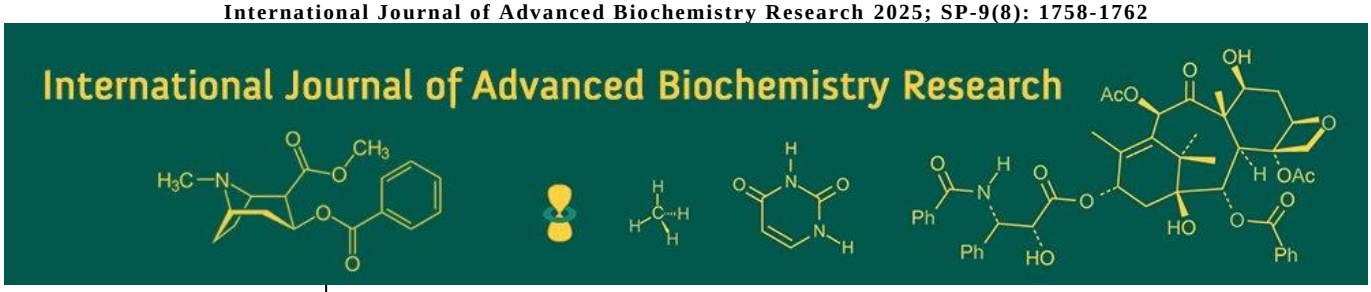

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Effect of chemical treatments on pebrine spore liberation for Tasar mother-moth examination

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Abstract
The Tasar silkworm (*Antheraea mylitta*) is highly vulnerable to a range of diseases, of which pebrine, virosis, bacteriosis, and muscardine are most common. Among these, pebrine is considered the most destructive, as it alone is responsible for nearly 30-35% of annual cocoon crop losses, and in extreme cases, it can wipe out the entire crop. The disease is primarily transmitted through vertical transmission, wherein the pathogen passes directly from the mother moth to its progeny. Hence, ensuring the production of disease-free layings (DFLs) is of utmost importance in sericulture, which is achieved through systematic microscopic examination of the mother moths. For this purpose, it becomes essential to improve the visibility of pebrine spores under the microscope. This requires the complete liberation of spores from the moth tissues, removal of fat bodies, dissolution of debris, and appropriate staining to provide a clear microscopic field.

In the present investigation, several chemical agents were evaluated for their effectiveness in liberating pebrine spores from moth tissues. The results demonstrated that treatments with Sarcosyl, formalin, and ethanol provided superior clarity in the microscopic field, with almost no debris observed. At a concentration of 0.5%, Sarcosyl and ethanol were found to release 212, 000 and 54, 000 more pebrine spores per cm^3, respectively, when compared with potassium carbonate (K_2CO_3), which served as the control. At 1% concentration, Sarcosyl treatment liberated 122, 000 additional spores per cm^3 over the control. When the concentration was increased to 2%, citric acid, ethanol, and Sarcosyl treatments showed significant improvement, releasing 300, 000, 100, 000, and 500, 000 more spores per cm^3, respectively, compared with K_2CO_3. Similarly, at 5% concentration, Sarcosyl, ethanol, and formalin treatments liberated 160, 000, 70, 000, and 100, 000 additional spores per cm^3, respectively, over the control.

From these findings, it is evident that Sarcosyl, formalin, ethanol, and citric acid were consistently more effective than K_2CO_3 in liberating a higher number of pebrine spores, thereby ensuring improved visibility and reliability in mother moth examinations. Among the tested chemicals, Sarcosyl was the most effective across concentrations, followed by ethanol, formalin, and citric acid. These results highlight the importance of adopting appropriate chemical treatments in mother moth examinations to ensure the production of pebrine-free generations of Tasar silkworms.

Keywords: *Antheraea mylitta* D., pebrine, mother moth examination, Sarcosyl

Introduction
The Tasar silkworm (*Antheraea mylitta* D.) is a wild silkworm of considerable economic value, reared mainly in outdoor conditions on host trees such as *Terminalia arjuna* (Arjuna), *Terminalia tomentosa* (Asan), and *Shorea robusta* (Sal) (Kiran Kumar *et al.*, 2017) [6]. Tasar silk has long been recognized for its natural texture and durability; however, production is frequently constrained by the outbreak of diseases that affect larval survival and cocoon yield. Major diseases of Tasar silkworms include Pebrine, Virosis, Bacteriosis, and Muscardine, which together account for an estimated 20-40% crop loss annually in India (Sahay *et al.*, 2000) [8].

