increase in the inhibition zone diameter to be equivalent to the free culture system (data not shown). Augmenting the concentration of each ingredient by 3-fold or 4-fold of its original value resulted in a 50% decrease in the inhibition zone diameter. On the other hand, the original strength or its double did not change the final pH value significantly, whereas the rest of the trials showed an adverse effect on the pH. Moreover, the maximum meroparamycin production occurred at the initial pH adjusted to 7.0. As the metabolic activities of microorganisms are very sensitive to minor changes in pH, meroparamycin production was negatively affected if the pH level was lower or higher than the

Table 1. Effect of solid substrate on the antibacterial activity and final pH of the culture filtrate of *Streptomyces* sp. strain MAR01.

Solid substrate	Final pH	Residual substrate (gm)	Inhibition zone diameter (mm)
Bread	6.87 [f]	5.67 [d]	18.07 [d]
Ground corn	6.81 [e]	6.48 [f]	17.58 [c]
Rice	6.49 [c]	4.30 [a]	0.00 [a]
Quaker	6.61 [d]	6.23 [e]	18.50 [e]
Wheat bran	6.40 [b]	4.27 [b]	24.14 [f]

* Initial pH of the culture medium of *Streptomyces* sp. strain MAR01 was adjusted to 7.0.
** Different letters indicate the significant difference at the probability level of 0.05 as evaluated by one-way ANOVA.
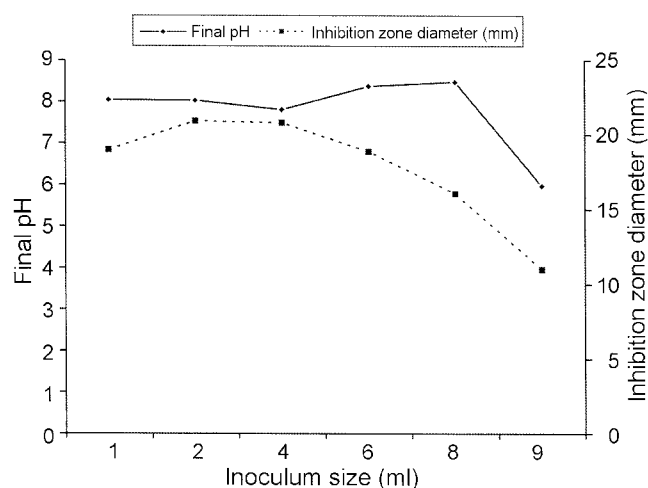
Fig. 3. Effect of inoculum size on final pH and antibacterial activity in Erlenmeyer flask solid-state cultures of *Streptomyces* sp. strain MAR01.
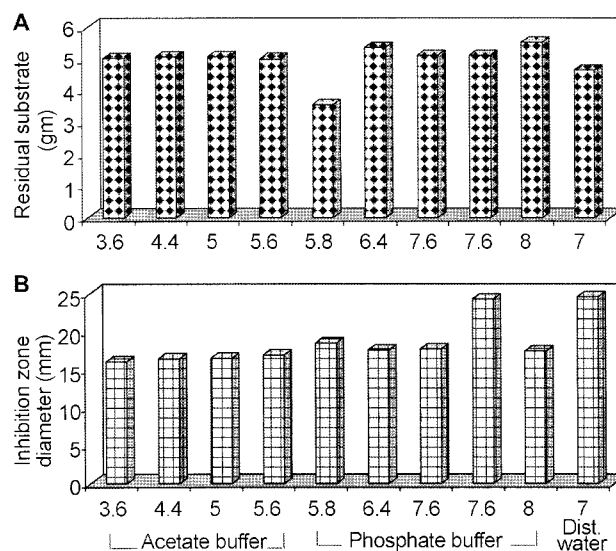

Fig. 4. Effect of buffers and water on the extraction of antibiotic from a solid-state culture of *Streptomyces* sp. strain MAR01.
A. Residual substrate; B. Antibacterial activity.