Of these, Pebrine remains the most destructive, responsible for nearly one-third of total losses and occasionally wiping out entire crops (Sahay *et al.*, 2000; Chakrabarty *et al.*, 2012) [8, 4]. The disease is caused by microsporidian parasites belonging to the family Nosematidae and is notorious for its vertical transmission, wherein infected moths pass the spores directly to their progeny. Infected larvae often die in the early instars, while those surviving to later stages display poor growth, reduced cocoon formation, and compromised silk quality (Singh *et al.*, 2018) [12].

If infection persists into the adult stage, contaminated eggs are laid, perpetuating the disease cycle and endangering silkworm seed supply.

To safeguard seed production, the microscopic examination of mother moths is a critical preventive measure (Singh *et al.*, 2021) [13]. Yet, even with careful screening and rejection of infected layings, Pebrine remains a persistent problem. This difficulty may be attributed to the presence of vegetative stages of Nosema that escape detection, as well as incomplete release of spores during tissue preparation. Additionally, fat bodies and residual tissue fragments can obscure spores under the microscope, complicating diagnosis.

Recent studies have emphasized improving the detection and management of Pebrine disease through advanced methodologies (Bhattacharya *et al.*, 1995; Datta *et al.*, 1998; Samson *et al.*, 1998; Patil and Sharadamma, 1999) [3, 5, 9, 7]. One of the effective approaches reported is the use of chemicals to induce the liberation of Pebrine spores from infected moths or eggs. Similar experiments were carried out by Thangavelu *et al.* (1995) [14] and Singh *et al.* (2018) [12], showing that chemical treatment can weaken or disrupt the protective layers around the spores, making them more easily visible. This enhanced visibility supports accurate diagnosis and improves overall disease management.

In addition, several researchers (Singh *et al.*, 2002; Aneja, 2003; Singh *et al.*, 2005) [10, 11] have explored different chemicals to increase the release of spores, refine staining methods, and remove interfering materials such as fat bodies and tissue debris. These improvements are important because debris often reduces clarity during microscopic examination. By ensuring proper staining and cleaning, the detection process becomes more precise and efficient.

Therefore, to achieve reliable diagnosis, it is necessary to ensure complete liberation of Pebrine spores, followed by effective staining, cleaning of preparations, and removal of fat bodies and other tissue residues. These refinements strengthen existing diagnostic and control measures, making them more effective in addressing the persistent problem of Pebrine in Tasar silkworm culture.

Materials and Methods

Tasar silkworm (*Antheraea mylitta* D.) moths were obtained from the Regional Sericulture Research Station (RSRS), Warangal, Telangana. The experimental work was carried out at the Department of Sericulture, Sri Krishnadevaraya University, Anantapur, Andhra Pradesh, during 2021-22.

Selection of Chemicals

A set of chemicals known for their potential to aid in spore liberation, tissue dissolution, and removal of fat bodies or debris from microscopic smears were selected based on earlier reports and published literature (Table 1). The chemicals chosen for evaluation included:

Sulphuric acid, Acetic acid , Hydrochloric acid, Ethanol, Citric acid, Sodium acetate, Formalin, Sodium hydroxide, Sodium Dodecyl Sulphate (SDS), Sarcosyl.

These chemicals were selected for their ability to facilitate the release of Pebrine spores from moth tissues and to improve smear clarity during microscopic examination.

Preparation of Stock Solutions

Stock solutions (10% concentration) of each selected chemical were prepared separately using analytical-grade reagents. For preparation, the required quantity of each chemical was measured accurately and dissolved in double-distilled water. In the case of acids, dilution was carried out carefully by adding the concentrated acid slowly into distilled water with constant stirring to avoid overheating or splashing. The prepared solutions were stored in properly labeled sterile containers at room temperature until further use.

Table-1: Selected chemicals for the study

S. No	Name of the chemical
1.	Sodium hydroxide
2.	Formalin
3.	Sodium acetate
4.	Citric acid
5.	Ethanol
6.	Hydrochloric acid
7.	Acetic acid
8.	Sulphuric acid
9.	SDS
10.	Sarcosyl

Preparation of Solid State Chemicals Stock Solution

Calculation in Percentage (weight/volume)

$$10\% \text{ of } 100 \text{ mL of chemical} = \frac{100 \text{ mL of the chemical} \times 10 \text{ g}}{100}$$

The obtained amount of chemical is then dissolved in 100 mL of distilled water.

Preparation of Liquid State Chemicals Stock Solution

Calculation in Percentage (volume/volume)

$$10\% \text{ of } 100 \text{ mL of chemical} = \frac{100 \text{ mL of the chemical x } 10 \text{ mL}}{100}$$

The obtained volume of that chemical (10 mL) is dissolved in 90 mL of distilled water.

Serial Dilution

To prepare the dilutions, 9 mL of distilled water was first measured into a clean measuring cylinder, followed by the addition of 1 mL of the desired chemical solution. This step yielded a solution with a 10% concentration of the original sample. Using the same procedure, a total stock volume of 100 mL was prepared for each of the selected chemicals.

For further dilution, 1 mL of the prepared stock solution was transferred into a test tube with the help of a pipette, and the final volume was adjusted to 10 mL by adding distilled water. This process produced a solution that was diluted by a factor of 10. The same procedure was repeated for all other selected chemicals, with the volume of distilled water adjusted proportionally, depending on the desired concentration of each specific solution.

Selection of Chemical Concentrations

To evaluate the effect of different chemical strengths on Pebrine spore liberation, a range of concentrations was tested. Working solutions of 0.5%, 1%, 2%, 3%, 4%, 5%, 7.5%, and 10% were prepared from the respective stock solutions and used for the experimental analysis.

Identification of Pebrinized Moths and Their Collection

Pebrine-infected moths were identified based on characteristic morphological deformities (Anonymous, 2023). Typical symptoms included crumpled or malformed wings, reduced or detached scales on the abdomen, and easily detachable wing scales. Infected moths also exhibited reduced mating efficiency and poor egg-laying capacity. Moths showing these signs were collected in sterile paper boxes and brought to the laboratory for further examination (Figure 1).

Fig 1: Pebrinized moths showing crumpled wings and scaleless abdomens

Examination of Tasar Moths

1. Pebrinized moths were dissected, and tissues from the 4th to 7th abdominal segments were excised.
2. The tissue sample was divided into two equal parts by weight.
3. Each portion was placed in a separate mortar and labeled as Sample A and Sample B. Both samples were crushed identically with separate pestles.
   Sample A: Treated with the test chemical solution.
   Sample B: Treated with potassium carbonate (K_2CO_3) solution, used as the control.
4. Smears prepared from both samples were observed under a student microscope at 600× magnification, and spore visibility was recorded.

Counting of Pebrine Spores

1.5 mL of all the samples were taken in different Eppendorf tubes and well labeled and then subjected to centrifuge at 3000 rpm for 5 min.

The supernatant was then discarded and 1mL of distilled water was added to each Eppendorf tube and then vortexed for 3 min. for proper mixing of the pallet with distilled water.

Then one 10 µL of the sample were taken in a haemocytometer and then subjected to counting of spores.

All the observations were noted down and tabulated in table 2, 3 and 4.

No. of Spore = average of all five readings × 104 spores cm^3 formula was used to calculate the total number of spores present in the taken sample.

Results and Discussion

The effectiveness of various chemicals in liberating Pebrine spores and improving microscopic field clarity was evaluated, and the observations are presented in Tables 2-4. Considerable variation was recorded among the tested chemicals, both in terms of spore release and smear clarity.

Microscopic field clarity

Initial qualitative observations showed that chemicals such as sodium hydroxide, sodium acetate, citric acid, acetic acid, and SDS did not produce any remarkable improvement when compared to the control (K_2CO_3). These smears often retained tissue debris, fat globules, and cell remnants that obscured the spores. In contrast, treatments with Formalin and Ethanol produced clearer fields with distinct spores that exhibited a fluorescent-like shine. Sarcosyl treatment resulted in the clearest background, with debris almost completely removed, which allowed for precise spore visualization. This indicates that certain chemicals not only dissolve interfering materials but also enhance contrast, thereby improving the accuracy of diagnosis.