optimum. On the other hand, the results in Fig. 3 showed that 2 ml of spore suspension (1 ml contains 5×10^9 spores/ml) was the optimum inoculum size for antibiotic production. Mudgett [17] recommended the need to optimize this parameter, since a very low inoculum density can give insufficient biomass and allow growth of contaminants. As occurs with other bacteria, *Streptomyces* cultures are niches where quorum-sensing phenomena take place [3], particularly in the relatively crowded conditions normally employed in the laboratory for growing surface cultures. Many signals of this type have been reported in the *Streptomyces* genus [5]. In the diluted inoculum, conditions might be related to the lack of appropriate signals. Another simple alternative explanation is due to late-germinating spores that are logically less common in highly diluted inocula; it can be hypothesized that when the mycelial layer is dense enough to produce the signals, no late-germinating spores remain. The phenomena described here are clearly different from other surface

phenotypes, such as the previously described *Streptomyces* "pocks" [23]. The pocks are caused by the presence of conjugative plasmids and their appearance is due to the slower rate of mycelial growth at these points [13, 23].

Since, the leaching process is an important unit operation for product recovery from the thin film covering the solid support, which determines the economics of a solid-state fermentation process, antibiotic extraction from the solid-state culture using buffer and water was investigated. Acetate (pH 3.6 to 5.6) and phosphate (pH 5.8 to 8.0) buffers were used. The results obtained (Fig. 4) clearly revealed that water was the best eluent. At pH 7.6, phosphate buffer proved useful as an eluent and gave the highest antibacterial activity, whereas the acetate buffer recorded the highest antibacterial activity only when its pH was originally adjusted to pH 5.6. The time course was investigated to find out the optimum time for water extraction. It was found that 10 min was enough to extract the antibiotic

Table 2. Effect of time course and extraction method on leaching of the produced antibiotic from solid-state culture of *Streptomyces* sp. strain MAR01.

| Time course (min) | Using water as an eluent, antibacterial substance was eluted using: | | | | | |
| | Shaker incubator | | | Mixer[b] | | |
	Final pH[a]	Residual substrate (gm)	Inhibition zone diameter (mm)	Final pH[a]	Residual substrate (gm)	Inhibition zone diameter (mm)
5	6.44	5.09	18.43	6.53	4.72	24.25
10	6.53	5.08	19.09	6.65	5.10	24.75
15	6.61	5.22	24.38	6.54	4.23	22.75
30	6.49	4.36	22.50	6.53	4.40	22.50
60	6.49	4.68	22.88	ND	ND	ND
120	6.42	4.57	22.38	ND	ND	ND

[a]Initial pH of the culture medium of *Streptomyces* sp. strain MAR01 was adjusted to 7.0.
[b]The mixer could not be used for more than 30 min. ND = Not determined

Table 3. Re-use of *Streptomyces* sp. strain MAR01 cells in percolation vessel for semi-continuous antibiotic production in a wheat bran solid state culture.

Run No.	Final pH	Inhibition zone diameter (mm)
First	7.10 [b]	23.15 [c]
Second	7.32 [c]	22.83 [b]
Third	6.95 [a]	22.83 [b]
Fourth	7.34 [c]	22.21 [a]

Initial pH of the culture medium of *Streptomyces* sp. strain MAR01 was adjusted to 7.0.
Different letters are indicative to the significant difference at the probability level of 0.05 as evaluated by one-way ANOVA. Each run was maintained for 7 days.

using a mixer, whereas 60 min was required when a stirrer (Stirrer IKA RW20, Janke & Kunkel GmbH, Germany) was used. It is interesting to mention that the mixer (Mixer IKA RW20; Janke & Kunkel GmbH, Germany) could not be used for more than 30 min (Table 2).
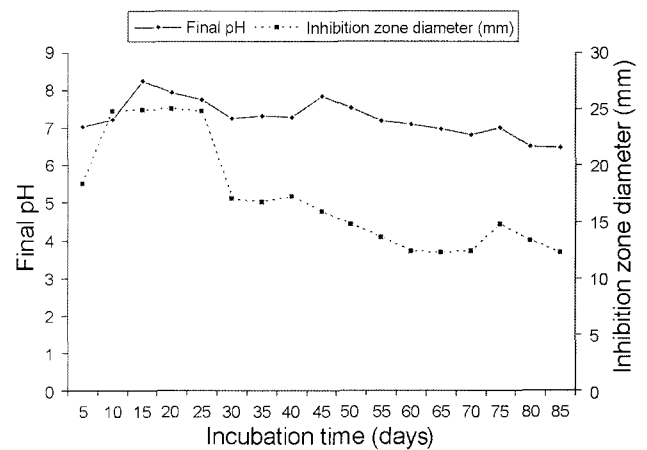
In a semicontinuous culture operated in a percolation vessel, the results obtained (Table 3) demonstrated a more or less constant antibacterial activity (450–480 μg/ml). A statistically significant difference was noticed between the first (480 μg/ml) and fourth (450 μg/ml) runs, whereas, no significant difference was present between the second and third (450 μg/ml) runs. In this experiment, *Streptomyces* sp. strain MAR01 was reused 4 times and retained its activity for a period of 28 days.