Performance at lower concentrations (0.5% and 1%)

At 0.5% concentration, the number of liberated spores ranged from 110, 000 (acetic acid) to 638, 000 (Sarcosyl), while the control showed 426, 000 spores/cm^3. Most chemicals released over 200, 000 spores, except acetic acid, sodium acetate, and hydrochloric acid. Ethanol liberated 54, 000 more spores and Sarcosyl liberated 212, 000 more spores than the control, highlighting their superior efficiency at low concentrations. At 1%, the trend was similar, with Sarcosyl (812, 000 spores/cm^3) showing the highest release, surpassing the control (690, 000) by 122, 000 spores. Hydrochloric acid showed the lowest release at this concentration. These results demonstrate that Sarcosyl maintains its efficiency even at dilute concentrations, whereas strong acids such as HCl appear to damage tissue structure without aiding spore release.

Performance at moderate concentration (2%)

At 2% concentration, spore liberation was most effective, ranging from 160, 000 (acetic acid) to 1, 400, 000 (Sarcosyl). The control recorded 900, 000 spores/cm^3. Here, Sarcosyl liberated 500, 000 additional spores compared to the control, while citric acid (+300, 000) and ethanol (+100, 000) also showed higher efficiency. The results suggest that around this concentration, certain chemicals reach their optimal level of action, dissolving fat bodies and breaking down tissue remnants to free the spores more effectively. The outstanding performance of Sarcosyl at this concentration highlights its detergent-like property, which efficiently solubilizes lipids and proteins, thereby releasing spores trapped within cellular material.

Performance at higher concentration (5%)

At 5% concentration, the number of liberated spores decreased compared to the 2% treatment, ranging from 179, 000 (sodium hydroxide) to 480, 000 (Sarcosyl). The control showed 320, 000 spores/cm^3. Formalin (+100, 000), Ethanol (+70, 000), and Sarcosyl (+160, 000) were more effective than the control. However, the overall reduction in liberated spores at this concentration suggests that stronger chemical exposure may alter spore integrity or reduce visibility by forming artefacts in the smear. For example, sodium hydroxide, a strong base, may have damaged or dissolved the spores themselves, leading to underestimation. This highlights the need for careful optimization of chemical concentration to maximize spore release without compromising structural integrity.

Comparative performance of chemicals

Across all concentrations tested, Sarcosyl consistently outperformed all other chemicals, both in terms of spore liberation and field clarity. Ethanol also performed well, particularly at lower and moderate concentrations, possibly due to its ability to dissolve lipids and dehydrate tissues, thereby releasing spores. Formalin, known for its protein-

fixing properties, showed better results at higher concentrations, producing clear smears with enhanced spore contrast. Citric acid was moderately effective at 2%, where it released substantially more spores than the control, though its performance declined at higher concentrations.

Implications for Pebrine detection

The consistent superiority of Sarcosyl suggests that it is the most promising chemical for preparing mother moth smears in Pebrine diagnosis. Its detergent nature allows it to efficiently dissolve fat bodies and tissue debris, freeing spores for easy detection under the microscope. Ethanol, Formalin, and Citric acid can serve as alternative or supplementary agents, particularly where Sarcosyl may not be readily available. The ability of these chemicals to enhance field clarity and spore visibility has direct implications for sericulture, as improved detection of Pebrine ensures the elimination of infected moths, thereby reducing vertical transmission and safeguarding seed production.

Overall evaluation

The findings of this study demonstrate that chemical treatment plays a pivotal role in improving the efficiency of Pebrine detection. Sarcosyl ranked first, followed by Ethanol, Formalin, and Citric acid, in enhancing spore liberation. These results are in agreement with earlier reports highlighting the importance of chemical aids in microscopic diagnosis of Pebrine (Singh *et al.*, 2002; Singh *et al.*, 2018) [10, 12]. Adopting such chemical treatments in routine mother moth examination protocols can significantly reduce diagnostic errors, ensure the production of disease-free layings, and ultimately contribute to the sustainability of Tasar sericulture.

Table 2: Visual observations of field treated with different chemicals

S. No	Name of the chemical	Observation
1.	Sodium hydroxide	No significant difference was observed.
2.	Formalin	Field was clear and aggregation of tissues was observed.
3.	Sodium acetate	No effect was observed.
4.	Citric acid	No significant difference was observed but the refractivity of spores was reduced.
5.	Ethanol	Field was clear and did not affect its basic structure.
6.	Hydrochloric acid	Clumps formation of cellular debris was observed.
7.	Acetic acid	No significant difference was observed.
8.	Sulphuric acid	Desiccation of all the other cells was observed along with inhibition of Brownian movement of spores.
9.	SDS	No significant difference was observed.
10.	Sarcosyl	Clearance of the field was observed to good extent and the amount of liberation of spores were very much high.
Control	K_2CO_3	The field was good with normal amount of liberation of spores.

Table 3: Performance of different chemicals at 0.5% and 1.0% concentrations

S. No	Name of the chemical	Chemical concentration			
		0.5%		1.00%	
		Liberation of spores (No)	Spores more than control (No)	Liberation of spores (No)	Spores more than control (No)
1.	Sodium hydroxide	360, 000	-66, 000	525, 000	-165, 000
2.	Formalin	410, 000	-16, 000	673, 000	-17, 000
3.	Sodium acetate	190, 000	-236, 000	390, 000	-300, 000
4.	Citric acid	395, 000	-31, 000	411, 000	-279, 000
5.	Ethanol	480, 000	+54, 000	656, 000	-34, 000
6.	Hydrochloric acid	175, 000	-251, 000	198, 000	-492, 000
7.	Acetic acid	110, 000	-316, 000	356, 000	-334, 000
8.	Sulphuric acid	410, 000	-16, 000	473, 000	-217, 000
9.	SDS	310, 000	-116, 000	580, 000	-110, 000
10.	Sarcosyl	638, 000	+212, 000	812, 000	+122, 000
Control	K_2CO_3	426, 000	0	690, 000	0

Table 4: Performance of different chemicals at 2% and 5% concentrations

S. No	Name of the chemical	Chemical concentration			
		2.00%		5.00%	
		Liberation of spores (No)	Spores more than control (No)	Liberation of spores (No)	Spores more than control (No)
1.	Sodium hydroxide	500, 000	-400, 000	179, 000	-141, 000
2.	Formalin	210, 000	-690, 000	420, 000	+100, 000
3.	Sodium acetate	360, 000	-540, 000	273, 000	-47, 000
4.	Citric acid	1, 200, 000	+300, 000	197, 000	-123, 000
5.	Ethanol	1, 000, 000	+100, 000	390, 000	+70, 000
6.	Hydrochloric acid	420, 000	-480, 000	180, 000	-140, 000
7.	Acetic acid	160, 000	-740, 000	220, 000	-100, 000
8.	Sulphuric acid	250, 000	-650, 000	290, 000	-30, 000
9.	SDS	830, 000	-70, 000	310, 000	-10, 000
10.	Sarcosyl	1, 400, 000	+500, 000	480, 000	+160, 000

Control	K_2CO_3	900, 000	0	320, 000	0

Conclusion

Among the different chemicals tested, Sarcosyl proved to be the most effective in liberating Pebrine spores from the homogenized moth tissues. At 0.5% concentration, Sarcosyl-treated samples released 638, 000 spores/cm³, which is 212, 000 spores/cm³ higher than the control (K_2CO_3, 426, 000 spores/cm³). The increased number of liberated spores not only enhanced the accuracy of microscopic detection but also provided a clear microscopic field free from debris, ensuring reliable identification.

Based on these findings, 0.5% Sarcosyl can be recommended as an efficient chemical aid for Pebrine spore detection in routine mother moth examinations. Its application in commercial grainages, private seed production units, and research institutes will help in early and precise diagnosis, thereby preventing vertical transmission of the disease and contributing to the production of disease-free layings (DFLs). This, in turn, will strengthen seed health management and improve the sustainability of sericulture.

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