The optimization of wheat bran weight (10, 15, and 30 g per column) for the fixed-bed bioreactor was considered (at 30°C) for the better antibiotic yield in a semicontinuous mode. It was found that 30 gm wheat bran (60% moisture content) is optimum for the antibiotic production. The optimum inoculum size was found to be 6 ml of spores (5×10^9 spores/ml) per column. Finally, the double-strength production medium was used as the optimum culture medium (Table 4). It is interesting to note that the double-strength medium reduced the incubation period to 5 days instead of 7 days for the original strength. Under the previous optimized conditions, the maximum activity was obtained at the end of run No. 4 (510 μg/g) and then activity was more or less fluctuating and then decreased. The overall process continued for 85 days (Fig. 6).

Fig. 5. Semicontinuous production of antibiotic in a solid-state culture of *Streptomyces* sp. strain MAR01 using a fixed-bed bioreactor.
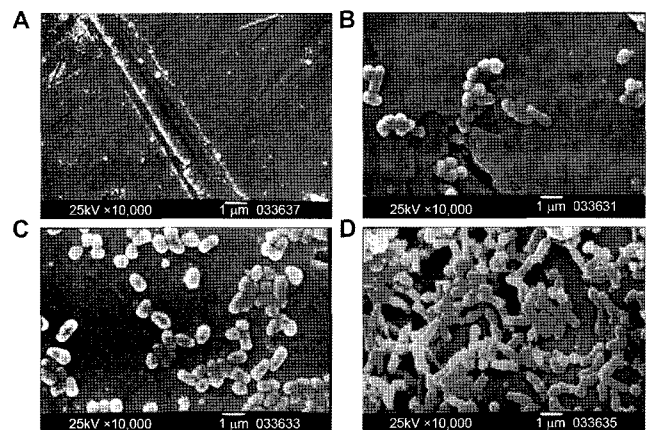
Fig. 6. Growth of *Streptomyces* sp. MAR01 during semicontinuous culture in fixed-bed bioreactor conditions.
A. Control glass matrix; B. Growth after 10 days; C. Growth after 30 days, and D. Growth after 60 days. Magnification × 1,0000, and the bar size is 1 μm.

The scanning electron microscope is a useful tool to observe the pattern of growth in SSF in the fixed-bed bioreactor. Fig. 6A–6D show the glass rings before cultivation and the growth of *Streptomyces* sp. strain MAR01 on the

Table 4. Effect of the strength of the production medium components on the antibacterial activity of solid-state culture of *Streptomyces* sp. strain MAR01 as operated in a wheat bran fixed-bed bioreactor.

Medium strength	Run duration (days)	Final pH	Volume of dist. H_2O added (ml)	Volume extracted (ml)	Inhibition zone diameter (mm)
1	7	7.53	160	196	18.73
	5	7.36	150	187	19.34
2	7	6.53	285	237	18.00
	5	7.34	175	194	24.16
1/2	7	7.33	150	173	11.75
	5	7.34	150	188	15.90

Initial pH of the culture medium of *Streptomyces* sp. strain MAR01 was adjusted to 7.0. At the end of the experiment, the residual substrate amounted to 13.73 gm. Medium strength: 1) original strength; 2) double the original strength; 1/2) half the original strength.

solid support during the semicontinuous mode. Specimens were taken under aseptic conditions at 10, 30, and 60 days of incubation. The photographs showed the directly proportional sporulation over the designated periods.

In conclusion, the optimum meroparamycin production was achieved *via* optimization of the environmental parameters in the flask and bioreactor solid-state cultures using wheat bran as an inexpensive solid substrate, which may be considered as a positive option for the industrial production of low-volume high-cost products like antibiotics.

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